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Reactive oxygen species-independent apoptotic pathway by gold nanoparticles in *Candida albicans*

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ABSTRACT

Candida albicans is the most common pathogenic fungus in humans, causing cutaneous and life-threatening systemic infections. In this study, we confirmed using propidium iodide influx that gold nanoparticles (AuNPs), which are promising materials for use as antimicrobial agents, did not affect the membrane permeability of *C. albicans*. Thus, the fungal cell death mechanisms induced by AuNPs were assessed at intracellular levels including DNA damage, mitochondrial dysfunction, and reactive oxygen species (ROS) overproduction. AuNPs interacted with *C. albicans* DNA leading to increased nuclear condensation and DNA fragmentation. Changes in the mitochondria induced by AuNPs involving mass, Ca^{2+} concentrations, and membrane potential indicated dysfunction, though the level of intracellular and mitochondrial ROS were maintained. Although ROS signaling was not disrupted, DNA damage and mitochondrial dysfunction triggered the release of mitochondrial cytochrome *c* into the cytosol, metacaspase activation, and phosphatidylserine externalization. Additionally, the AuNPs-induced apoptotic pathway was not influenced by *N*-acetylcysteine, an ROS scavenger. This indicates that ROS signaling is not linked with the apoptosis. In conclusion, AuNPs induce ROS-independent apoptosis in *C. albicans* by causing DNA damage and mitochondria dysfunction.

1. Introduction

The common pathogenic fungi, *Candida albicans* resides as a commensal organism in the mucocutaneous cavities of the skin, vagina and intestine of humans. It can cause infections under certain pathological and physiological conditions such as infancy, diabetes, pregnancy, steroidal chemotherapy, and prolonged broad spectrum antibiotic administration as well as acquired immunodeficiency syndrome (AIDS) (Manohar et al., 2001). Resistance to conventional drugs is rapidly emerging, and the decreased activity of these drugs against *C. albicans* has been observed on some level for every currently used drug class (Sanglard, 2017). Thus, development of novel and effective antifungal agents against *C. albicans* has gained major interest.

Nanoparticles, defined as being between 10 and 100 nanometers in size, are promising materials owing to their wide variety of potential biological, biomedical, catalytic, optoelectronic, and pharmaceutical applications (Mohanraj and Chen, 2006). Previous studies have shown that nanoparticles can act as antimicrobial agents because of their ability to interact with microorganisms (Albanese et al., 2012). Because of these characteristics, various metal nanoparticles have been studied to determine their unique antimicrobial properties and their potential usage in a wide range of applications such as medical instruments, textiles, and purifications (Sondi and Salopek-Sondi, 2004; Espitia et al., 2012).

Gold nanoparticles (AuNPs) are a well-studied material and their unique chemical and physical properties make them promising therapeutic agents without inherently toxic to human cells (Wang et al., 2008; Alkilany et al., 2012; Conde et al., 2014). AuNPs have been widely used in cancer treatments as a drug delivery system and thermal therapy (Huang et al., 2008; Brown et al., 2010). Furthermore, in previous studies, AuNPs showed that antimicrobial activity against various pathogens including *Escherichia coli*, *Streptococcus mutans*, and *Candida* species (Hernandez-Sierra et al., 2008; Lima et al., 2013; Wani et al., 2013). However, while their antimicrobial activity has been the focus of numerous studies, and there has yet been no study demonstrating their mechanism against *C. albicans*. Therefore, in this context, this study aims to confirm the mode of fungicidal action of AuNPs on *C. albicans*.

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Table 1
The antifungal effect of AuNPs and H_2O_2.

Fungal strains	MIC (μg/ml)	
	AuNPs	H_2O_2
C. albicans ATCC 90028	32	20

2. Materials and methods

2.1. Nanoparticle preparation and antifungal activity of AuNPs

C. albicans (ATCC 90028) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in aerated YPD (yeast extract-peptone-dextrose) broth (BD Bioscience, Franklin Lakes, NJ, USA) at 28 °C. Gold nanoparticles (AuNPs) were purchased from Sigma-Aldrich. The particles were 30 nm in diameter and a stabilized suspension in phosphate-buffered saline (PBS). H_2O_2 (Sigma-Aldrich) was used as a positive control for comparisons of the physiological responses caused by AuNPs.

C. albicans cells were inoculated into YPD and then dispensed into microtiter plates. The minimum inhibitory concentration (MIC) was determined by a standard microdilution method. After incubation with AuNPs for 12 h, the growth was measured using a microtiter ELISA Reader (Molecular Devices Emax, CA; Table 1).

2.2. Membrane permeabilization detection

Membrane permeabilization was detected using propidium iodide (PI). The cells were washed with PBS (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) and incubated with 32 μg/mL AuNPs for 4 h at 28 °C. The fluorescent intensity was measured using a FACSverse flow cytometer (BD Biosciences) (Lecoeur et al., 2001).

2.3. DNA condensation assay

A DNA-specific fluorescent dye, 4′-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used to analyze nuclear condensation. *C. albicans* cells were washed with PBS buffer and incubated with AuNPs for 4 h at 28 °C. After incubation, the cells were resuspended in PBS and treated with DAPI for 20 min. Next, the cells were washed, and the fluorescence was analyzed using a spectrofluorophotometer (Shimadzu RF-5301PC; Shimadzu, Kyoto, Japan) and visualized using a fluorescence microscope (Nikon Eclipse Ti-S; Nikon, Japan) (Madeo et al., 1997).

2.4. DNA fragmentation assay

For the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, a method for assessing DNA damage, the cells were washed with PBS and incubated with AuNPs for 4 h at 28 °C. Next, the cells were fixed with 2% paraformaldehyde for 1 h and incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) on ice for 2 min. Then, the cells were incubated with the TUNEL reaction mixture from an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) for 1 h at 28 °C. Finally, the cells were washed, and the fluorescence was assayed using a spectrofluorophotometer (Madeo et al., 1997; Valipour, 2012).

2.5. Interactions of AuNPs with C. albicans DNA

To investigate interactions of DNA with AuNPs, fluorescence measurements were evaluated by the process described by Atay et al. (2009) and Sung and Lee (2007), with slight modification. we prepared genomic DNA that was extracted from *C. albicans* cells (Hanna and Xiao, 2006). Fluorescence changes in the AuNPs after the addition of genomic DNA were recorded using a RF-5301PC spectrofluorometer. An excitation wavelength of 527 nm (approximate absorption maximum) was used for the AuNPs. The emission spectra were recorded in the wavelength range from 460 nm to 600 nm. The fluorescence spectra of AuNPs were determined at room temperature in the presence of 1 μg of genomic DNA (An and Jin, 2012).

2.6. Mitochondrial mass detection

To evaluate mitochondrial mass, Mitotracker Green FM fluorescent probe (Invitrogen, Carlsbad, CA) was used. Harvested *C. albicans* cells were washed with PBS and incubated with AuNPs for 4 h at 28 °C. Then, AuNP-treated cells were resuspended in PBS and incubated with 100 nM Mitotracker Green FM for 45 min. Next, the cells were washed twice, and the fluorescence was measured using a spectrofluorophotometer (Blanquer-Rossello et al., 2017).

2.7. Mitochondrial Ca^{2+} concentration detection

To assess mitochondrial Ca^{2+} levels, a Rhod-2AM (Molecular Probes) assay was used. Harvested *C. albicans* cells were washed in PBS. Next, the cells were incubated with AuNPs or H_2O_2 for 4 h at 28 °C. After incubation, the cells were centrifuged twice at 12,000 rpm (Sorvall Biofuge Fresco) for 5 min with Krebs buffer (pH 7.4; 4 mM KCl, 132 mM NaCl, 1.4 mM $MgCl_2$, 10 mM HEPES, 6 mM glucose, 10 mM $NaHCO_3$, and 1 mM $CaCl_2$) and treated with 1% bovine serum albumin and 0.01% pluronic acid F-127. The cells were then incubated with a Ca^{2+}-sensitive fluorescent dyes (Rhod-2AM; Molecular Probes) for 30 min and washed three times with Ca^{2+}-free Krebs buffer. The fluorescence intensity of Rhod-2AM was examined using a spectrofluorophotometer (excitation/emission = 552 nm/581 nm) (Shimadzu RF-5301PC, Shimadzu) (Valipour et al., 2015).

2.8. Mitochondrial membrane potential detection

The collapse of the electrochemical gradient across the mitochondrial membrane was measured using a JC-1 dye (Molecular Probes). The fungal cells were washed with PBS and the cells were then incubated with AuNPs for 4 h at 28 °C. After centrifugation, the cells were resuspended in warm PBS and incubated with JC-1 for 10 min. The fluorescent intensity of the JC-1 monomer and aggregate (FL1 and FL2, green and red fluorescence, respectively) was measured using a FACSverse flow cytometer (Alonso-Monge et al., 2009).

2.9. Intracellular ROS formation assay

To detect intracellular ROS levels, an oxidation-sensitive fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) was used. The cells were washed with PBS buffer and incubated with AuNPs for 4 h at 28 °C. After centrifugation, the cells were incubated with H_2DCFDA for 1 h in the dark and washed twice. The fluorescent intensity was measured using a FACSverse flow cytometer (Echave et al., 2003).

Mitochondrial ROS were measured using MitoSOX Red (Molecular Probes). *C. albicans* cells were incubated with AuNPs for 4 h at 28 °C and centrifuged at 12,000 rpm (Sorvall Biofuge Fresco). The cells were suspended in 5 μM MitoSOX Red reagent at 28 °C for 30 min and then the stained cells were washed with PBS. The fluorescent cells were analyzed with a FACSverse flow cytometer (Bankapalli et al., 2015).

2.10. Detection of cytochrome c concentration

To confirm the effect of AuNPs on cytochrome *c* release, the levels of cytochrome *c* in the cytosol and mitochondria were measured as