Inhibitory effect of silver nanoparticles mediated by atmospheric pressure air cold plasma jet against dermatophyte fungi

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In an in vitro study with five clinical isolates of dermatophytes, the MIC50 and MIC100 values of silver nanoparticles (AgNPs) ranged from 5 to 16 and from 15 to 32 μg ml⁻¹, respectively. The combined treatment of AgNPs with atmospheric pressure-air cold plasma (APACP) induced a drop in the MIC50 and MIC100 values of AgNPs reaching 3–11 and 12–23 μg ml⁻¹, respectively, according to the examined species. *Epidermophyton floccosum* was the most sensitive fungus to AgNPs, while *Trichophyton rubrum* was the most tolerant. AgNPs induced significant reduction in keratinase activity and an increase in the mycelium permeability that was greater when applied combined with plasma treatment. Scanning electron microscopy showed electroporation of the cell walls and the accumulation of AgNPs on the cell wall and inside the cells, particularly when AgNPs were combined with APACP treatment. An in vivo experiment with dermatophyte-inoculated guinea pigs indicated that the application of AgNPs combined with APACP was more efficacious in healing and suppressing disease symptoms of skin as compared with the application of AgNPs alone. The recovery from the infection reached 91.7 % in the case of *Microsporum canis*-inoculated guinea pigs treated with 13 μg ml⁻¹ AgNPs combined with APACP treatment delivered for 2 min. The emission spectra indicated that the efficacy of APACP was mainly due to generation of NO radicals and excited nitrogen molecules. These reactive species interact and block the activity of the fungal spores in vitro and in the skin lesions of the guinea pigs. The results achieved are promising compared with fluconazole as reference antifungal drug.

INTRODUCTION

Dermatophytozes or tineas are a group of superficial mycoses caused by fungi that parasitize keratin-rich structures such as the outer layer of the epidermis (stratum corneum), hair and nails. Species of dermatophytes are capable of producing several clinical manifestations in different parts of the body and hence are divided into clinical forms according to the regions of the body they commonly affect. Infectious diseases, particularly skin and mucosal infections, are common in most of the tribal inhabitants owing to lack of adequate sanitation and of awareness regarding effective personal hygiene practices. Abanmi et al. (2008) stated that in the Kingdom of Saudi Arabia (KSA) onychomycosis is the most frequent form of dermatophyte infection (40.3 %), followed by tinea capitis (21.9 %), tinea pedis (16 %), tinea cruris (15.1 %) and tinea corporis (6.7 %).

In the last few years, the numbers of immune-suppressed and immunocompromised patients, who frequently develop superficial infections such as dermatomycosis, candidiasis, fungal infections, etc., have increased. This is mainly due to the non-availability of effective antifungal medications for these infections and the risk associated with long-term treatment with antifungal drugs. The azole-containing medicines, for example, may induce several side effects, including interference with the activity of hepatic microsomal enzymes,
the central nervous system, sex and thyroid hormones, and testosterone biosynthesis (del Palacio et al., 2000). Moreover, the emergence of pathogenic strains resistant to conventional antifungal drugs has motivated medical mycologists to search for new therapeutic agents such as nanoparticles (Zharov et al., 2006). Nanoparticles, as a new generation of antimicrobials, have recently been discussed by several investigators. Nanoparticles are clusters of atoms in the size range 1–100 nm. The use of nanoparticles is gaining impetus in the present century as they possess defined chemical, optical and mechanical properties. Metallic nanoparticles are most promising as they show good antimicrobial properties, of current interest to researchers owing to the growing microbial resistance against metal ions and antibiotics (Gong et al., 2007). Different types of nanomaterials, like copper, zinc, titanium, magnesium, gold and silver, have been developed, but silver nanoparticles (AgNPs) have been shown to be the most effective, having considerable antimicrobial efficacy against several micro-organisms.

The cell wall is the first and main target of the nanoparticles. It has been reported that nanoparticles are less effective in inactivating fungi than bacteria, mainly owing to the thicker fungal cell wall structure consisting of rigid chitin as compared with the peptidoglycan of bacterial membrane. To achieve higher antifungal efficacy of nanoparticles, moderation, attenuation or erosion of the cell wall probably will enhance the effect of nanoparticles and facilitate their interaction with cellular components. In this study, atmospheric-pressure-air cold plasma (APACP) was employed for this purpose.

Recently, atmospheric-pressure cold plasmas have received much attention as a physical device capable of microbial decontamination and sterilization through their interaction with surface layers, including the microbial cell wall. Plasma is a highly energized fourth state of matter composed of excited atoms and molecules, ionized gases, radicals and free electrons (Moisan et al., 2001). Non-thermal plasmas have been used as a sterilization method against various micro-organisms (Laroussi & Leipold, 2004; Ouf et al., 2015 a, b). Many plasma devices that generate reactive species have been examined for decontamination of thermally sensitive tools and for medical applications (Kolb et al., 2008; Laroussi et al., 2008).

Although the literature reports several researches related to the antimicrobial activity of nanoparticles, to the authors’ knowledge there are no studies concerning the effect of these particles associated with cold plasma. Thus, the aim of the current investigation was to evaluate the use of AgNPs combined with APACP against some dermatophytes through in vitro and in vivo experiments.

**METHODS**

**Test organisms.** Five dermatophyte species were isolated from patients with skin, nail and hair infections submitted to the dermatological and microbiological laboratory of King Fahd Hospital, Almadinah Almunawwarah, KSA. The isolated species were identified as Epidermophyton floccosum, Microsorum canis, Tri-chophyton mentagrophytes and T. rubrum. All dermatophytes were stored in Sabouraud dextrose (SD) broth (Oxoid) with 20 % glycerol at ~80 °C and propagated on potato dextrose agar (PDA; Oxoid) plates incubated at 28 °C before each test. The inocula were prepared from 7-day-old cultures.

**AgNPs.** AgNPs were purchased from Nanotech Egypt and were at least 99.95 % pure. The particles were prepared by a chemical reduction method as reported by Solomon et al. (2007). A solution of AgNO₃ was used as the Ag⁺ ion precursor. Sodium borohydride was used as a mild reducing and stabilizing agent. The color of the AgNP solution was greyish yellow, indicating the reduction of Ag⁺ ions to AgNPs. The particles were spherical in shape, with 15 ± 3 nm mean diameter, and had an optical absorption peak at 410 nm.

**Air plasma jet experimental setup.** Figure 1 is a schematic representation of the atmospheric-pressure air plasma jet experimental setup. The system consisted of a sharp stainless steel capillary needle electrode, 70 mm in length with 0.45 and 0.84 mm internal and external diameters, respectively. A 25 kV, 30 kHz sinusoidal waveform alternating current high voltage power supply was connected to the needle electrode through a current-limiting resistor of 33.33 kΩ. The needle had a sleeve of alumina tube insulator with 0.84 and 1.63 mm inner and outer diameter, respectively. The alumina tube nozzle was covered by an earthed hollow-cup copper alloy (brass) electrode with central inner diameter 1 mm.

A high-voltage electric probe (Tektronix P6015) and Pearson current monitor probe (model 6585) were used to measure the voltage and current waveforms, respectively. The probes were connected to a 1 GHz, 5 GS s⁻¹ digital phosphor oscilloscope (DPO 4104B; Tektronix) as shown in Fig. 1. In this work, the high-voltage waveform signals were measured at the high-voltage electrode and the current waveforms were measured at the high-voltage electrode (needle) and the earth electrode (hollow cup). The jet was formed by blowing air through the capillary needle while a high-voltage signal was applied between the needle electrode and the hollow-cup electrode. The discharge started between the needle tip and nearest point of the earth cup electrode. Then, the plasma spread on the electrode surface, entering the cup hole with higher current values.

**Fig. 1.** Setup of the APACP jet (left) and image of the generated plasma (right). HV AC, high-voltage alternating current; V, voltage; I, current.
The plasma jet emission spectra were recorded using a 500 mm spectrophotograph with 1800 g mm\(^{-1}\) grating blazed at 500 nm. The emission spectra were collected from a fibre-optics bundle using a plano-convex UV fused silica lens of 75 mm effective focal length. The spectra were collected from the gap between the two electrodes where end-on measurements were performed. The generated plasma jet formation was measured using an AVT Manta G-504 colour CCD camera.

**Plasma treatment.** Preliminary tests revealed that the optimum conditions for APACP generation producing maximum inhibition for the tested dermatophytes were: sustained voltage of 0.6 kV, 15 mA current, 21 kHz repetition rate and 21 SLPM (standard litres min\(^{-1}\), where standard refers to 0 °C and 101325 Pa) air controlled by a flow meter (ALICAT MC-205LPM-D/5). The distance between the jet nozzle and the surface of the treated sample was 1.0 cm and the duration of treatment of the sample was 2 min. For testing MIC, the inoculum spore suspension was placed in Eppendorf tubes, and for investigation of keratinase activity and cellular leakage the treatment was carried out directly on the mycelium inoculum in 6 cm diameter sterile glass Petri dishes. In pathogenicity experiments, the source of APACP was movable and introduced through the top of the guinea pig cages such that the *M. canis*-inoculated area of the animal was never more than 2 cm away from the cold plasma source.

**MIC values.** The MIC for the different clinical isolates of dermatophytes was determined by a broth microdilution assay in accordance with the guidelines of the National Committee for Clinical Laboratory Standards as defined in document M38-A for filamentous fungi (CLSI, 2002).

The inoculum suspensions of the test dermatophytes were prepared by covering the fungal colonies, previously grown on 9 cm potato dextrose agar (PDA) plates for 7 days at 28 °C, with 10 ml distilled water and then scraping the colony surfaces with the tip of a sterile loop. The resulting suspension containing conidia and hyphal fragments was transferred to sterile agar plates and allowed to stand for 15 min at room temperature to allow the heavy particles and fragments to settle. The suspension was adjusted using a double-beam UV–visible spectrophotometer (model UV 2150; UNI CO) to 75 to 80 % transmittance. The number of c.f.u. ml\(^{-1}\) was quantified using a haemocytometer and by plating 10 ml suspension on Sabouraud dextrose agar and counting the colonies that developed. The concentration of the inoculum suspension was then adjusted to 10\(^5\) cells ml\(^{-1}\) using RPMI 1640 medium (with glutamine and without sodium bicarbonate; Sigma). Aliquots of 100 μl of the resulting suspension, treated or untreated with APACP, for 2 min, were inoculated into U-bottomed 96-well microdilution plates (Brand 781660; Wertheim) containing 100 μl twofold dilution of AgNPs. Inoculated RPMI 1640 medium free of AgNPs was used as a control. Fluconazole (Sigma-Aldrich) run in the same way was employed as antifungal reference standard for comparison. The microdilution plates were incubated without agitation at 28 °C and readings were made visually every 24 h until growth appeared in the AgNP-free control well. Each assay was run in duplicate. MIC\(_{50}\) and MIC\(_{100}\) values were, respectively, the concentrations required to inhibit dermatophyte growth by 50 and 100 % (total inhibition of growth).

**Keratinase assay.** In this experiment, 10 mg ml\(^{-1}\) AgNPs were used as mean inhibitory dose that allow considerable growth of the most tested species. The effect of nanoparticles either individually or combined with APACP on keratinase activity was studied. The AgNP-treated inoculum of each fungus species was exposed to APACP for 2 min at 3.5 SLPM and then was incubated with keratinase induction medium (Muhsin & Aubaid, 2001). Inocula treated only with AgNPs were used for comparison and others treated with fluconazole were used as reference control. The cultures were allowed to stand for 5 days at 28 °C and then were shaken for 5 days in a shaking incubator (model JSSI-100C; JS Research). At the end of the growth period, the mycelium was removed from the flasks by filtration and the culture filtrates were assayed for keratinase activity using a modification of the method of Muhsin & Aubaid (2001). In brief, 500 ml cell-free supernatant was mixed with 50 mg guinea pig hair in 5 ml 0.03 M phosphate buffer (pH 7.8) and incubated at 37 °C for 3 h. The sample under investigation was replaced by buffer to run the control. The reaction mixture was stopped by adding 5 ml 10 % TCA, kept on ice for 30 min and centrifuged at 2236 g for 30 min. A boiled enzyme solution was used in the same way as a control. The hair was removed by filtration and the absorbance was read at 280 nm using a double-beam UV–visible spectrophotometer. The enzyme activity was expressed as U ml\(^{-1}\), where 1 U keratinolytic activity was defined as the amount of enzyme required to liberate 1 mmol tyrosine under these conditions.

**Leakage measurement.** The release of cell constituents into the supernatant was measured according to the method described by Paul et al. (2011), with minor modifications. Briefly, mycelium from a 100 ml Sabouraud glucose broth culture of the tested dermatophytes was collected by centrifugation at 4000 g for 20 min, washed three times with distilled water, and then resuspended in 100 ml PBS (pH 7.0) amended with 10 μg ml\(^{-1}\) AgNPs. A parallel experiment was carried out in the same way except that the AgNP-treated mycelium was exposed to APACP for 2 min. Fluconazole at 100 μg ml\(^{-1}\) was employed as reference antifungal drug. The treated mycelium in saline was incubated at 25 °C with agitation in a shaking incubator for 30 min. At the end of the incubation time, 2 ml sample was collected and centrifuged at 12 000 g for 2 min. To determine the concentration of the released constituents, 1 ml supernatant was used to measure the absorbance at 260 nm with a spectrophotometer. SDS (2 %) was used to calculate 100 % cellular leakage. Results are expressed as the mean of percentage cellular leakage values from three independent assays.

**SEM.** The effect of APACP on spores of *M. canis* as a representative dermatophyte was examined using SEM. The spores were detached from agar discs (1 cm diameter) of inoculated Sabouraud glucose agar that had been incubated for 12 days at 25 °C. The spores were suspended in demineralized sterile water. The suspension was centrifuged for 15 min at 7000 g and the pellet was resuspended in 25 ml sterile demineralized water. This operation was performed twice. Then, the pellet was resuspended and centrifuged twice at 5000 g and twice at 4000 g for 15 min each. After the last wash, the purified suspension was collected in demineralized sterile water and distributed in eight Eppendorf tubes, each containing 1 ml suspension. Two tubes were treated with APACP, two with 10 μg ml\(^{-1}\) AgNPs and two with 10 μg ml\(^{-1}\) AgNPs followed by APACP; the spore suspension in the last two tubes remained without treatment and was employed as control. Twenty five microlitre aliquots of the treated and untreated spore suspensions were sprayed on 1.0 cm diameter polystyrene sheets and dried for 24 h at room temperature in a laminar flow hood. The sheets were then pasted on a metallic support and gold-plated before observation by SEM at 20.0 kV with an FEI/Phillips XL30 microscope.

**Pathogenicity test.** This experiment was conducted to investigate the possibility of using the combined application of APACP and AgNPs in curing the *M. canis*-inoculated guinea pigs. Animal housing and treatment conditions were approved by the ethics committee of Taibah University, KSA that determines ethical and animal welfare practices.

The test species (*M. canis*) was grown on plates of Sabouraud dextrose agar (Oxoid) for 12 days at 25 °C. The growth in each plate was scraped off with a sterile needle and placed into 9 ml tubes of honey, diluted 1 : 3 in water. The suspension was briefly homogenized with an Ultra-Turrax apparatus, and 1 ml volumes (1 × 10\(^5\) spores ml\(^{-1}\)) were smeared on three separate areas of the shaved and lightly.
scarified dorsa of the albino guinea pigs. Adult guinea pigs with body weights between 500 and 600 g were used in this study. The animals were kept in pairs in stainless steel cages and allowed to adapt to the conditions of the animal house for 14 days before the experiments. The animals were maintained at 25 ± 2 °C with intervals of 12 h light and 12 h dark. Animal care was performed according to the KSA national legislation for the care and use of laboratory animals and approved by the Taibah University animal ethics committee (approval no. 3006/434). Each animal was identified by numbered ear-tag.

For the inoculated animals, the above procedure generated crusted, erythematous lesions approximately 4 to 5 cm in diameter, which were well-developed 3 days after inoculation. Starting from the fifth day after inoculation, 1 ml 13 μg ml⁻¹ AgNPs dissolved in polyethylene glycol 200 was applied following treatment of the infected region with APACP for 2 min. Parallel treatment testing the application of AgNPs without APACP was carried out. Fluconazole at a concentration of 152 cation of AgNPs without APACP was carried out. Fluconazole at a flow rate of 3.5 SLPM. Blue line, total current; red line, earth current.

RESULTS AND DISCUSSION

**Statistical analysis.** All data were expressed as the mean ± SD of three independent replicates. Analysis of variance using one-way ANOVA was performed to test the significance of differences between data at the 5 % level.

**Plasma jet current—voltage waveforms**

Figure 2 shows a typical current–voltage waveform of the atmospheric-pressure-air plasma jet at 3.5 SLPM air-flow rate. The applied high voltage had a sinusoidal waveform which was measured at the needle electrode. The total current was the current passing through the needle electrode, while the earth current was the current through the earthed electrode. The electrodes in our setup are a modified version of the microhollow cathode discharge electrodes studied previously by several authors (Mohamed et al., 2010; Becker et al., 2006; Kolb et al., 2008). In this work a sharp-needle electrode was used to enhance the electric field at its tip to reduce the breakdown voltage. The typical discharge breakdown voltage for a parallel plate electrode is 30 kV cm⁻¹, which indicates that the expected breakdown voltage for the 1 mm gap used in this work was 3000 V. However, the measured breakdown voltage in this experiment was in the region of 1200 V, which was less than the expected value by 1800 V owing to the use of a hollow-electrode system and the sharp-needle electrode. The current waveforms showed an oscillation at the discharge breakdown. The oscillation in current was followed by a sustained current in the region of 15 mA while the peak current in the oscillation reached up to 65 mA. In similar work, Hong & Uhm (2007) observed damping oscillations in an air plasma jet with about 15 mA current and a breakdown voltage in the region of 3.5 kV for a gap of 1.5 mm.

**Air plasma jet emission spectra**

In the discharge zone of the atmospheric-pressure plasma jet, electrons are accelerated by the electric field and impact with plasma species. The electron impacts excite atoms and molecules to higher electronic states, which decay and emit photons at defined wavelengths. The set of emitted wavelengths forms the emission spectrum of the generated plasma in the detected range. The emission intensity of given wavelength is proportional to the concentration of the species from which the excited state decays.

The emission spectrum of the APACP jet in the range 200–850 nm showed a significantly dominant N₂ second positive band system (C²Π_u→B¹Π_g) (Fig. 3). The spectrum indicates the generation of NO⁺ (A¹Σ_u⁺→X²Π_g) radicals in the UV range 200–280 nm, as illustrated in the inset of Fig. 3. The generation of NO in the UV region in cold plasma has been elucidated by several authors (Machala et al. 2007; Hao et al. 2014). The spectrum was measured using a 1800 g mm⁻¹ grating blazed at 500 nm. Therefore, the NO spectrum was detected at the end of the sensitive range of the grating. The real NO intensity must have a higher ratio with respect to the 0–0 transition at 337.2 nm N₂ second positive band system (C²Π_u→B¹Π_g). This indication was confirmed when the emission spectrum was measured using a 3600 g mm⁻¹ grating blazed at 240 nm. The spectrum of the N₂⁺ first negative system (B²Σ_g⁺→X²Σ_g⁻) was measured at 391 nm, indicating the presence of a high electron temperature in the generated plasma (Machala et al. 2007). In earlier work, Hong &
with MIC50 and MIC100 values recorded as 16 and 32 µg ml⁻¹, respectively. *M. canis* and *M. gypseum* were less sensitive, while *T. mentagrophytes* was the least tolerant fungus.

Although the bactericidal effects of nanoparticles are well-known, their antifungal activities have received only marginal attention and just a few investigations on this topic have been reported (El-Rafie et al., 2010; Gogoi et al. 2006; Zeng et al., 2007; Roe et al., 2008). The difference in the MIC values (µg ml⁻¹) of AgNPs between the fungal species has been documented for *Candida*, being 2–4 for *C. albicans*, 1–7 for *C. glabrata*, 2–25 for *C. parapsilosis*, 13 for *C. krusei* and 7 for *C. tropicalis* (Kim et al. 2008). Even with the same species, the MIC (µg ml⁻¹) of *C. albicans* was 0.4–1.6 as reported by Monteiro et al. (2011), 0.052–0.1 as reported by Panáček et al. (2009), and 2–4 as reported by Kim et al. (2008). Considering the effect of nanoparticles on filamentous fungal pathogens of skin, several authors indicated lower MIC values than those reported in the present research. Kim et al. (2008) indicated potent activity of AgNPs against clinical isolates of *T. mentagrophytes*, recording 1–4 µg ml⁻¹ for IC80, which was comparable to that of amphotericin B applied at 1–2 µg ml⁻¹ and superior to that of fluconazole applied at 20–30 µg ml⁻¹. Noorbakhsh et al. (2011) demonstrated that AgNPs can inhibit *T. rubrum* at 10 µg ml⁻¹ as compared with 0.8 µg ml⁻¹ for griseofulvin and 40 µg ml⁻¹ for fluconazole. The variation in AgNP toxicity level found for a fungal species in this research compared with the same species investigated by other authors may be related to the difference in susceptibility pattern of the strains or may be explained by differences in the nanoparticle synthesis method and subsequent stabilization (Monteiro et al., 2011).

The effect of AgNPs, as indicated by the lower values of MIC, was greater when combined with APACP treatment. The MIC50 and MIC100 recorded were, respectively, 3 and 12 µg ml⁻¹ in the case of *E. floccosum*, and 11 and 23 µg ml⁻¹ in the case of *T. rubrum*. The application of AgNPs alone or combined with APACP was superior as an anti-dermatophytic agent compared with fluconazole as reference antifungal drug. In the literature, the

**Table 1.** MIC50 and MIC100 (µg ml⁻¹) of nanoparticles in combination with APACP jet for growth of dermatophytes in comparison with fluconazole as reference antifungal drug

<table>
<thead>
<tr>
<th>Dermatophytes</th>
<th>MIC50</th>
<th>MIC100</th>
<th>MIC50</th>
<th>MIC100</th>
<th>MIC50</th>
<th>MIC100</th>
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<tr>
<td></td>
<td>AgNPs</td>
<td>AgNPs + cold plasma</td>
<td>AgNPs</td>
<td>AgNPs + cold plasma</td>
<td>Fluconazole</td>
<td></td>
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<tr>
<td><em>E. floccosum</em></td>
<td>5</td>
<td>3</td>
<td>15</td>
<td>12</td>
<td>57</td>
<td>144</td>
</tr>
<tr>
<td><em>M. canis</em></td>
<td>9</td>
<td>5</td>
<td>21</td>
<td>13</td>
<td>53</td>
<td>152</td>
</tr>
<tr>
<td><em>M. gypseum</em></td>
<td>8</td>
<td>5</td>
<td>19</td>
<td>13</td>
<td>66</td>
<td>172</td>
</tr>
<tr>
<td><em>T. mentagrophytes</em></td>
<td>12</td>
<td>9</td>
<td>25</td>
<td>19</td>
<td>102</td>
<td>197</td>
</tr>
<tr>
<td><em>T. rubrum</em></td>
<td>16</td>
<td>11</td>
<td>32</td>
<td>23</td>
<td>113</td>
<td>224</td>
</tr>
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</table>
synergistic effects between cold plasma and nanoparticles are extremely limited. The significantly lower MIC values of AgNPs combined with APACP treatment indicate that some reactive radicals may be carried by nanoparticles and so be longer-lived (Kong et al. 2011). Moreover, the cell proliferation and cell wall cracks brought about and promoted by cold plasma probably facilitate penetration of the nanoparticles into the cytoplasm and/or cell organelles.

**Keratinase activity**

In this experiment a concentration of 10 μg ml⁻¹ AgNPs was used in order to allow fungal growth and to test for differences in keratinase activity. Under control conditions, the data in Table 2 show variation in the enzyme activity of the different species: *M. gypseum* was the most powerful keratinase producer (38.0 U mg⁻¹ dry weight), followed by *M. canis* and *E. floccosum* (18.1 and 16.5 U mg⁻¹ dry weight, respectively). *T. mentagrophytes* and *T. rubrum* showed lower keratinase activities (8.5 and 7.3 U mg⁻¹ dry weight). Treatment with 10 μg ml⁻¹ AgNPs induced variable reduction in keratinase activity, ranging from 23.3 % in the case of *T. rubrum* to 62.4 % for *M. gypseum*. A negative effect of AgNPs on the activities of exoenzymes, including urease, acid phosphatase, β-glucosidase and dehydrogenase, was reported by Shin et al. (2012). The inhibitory effect of AgNPs on keratinase is probably due to their preferential adsorption or attachment on the surface of enzyme (Fischer et al. 2002; Bayraktar et al. 2006), which induces changes in function, regulation and configuration of the enzyme (Wu et al. 2009), or may possibly limit the access of substrate to the active site of the surface-bound enzyme (Bhindar & Dadra, 2009). Moreover, it was also proposed that silver ions (particularly Ag⁺) released from AgNPs may react with sulfur-containing proteins, resulting in the inactivation of enzyme functions (Gupta & Silver, 1998; Matsumura et al., 2003).

Further reduction of keratinase activity was recorded on the application of AgNPs in combination with APACP treatment, when the reduction ranged from 48.3 % for *T. mentagrophytes* to 73.2 % for *M. gypseum*. Although few researchers have reported antioxidant potential and free-radical scavenging activities of AgNPs (Banerjee & Narendhirakannan, 2011; Konwarh et al., 2011), it seems that there is an inhibition complementarity between the AgNPs and APACP. It is hypothesized that NO and OH radicals generated from APACP probably bind to AgNPs, leading to production of reactive oxygen species (ROS) that induce oxidative damage to the cellular proteins and other components. This may result in reduction in viability of the fungal cells and consequently diminish keratinase production. The literature proves the importance of reactive particle surface in ROS generation (Vallyathan & Shi, 1997; Schins, 2002). ROS are produced from the surface of nanoparticles when both the oxidants and free radicals attach to the particle surface. It has also been suggested that there can be release of silver ions by the nanoparticles (Feng et al., 2000) and these ions can interact with the essential serine residue at or near the active site of the keratinase and inactivate the enzyme. In other cases, the charged silver ions may ultimately attach with the sensing surface of the C-terminal residue of amines in keratinase, causing its modification. It is also possible that the reactive species generated from APACP act against the extensive disulfide linkages of keratin, resulting in substrate breakdown and consequently diminishing the enzyme activity (Brandelli et al., 2010).

It has to be mentioned that all reduction percentages, even for treatment with AgNPs alone, were significantly higher than that recorded in the case of fluconazole applied at 100 μg ml⁻¹.

**Leakage of substances absorbing at 260 nm**

The loss of membrane integrity of the investigated fungi was evaluated by determining the leakage of 260 nm-absorbing materials after 30 min. SDS at 2 % was used to produce 100 % cellular leakage. The percentage cellular leakage after 30 min compared with SDS varied according to the test species, with maximum release recorded for *M. gypseum* (38.0 %) and minimum for *T. rubrum* (7.3 %)

<table>
<thead>
<tr>
<th>Dermatophytes</th>
<th>Keratinase activity [U (mg mycelial dry weight)⁻¹]</th>
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<tbody>
<tr>
<td></td>
<td>Control Activity Reduction (%)*</td>
<td>AgNPs Activity Reduction (%)*</td>
</tr>
<tr>
<td><em>E. floccosum</em></td>
<td>16.5 ± 1.5 10.8 ± 1.1 36.8</td>
<td>7.9 ± 0.8 52.1</td>
</tr>
<tr>
<td><em>M. canis</em></td>
<td>18.1 ± 2.3 10.7 ± 1.4 40.9</td>
<td>8.5 ± 0.8 53.0</td>
</tr>
<tr>
<td><em>M. gypseum</em></td>
<td>38.0 ± 4.2 14.3 ± 1.7 62.4</td>
<td>10.2 ± 1.0 73.2</td>
</tr>
<tr>
<td><em>T. mentagrophytes</em></td>
<td>8.5 ± 0.9 6.2 ± 0.8 27.1</td>
<td>4.4 ± 0.5 48.3</td>
</tr>
<tr>
<td><em>T. rubrum</em></td>
<td>7.3 ± 0.9 5.6 ± 0.6 23.3</td>
<td>2.9 ± 0.3 60.3</td>
</tr>
</tbody>
</table>

*As compared with control.
AgNPs are mostly compatible with those obtained from treatment with fluconazole as reference antifungal drug.

**Fig. 4.** Effect of 10 µg ml⁻¹ AgNPs combined with APACP jet on the percentage cellular leakage (in relation to 100 % leakage caused by SDS) after 30 min, measured at 260 nm, of dermatophytes previously grown for 12 days on Sabouraud glucose broth as compared with 100 µg ml⁻¹ fluconazole as reference antifungal drug.

(Fig. 4). Treatment with 10 µg ml⁻¹ AgNPs induced significant increase in leaked materials, reaching a maximum value of 48 % in the case of *M. gypseum*. The least leakage (21.3 %) was estimated in the case of *E. floccosum* AgNP-treated mycelium. The data for cellular leakage with 10 µg ml⁻¹ AgNPs are mostly compatible with those obtained from treatment with 100 µg ml⁻¹ fluconazole. Li et al. (2010) related the increase in cellular leakage of AgNP-treated bacteria to the formation of pits in the cell walls that facilitate the penetration of the nanoparticles into the periplasm and destroy the cell membrane. AgNPs may bind to and coagulate cytoplasmic material in damaged cells, resulting in the leakage of cytoplasmic components and the eventual death of the cells. Besides this, the moderate antifungal activity of AgNPs against *Aspergillus niger* and *Trichoderma* sp. is due to formation of insoluble compounds by inactivation of thiol groups in the fungal cell wall and disruption of the potential membrane-bound enzymes and lipids, which causes cell lysis (Fatima et al. 2015).

Furthermore, the application of AgNPs for 30 min combined with APACP treatment caused a sharp increase in cellular leakage, resulting in 92.4, 81.3, 72.7, 68.9 and 65.9 % increases in leakage in the case of *M. canis*, *M. gypseum*, *T. mentagrophytes*, *E. floccosum* and *T. rubrum*, respectively, compared with the SDS control. It is suggested that APACP acts synergistically with AgNPs, causing increase in cellular leakage. The generated reactive species, such as NO⁻, OH⁻ and N₂, generated during plasma discharge are assumed to play a crucial role in loss of the original morphological features and degradation of cell components. The degradation probably involves processes such as rupture, distortion, proliferation and shrinking of the outer layer that possibly enhance the leakage of intracellular constituents. The hydroxyl radicals generated from APACP may contribute to membrane lipid peroxidation, causing an increase in membrane permeability (Ryu et al., 2013; Mai-Prochnow et al., 2014). It is evident from several related studies that the direct permeabilization of the cell membrane or wall is one of the modes of action in the toxicity of cold plasma for microbial cells (Kvam et al., 2012; Moreau et al., 2007).

**SEM**

SEM analysis was used for assessment of structural alterations that occurred in *M. canis* spores. The SEM images revealed variation in reactions to the different treatments (Fig. 5). The cold plasma-treated spores appeared irregular, with obvious electroporation, necrotic lesions and disruptions in the walls (Fig. 5b). The 10 µg ml⁻¹ AgNP-treated spores showed the accumulation of nanoparticles on the spore surfaces causing its deformation. The spores appeared to have lost their smoothness, and unusual protuberances appeared on the surface of spores (Fig. 5c), indicating that AgNPs inhibited the growth of *M. canis* by deforming the spore structures. The use of nanoparticles combined with plasma induced distortion, complete damage and rupture of spores (Fig. 5d). The spores looked flaccid, plasmolysed and morphologically collapsed.

Our previous study (Ouf et al., 2015a, b) indicated that the difference in spore reaction of *Alternaria alternata* and *A. niger* caused by cold plasma was due to the chemical structure of the cell wall. Cold plasma treatment produced irregularities, malformation and septum damage in the spores of *A. alternata*, while electroporation was seen in the walls of *A. niger* spores. Moisan et al. (2001) indicated that, in micro-organisms subjected to intense electron or ion bombardments, spore coatings or cell wall materials are eroded and occasionally rupture, resulting in fatal outcome. Our SEM images established drastic destruction of *M. canis* spores treated with AgNPs, particularly when the nanoparticles were combined with APACP.

**Pathogenicity test**

The treatment of *M. canis*-inoculated guinea pigs with 13 µg ml⁻¹ AgNPs induced 70.8 % healing after 45 days of inoculation as compared with 79.2 % in the case of fluconazole applied at 152 µg ml⁻¹ (Table 3). More than 90 % healing was achieved on using 13 µg ml⁻¹ AgNPs combined with APACP generated at a flow rate of 3.5 SLPM for 2 min. The treatment of the inoculated guinea pigs with APACP alone induced significant healing, reaching 42.0 %, which is considerably lower than attained by AgNPs or fluconazole. By the end of the experiment, the percentage body weight gain of the guinea pigs showed no significant difference between control and the different treatment groups. Moreover, no toxic signs or
Histopathological abnormalities were observed in skin after 45 days (Fig. 6). Our results are coupled with the finding by Maneewattanapinyo et al. (2011) of safe administration of colloidal AgNPs to oral, eye and skin of the animal models for short periods of time. Moreover, Daeschlein et al. (2012) indicated that cold-plasma treatments are well-tolerated, do not disturb or damage the skin barrier or reduce skin moisture, and fulfil basic recommendations for safe use on human skin.

Considering the effect of the different treatments on the haematological values of *M. canis* inoculated guinea pigs after 45 days of inoculation, the results presented in Table 4 indicated significant decrease in haemoglobin, red blood cells, monocytes and lymphocytes, while neutrophils and eosinophils showed a marked rise. Neutrophils and eosinophils as classic characteristics of host defensive agents have been reported to accumulate at sites of cutaneous inflammation in guinea pigs experimentally infected with *Trixacarus caviae* (guinea pig mange mites) (Rothwell et al., 1991) or in response to infestation with *Amblyomma americanum* (lone star tick) (Brown & Askenase, 1982). It is believed that these cell types migrate from the inflammation site into the blood, with the potential progress of disease control initiating relatively high counts in blood. Regardless of the infection, Maneewattanapinyo et al. (2011) did not record any significant changes in haematological and blood chemistry analyses between guinea pigs treated with AgNPs and controls.

**Table 3.** Efficacy of 13 μg ml⁻¹ AgNPs, singly or combined with APACP jet generated at a flow rate of 3.5 SLPM for 2 min, in treatment of dermatomycosis due to *M. canis* inoculation of guinea pigs 45 days after inoculation as compared with fluconazole applied at 152 μg ml⁻¹

<table>
<thead>
<tr>
<th>Treatment of guinea pigs</th>
<th>Recovery (%) 45 days after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated, untreated</td>
<td>0.0</td>
</tr>
<tr>
<td>Inoculated, treated with APACP for 2 min</td>
<td>42.0 ± 3.1</td>
</tr>
<tr>
<td>Inoculated, treated with 13 μg ml⁻¹ AgNPs</td>
<td>70.8 ± 3.5</td>
</tr>
<tr>
<td>Inoculated, treated with 13 μg ml⁻¹ AgNPs combined with APACP</td>
<td>91.7 ± 4.6</td>
</tr>
<tr>
<td>Inoculated, treated with 152 μg ml⁻¹ fluconazole</td>
<td>79.2 ± 4.1</td>
</tr>
</tbody>
</table>

(Fig. 5. SEM images of *M. canis* spores treated with 10 μg ml⁻¹ AgNPs in conjunction with APACP jet generated at a flow rate of 3.5 SLPM for 2 min (d) as compared with spores solely treated with AgNPs (c) or APACP (b), and untreated spores (a). The input voltage was 2.0 kV. Scale bars, 5 μm.)
CONCLUSIONS

Over the last few years, most research that has been published on dermatophyte infections has focused on the use of azole compounds with different derivatives and formulations. However, there are several drawbacks to these drugs, including a decrease in testosterone, liver function, gastrointestinal problems and drug interactions. AgNPs have been successfully used in this study as an antidermatophytic agent against five dermatophytes. The effective applied dose for in vitro and in vivo treatment is lower than that of fluconazole as standard antifungal drug.

Table 4. Effect of 13 µg ml⁻¹ AgNPs combined with APACP jet on haematological values of M. canis-inoculated guinea pigs 45 days after inoculation as compared with 152 µg ml⁻¹ fluconazole as reference antifungal drug

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control (normal)</th>
<th>Inoculated non-treated guinea pigs</th>
<th>Inoculated and treated guinea pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>APACP</td>
</tr>
<tr>
<td>Haemoglobin (g dl⁻¹)</td>
<td>16.30 ± 3.35</td>
<td>13.80 ± 3.01</td>
<td>15.30 ± 3.39</td>
</tr>
<tr>
<td>RBCs (×10⁶ µl⁻¹)</td>
<td>4.54 ± 1.15</td>
<td>3.41 ± 0.54</td>
<td>4.22 ± 1.05</td>
</tr>
<tr>
<td>Leukocytes (×10⁹ µl⁻¹)</td>
<td>5.32 ± 1.15</td>
<td>13.47 ± 2.84</td>
<td>5.85 ± 1.00</td>
</tr>
<tr>
<td>Neutrophils (×10³ µl⁻¹)</td>
<td>0.58 ± 0.17</td>
<td>0.74 ± 0.21</td>
<td>0.62 ± 0.20</td>
</tr>
<tr>
<td>Eosinophils (×10³ µl⁻¹)</td>
<td>0.44 ± 0.12</td>
<td>0.71 ± 0.16</td>
<td>0.49 ± 0.17</td>
</tr>
<tr>
<td>Basophils (×10³ µl⁻¹)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Monocytes (×10⁶ µl⁻¹)</td>
<td>5.56 ± 1.22</td>
<td>4.05 ± 1.17</td>
<td>5.07 ± 1.35</td>
</tr>
<tr>
<td>Lymphocytes (×10⁹ µl⁻¹)</td>
<td>4.20 ± 0.79</td>
<td>1.39 ± 0.10</td>
<td>4.04 ± 0.64</td>
</tr>
</tbody>
</table>

RBC, red blood cell.
The use of AgNPs enhanced by cold plasma has potent activity and promises to be an alternative to the classic medicines used for treatment of skin diseases due to dermatophytes. Additionally, AgNPs in conjunction with cold plasma have advantages over conventional azole compounds, currently used for treatment, as they have a good safety profile and their adverse effects are limited, particularly when used topically to treat superficial mycotic infections. The clinical success of AgNPs will open the way to a new generation of wide-ranging Ag-containing therapeutic products for controlling and preventing further outbreak of diseases.

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