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ORIGINAL RESEARCH

Gold Nanoclusters as an Antibacterial Alternative Against *Clostridium difficile*

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Background: *Clostridium difficile* infection (CDI) has become one of the most important factors threatening human health, and about 20–30% antibiotic-related diarrhea cases and almost all pseudomembranous enteritis cases are related to CDI. The high recurrence of *Clostridium difficile (C. difficile)* and the emergence of drug resistance make clinical treatment of CDI difficult. Therefore, there is an urgent need to develop a non-antibiotic-alternative therapy against CDI. Gold nanoclusters (AuNCs) can better interact with bacteria due to its ultrasmall size. The aim of the present study was to explore whether AuNCs could be used as an antibacterial agent to kill *C. difficile*.

Methods: AuNCs and *C. difficile* were co-cultivated in an anaerobic atmosphere to evaluate the bactericidal effect of AuNCs. The bacterial growth rate was estimated by using two concentrations of AuNCs (50 and 100 μM). The damage of AuNCs to *C. difficile* is detected by SYTOX Green staining methods and SEM image analysis. The mechanism of AuNCs on *C. difficile* was explored by reactive oxygen species (ROS) detection. The toxic effect of AuNCs on human cells was evaluated by MTT method.

Results: AuNCs (100 μM) killed *C. difficile* drastically. AuNCs increased the release of ROS by about 5 fold and destroyed the membrane integrity of *C. difficile* cells without causing significant toxic effect on human cells.

Conclusion: AuNCs showed great potential as an alternative to traditional antibiotics in killing *C. difficile* and may prove to be an alternative to treat CDI.

Keywords: *Clostridium difficile*, antibacterial effect, gold nanoclusters, reactive oxygen species

Introduction

C. difficile is a toxin-producing Gram-positive anaerobic spore-forming bacterium. It was discovered in 1978 that *C. difficile* was associated with bacterial-associated diarrhea. Since then, more CDI have been identified, revealing that about 25% antibiotic related diarrhea cases, 75% antibiotic-associated enteritis cases and nearly 100% pseudomembranous enteritis cases are caused by this bacterium.[1] *C. difficile* is also one of the most common causes of diarrhea infections in hospitalized patients in developed countries. In the United States, *C. difficile* has surpassed Methicillin-resistant Staphylococcus aureus as the first pathogen of hospital-acquired infections.[2,3] The current status of CDI in China is not optimistic, with an overall incidence of 3.4–36.9 per 10,000 hospitalized patients, which is significantly higher than 7.4 per 10,000 hospitalized patients released by the US Centers for Disease Control and Prevention (CDC).[4,5]

Prolonged administration of antibiotics, chemotherapeutic drugs or proton pump inhibitors would destroy the normal intestinal flora, when the body is more susceptible

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to CDI. Once established in the intestine, *C. difficile* can induce cytopathic and cytotoxic effects, causing *C. difficile* infection (CDI) and *C. difficile*-associated diarrhea (CDAD).[6] Metronidazole and vancomycin are two main drugs for the treatment of CDI at present. However, simultaneous administration of the two drugs is likely to cause intestinal disorders resulting in a relatively high recurrence rate of cured CDI due to the broad antibacterial spectrum.[7] Subsequent studies have shown that fidaxomicin can reduce the recurrence of CDI, but the drug is expensive and more preferable for patients with severe CDI recurrence.[8,9] It is therefore an urgent task to seek non-antibiotic methods for the treatment of CDI. Current non-antibiotic methods include antibody vaccines against virulence factors, fecal microbiota transplantation, and probiotics.[8] However, multiple factors such as reasonable safety and regulatory issues have hindered their clinical applications,[10,11] and a safer and more reliable non-antibiotic treatment is required.

Gold nanoclusters (AuNCs) have broad application prospects in biomedicine and have gained rapid development in recent years for the treatment of various cancers and microbial infections.[12,13] In terms of cancer, AuNCs are a promising carrier for combined therapy with anticancer stem cells, and have bright prospects in the treatment of lung cancer and pancreatic cancer.[14–16] Especially in terms of microbial infection, whether it is small or large molecule conjugated AuNCs, it seems that they can show antibacterial effects. It can effectively kill Gram-positive bacteria, Staphylococcus epidermidis and Bacillus subtilis, as well as Gram-negative bacteria, Escherichia coli and Pseudomonas aeruginosa.[17,18] For example, small molecule-conjugated AuNCs have a high specific surface area and high surface chemical activity. They can interact with bacterial membrane surface proteins after modification to affect the function of the bacterial membrane and destroy the integrity of the membrane.[19] When AuNCs enter cells, they can interfere with DNA transcription and replication, and cause aggregation of reactive oxygen species (ROS) by interfering with the metabolism of bacteria, thereby killing them.[17,20] More importantly, AuNCs are metal inert, stable in chemical properties, low in cytotoxicity, and have good biocompatibility.[21,22] Most importantly, studies have proven that AuNCs are difficult to cause bacterial resistance.[23] However, the antibacterial effect of AuNCs on *C. difficile* is not clear.

CDI poses a considerable threat to public health, especially when it develops resistance to antibiotics. Here, our study explored whether AuNCs could exert a bactericidal effect against *C. difficile* for the first time. In addition, we clarified the possible mechanism that drives the antimicrobial activity of AuNCs against *C. difficile*. In sum, AuNCs may provide an alternative method for the treatment of CDI.

Materials and Methods

Synthesis and Characterization of AuNCs

AuNCs with the 6-mercaptohexanoic acid (MHA) ligands were prepared according to a reported method.[14,20] In brief, $HAuCl_4$ (20 mM, 0.25 mL) and MHA (10 mM, 1 mL) were mixed with 3.35 mL water to form an Au-MHA complex, which was then dissolved by addition of NaOH solution (1 M, 0.3 mL). A freshly prepared $NaBH_4$ solution (112 mM, 0.1 mL) was added into the Au-MHA complex. AuNCs were collected after 3-h reaction, and then purified by Ultrafiltration in a Stirred Cell (model 8010, Millipore Corporation, USA) with a semipermeable membrane of 3 kDa molecular weight cutoff. The size and quality of the AuNCs were characterized by transmission electron microscopy (TEM) (JEM-2100, Japan), dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS90, UK) and an ultraviolet-visible (UV-Vis) spectrometer (Shimadzu, UV-3600, Japan).

Strains and Growth Culture

The *C. difficile* strains used in this study were American Type Culture Collection (ATCC) 43,255 and BNC 186,155, which were stored at −80 °C and those were resuscitated before use. The strains were inoculated into Brain Heart Infusion Broth (BHI) solid medium containing 3.7 g/mL BHI, 0.05 g/mL L-cysteine, 0.015 g/mL agar and deionized water, incubated at 37 °C for 48 h in an anaerobic environment, and diluted with sterile water into a bacterial suspension. After multiple dilutions, the plate was separated.

Antimicrobial Activity of AuNCs

For broth assays, bacteria were incubated for 48 h in BHI in an anaerobic environment. To determine whether AuNCs could inhibit bacterial growth, they were weighed out into bacterial culture tubes at concentrations of 0, 50, and 100 μM, and incubated for 30 min and 60 min in the anaerobic environment. The bacterial suspension was incubated, centrifuged, re-suspended, diluted, coated on the BHI solid plates, and then incubated in an anaerobic environment at 37 °C for 48 h. Finally, the number of

C. difficile cells on the plate was counted to quantify the antibacterial ability of AuNCs.

Detection of ROS Release

C. difficile was diluted at 1:50 and co-cultured with AuNCs at 0 and 100 μM for 30 min in 96-well plates, 100 μL per well, each with 3 replicate wells. After addition of DCFH-DA to make the final concentration of 40 μg/mL and 2.5 μL mother liquor, cells were incubated in the dark for 30 min and centrifuged to obtain the supernatant (20 μL), which was washed twice with PBS and re-suspended with 350 μL PBS. After fully removing the DCFH-DA that had not entered the cells, intracellular ROS was detected quantitatively by the fluorescence microplate reader at excitation wavelength of 485 nm and emission wavelength of 528 nm.

Bacteria Characterization with SEM

The effect of AuNCs on bacterial cell integrity was detected and the action mode of AuNCs was clarified by scanning electron microscopy (SEM). Briefly, 100 μM AuNCs-treated culture was fixed. Untreated *C. difficile* was placed at 4 °C for 12 h, washed with 0.1% phosphate buffer solution for 10–15 x 3, immersed in 1–2% osmium tetroxide for 1 h, rinsed with distilled water for 5–10 x 3, dehydrated with 70% ethanol/80% ethanol/90% ethanol/anhydrous ethanol/anhydrous ethanol for 12–15 min (2 times, isoamyl acetate, 15 min each time), dried, mounted, observed and photographed under a SEM (Hitachi-S4800, Japan).

Detection of Cell Viability by MTT Method

The inhibitory effect of AuNCs on the growth of human umbilical vein endothelial cells (EA. hy926, ATCC CRL-2922) and human colon cancer cell (Caco2, ATCC HTB-37) was measured by MTT colorimetry. Briefly, cells to be tested were seeded to a 96-well plate at a density of 100 mL/well and 5000 cells/well, each with 5 duplicate wells. After cell confluence, AuNCs in 0, 50 and 100 μM concentration groups were added and incubated for 24 h. Finally, 10 mL MTT solution (10%) was added to each well. After 4-h incubation, the supernatant was aspirated from the wells, and 150 mL DMSO was added to each well to dissolve the precipitate. After shaking at a low speed, the optical density (OD) was measured at 490 nm, and cell viability was calculated by comparing the different concentration gradients.

Immunofluorescence Analysis of of Dead Bacteria

C. difficile were incubated for 48 h in BHI in an anaerobic environment. To determine whether AuNCs could kill *C. difficile*, 1 x 10^6 C. difficile and 100 μM AuNCs co-culture for 60 min in the anaerobic environment. Subsequently, add 2 drops SYTOX Green Ready Flow Reagent in 1 mL C. difficile, incubate 15 min at 25 °C, and proceed with Immunofluorescence microscopy (Olympus, Tokyo, Japan). SYTOX Green enters the cell upon loss of membrane integrity and binds to DNA, thereby acting as a counterstain that can be analyzed when excited at 488 nm and the emission captured at a peak of 523 nm.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data are expressed as the mean±SD. Comparisons between two groups were assessed using a Student's *t*-test or Mann–Whitney test depending on whether the data were normally distributed. Statistical significance between multiple groups was tested using a one-way multiple analysis of variance (ANOVA) or Kruskal–Wallis. The level of statistical significance was set at $p < 0.05$.

Results

Characterization of AuNCs

AuNCs were synthesized by $NaBH_4$ reduction method.[14,19] The TEM images showed that AuNCs were spherical and well monodispersed (Figure 1A). The core size below 2 nm was observed by TEM. DLS showed that the mean hydrodynamic size was about 9 nm due to the presence of MHA surrounding the gold core (Figure 1B). The UV-vis absorption spectrum of AuNCs showed four distinct absorption peaks at 440, 550, 670 and 760 nm (Figure 1C), which corresponded to the characteristic absorption of $Au_{25}(MHA)_{18}$ according to previous reports.[24,25]

Antibacterial Activity of AuNCs

To evaluate the antibacterial activity of AuNCs, quantitative analysis was performed on bacterial viability using colony-forming unit (CFU) method. The antibacterial activity of different concentrations of AuNCs against

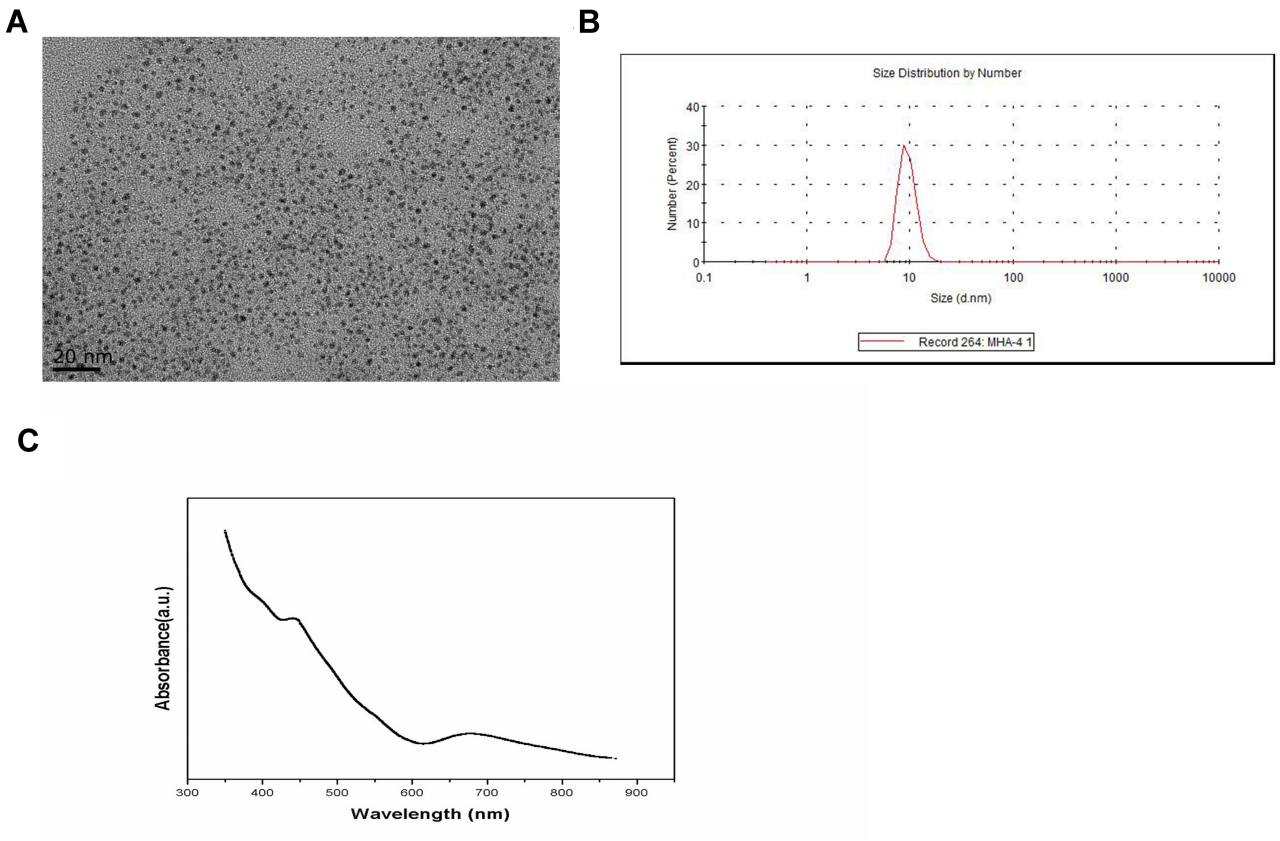

Figure 1 Characterization of AuNCs. (**A**) TEM image of AuNCs, (**B**) Dynamic light scattering diagram of AuNCs, (**C**) UV-vis absorption spectrum of AuNCs.

C. difficile at 30 min and 60 min is shown in Figure 2. The growth curve showed that the inhibitory effect of AuNCs on bacterial growth was almost 100% at the dose of 100 μM for 30 and 60-min exposure. At the same time, the growth of *C. difficile* was also significantly inhibited by 50 μM AuNCs after 60-min exposure (Figure 2B).

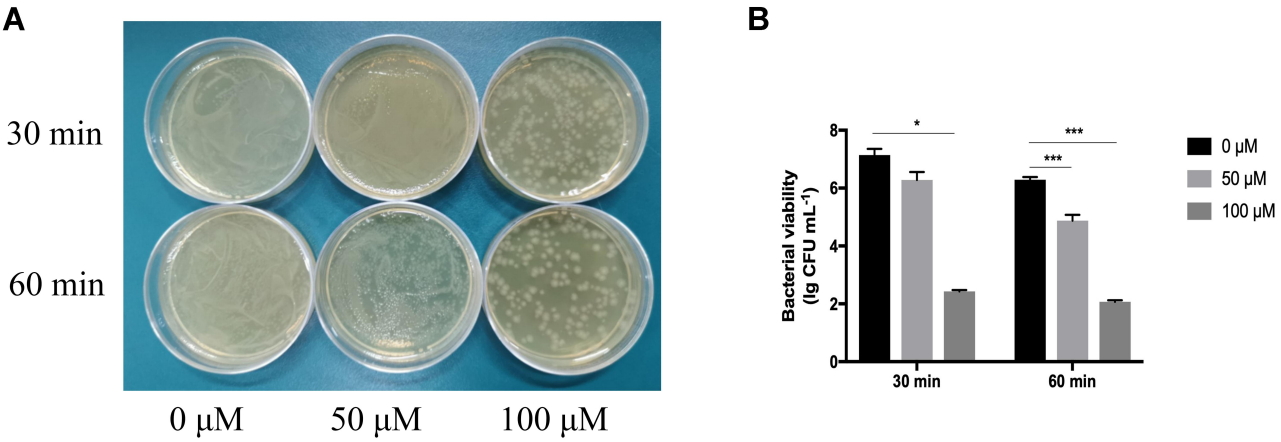

Figure 2 Antimicrobial activity of AuNCs against *C. difficile*. Antibacterial activity of AuNCs against *C. difficile* at different concentrations (0, 50, 100 μM), exposure time (30 and 60 min). (**A**) The Plates represent plate counting method to describe the colony-forming unit (CFU), (**B**) Cell viability of *C. difficile*. Data are means ± SD, n = 5, one -way Anova test, *$p < 0.05$ and ***$p < 0.001$.

The Killing Efficiency of AuNCs in *C. difficile*

We observed the live and dead *C. difficile* cells after treatment by 100 μM AuNCs under Immunofluorescence microscope. As shown in Figure 3, the fluorescence images suggest that a majority of the *C. difficile* population were killed by AuNCs (stained with SYTOX green), whereas those in the untreated control group were viable. We also noted more than 80% of the *C. difficile* population were killed following the AuNCs treatment with a dose of 100 μM.

The Antibacterial Action Mode of AuNCs

The morphological change of the bacterium represents the antibacterial action mode of either extra- or intracellular AuNC particles. As shown in Figure 4, the integrity and structure of *C. difficile* treated with 100 μM AuNCs were destroyed. The internalization is necessary for AuNCs to confer their antibacterial properties by inducing metabolic imbalance, increasing ROS release, down-regulating genes related to the surface membrane structure, and eventually causing damage to the bacterial membrane and loss of integrity,[17] which indirectly suggests the intracellular action mechanism of AuNCs.

The Effect of AuNCs on ROS Release

Generally speaking, ROS is the result of cell metabolism. If ROS exceeds its own tolerance level and oxygen stress in the environment is generated, bacterial proliferation will

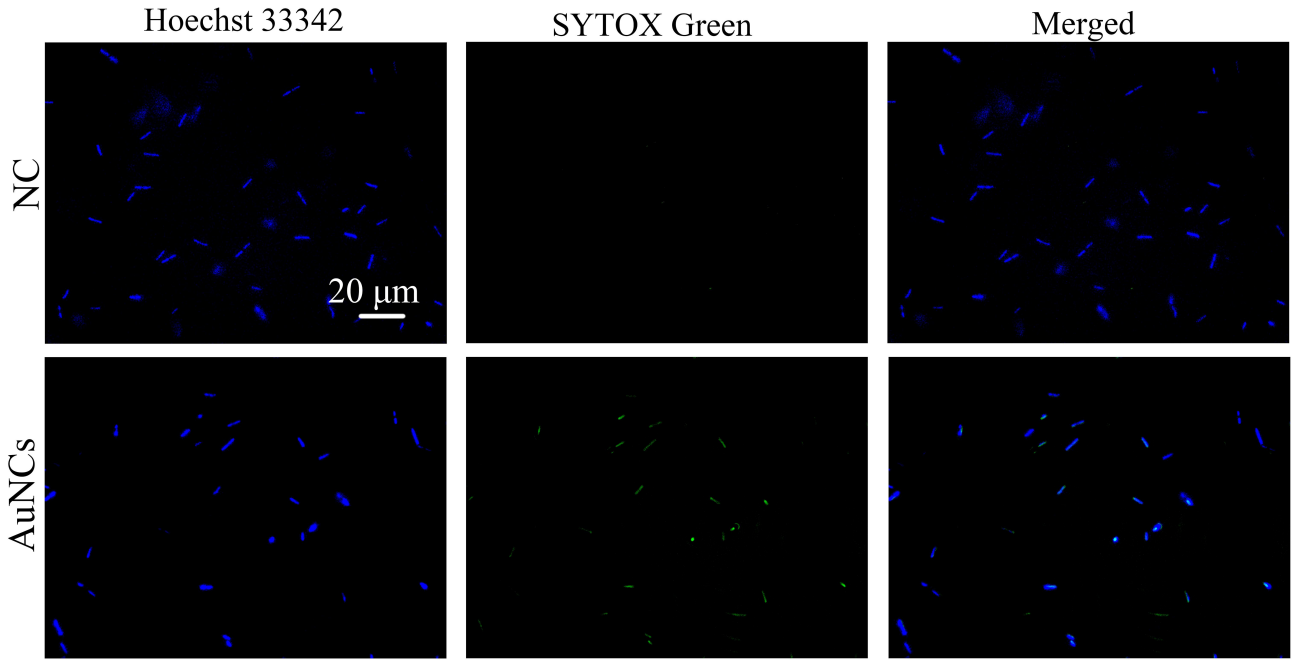

Figure 3 AuNCs showed high killing efficiency to *C. difficile*. Representative fluorescence images of the *C. difficile* after 60 min treatment. The dead cells were visualized by SYTOX green (false color: green), whereas the Hoechst 33,342 (blue) helped to identify all cells. Scale bar is 20 μm.

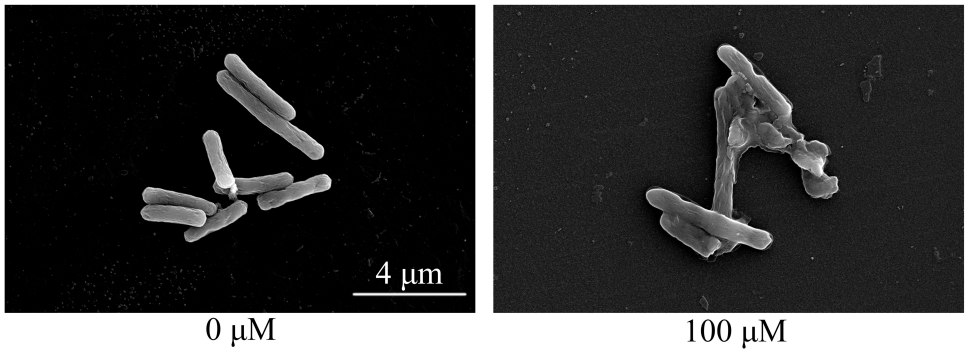

Figure 4 SEM images of the effect of AuNCs on *C. difficile*. Changes in bacterial cell membrane morphology under 0 μM and 100 μM AuNCs. Scale bar is 4 μm.

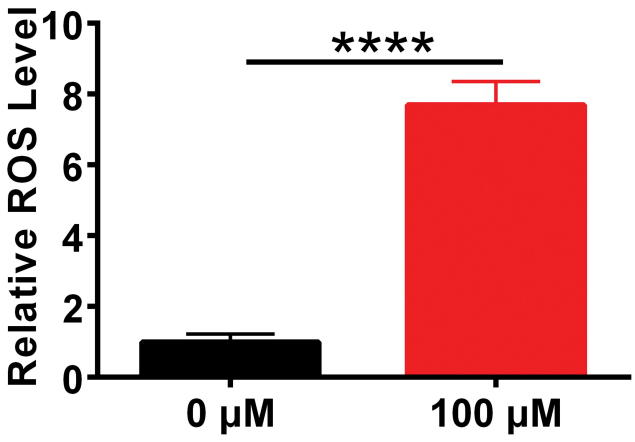

Figure 5 Changes in the release of ROS in *C. difficile* under the action of 0 and 100 μM AuNCs. Data are means ± SD, n = 6, Student 's *t*-test, ****$p < 0.0001$.

be significantly slowed down. Excess ROS will cause damage to DNA, RNA, lipids and proteins.[26]

Many antimicrobial compounds owe their capability to modulate the ROS production and eventually kill the bacterial cells.[17] As such, we investigated whether the antibacterial effect of AuNCs was also mediated by ROS. As shown in Figure 5, AuNCs induced about a 5-fold increase in the intracellular ROS production compared with the control group, indicating that when AuNCs interacted with bacteria, the active surface could induce metabolic imbalance, thus increasing the release of ROS and killing bacteria.[17] Overall, our results suggested that the intracellular ROS was the main factor that determined the antimicrobial property of AuNCs.

The Effect of AuNCs on Cell Viability

Any antibacterial agent may have certain drawbacks, but it must be safe for humans. Therefore, we studied the effect of AuNCs on human cells viability. Gold is inert, highly stable and not easily decomposed into ions.[21] Studies have shown that AuNCs are highly biocompatible in mammalian systems no matter in vitro or in vivo.[27] Knowing that the measurement of cell activity is the most direct measurement of cytotoxicity, we co-cultured different concentrations of AuNCs with human umbilical vein endothelial cells and human colon cancer cells, finding that the cell viability was not significantly affected (Figure 6), which demonstrated that AuNCs had no toxic and adverse effects on human cells.

Discussion

In this study, we used the NaBH4 reduction method to synthesize AuNCs as previously described,[17,24] knowing that this is a simple, fast and one-step reaction method to obtain AuNCs with high SERS activity and high stability without heating. DLS measurement showed that the mean size of the synthesized AuNCs is about 9 nm. TEM images showed good dispersibility and no aggregation, with the core size below 2 nm.

With the trend of longevity[28] and antibiotic abuse, CDI outbreaks have been frequently reported all over the world. However, the current medical methods cannot cure CDI. This study represents one of the first efforts to have discovered that AuNCs have a strong antibacterial activity against *C. difficile*, although there are reports that AuNCs can kill both Gram-positive and Gram-negative bacteria.[17] Besides, studies have shown that AuNCs can down-regulate membrane-related genes and destroy bacterial membranes,[17] so we explored changes in *C. difficile* cell membranes, knowing that the morphological changes of *C. difficile* will show the action mode of AuNCs. We used SEM to image and analyze *C. difficile* and found that the cell membrane of *C. difficile* was destroyed and lost its integrity after AuNCs treatment, while the cell membrane of *C. difficile* without AuNCs treatment remained intact, demonstrating that AuNCs had an effect on the membrane of *C. difficile*. Our SYTOX data further supports that AuNCs can kill *C. difficile*, which directly depends on the integrity of the membrane.

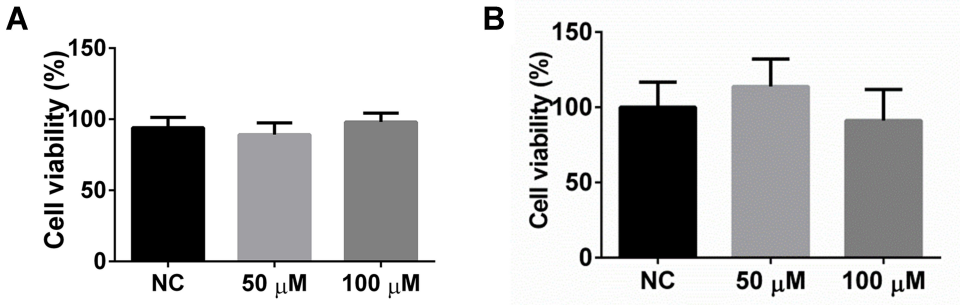

Figure 6 Comparison of cell viability of Caco-2 (**A**) and EA. hy926 (**B**) under 0, 50 and 100 μM AuNCs. Data are means ± SD, n = 5, one -way Anova test.

Then, we investigated the mechanism of AuNCs in killing *C. difficile*. Knowing that contacts between AuNCs and bacteria will produce two modes of action involving the cell membrane and intracellular mode,[29] we first explored the intracellular role of AuNCs by interacting them with bacteria. The result showed that AuNCs could interfere with the expression of metabolic genes and promote the sudden rise of ROS. Knowing that large amounts of ROS will cause intracellular protein aggregation and damage to DNA and lipids, thereby killing bacteria,[29,30] we detected the release of ROS in *C. difficile* after treatment with AuNCs, and found that ROS release increased by about 5-fold as compared with the negative control, indicating that the antibacterial effect of AuNCs on *C. difficile* was also mediated by ROS. Despite the antibacterial activity of AuNCs against *C. difficile*, whether they could be used as an antibacterial agent instead of conventional antibiotics without producing cytotoxic effects on our body remained to be defined. Hence, we explored the cell viability of human umbilical vein endothelial cells and human colon cancer cells in the presence of AuNCs. The result showed that the cell viability of these cell lines did not undergo significant changes after AuNCs treatment. Other experiments also proved that AuNCs had no significant toxic and adverse effects on humans and had a high degree of biocompatibility.[17,27] Therefore, AuNCs have the potential to kill *C. difficile* as an antibacterial agent.

Fecal microbiota transplantation may be a promising non-antibiotic treatment for CDI. However, it also has some limitations. Firstly, the appropriate donor of fecal bacteria transplantation is difficult to prepare for. Secondly, the safety of the fecal bacteria transplantation still needs further investigation, it is possible to be infected with other pathogens or adverse reactions after fecal bacteria transplantation.[31] Compared with the fecal bacteria transplantation, AuNCs are easy to produce largely and non-cytotoxicity on human cells. In addition, the treatment of AuNCs is easy to accept for patients and does not involve ethical issues.

The effect of AuNCs on CDI in vivo is still unclear. But the future development of AuNCs is beyond doubt. Its special physiochemical properties will attract us to continue to explore their clinical significance in CDI, and eventually can open up a new and safer path in the treatment of CDI.

Conclusion

CDI has become a global public health problem. On the one hand, traditional antibiotics are increasingly resistant to *C. difficile*, and on the other hand they can easily damage the intestinal flora and cause disease recurrence. The emergence of antibiotic resistance of *C. difficile* makes clinical treatment difficult. Our study demonstrated that AuNCs could be used as an alternative to conventional antibiotics to kill *C. difficile*. AuNCs confer their antimicrobial activity against *C. difficile* probably by destroying the integrity of cell membranes and promoting ROS release. Together, AuNCs showed no toxicity on human cells so as to provide an alternative method for the treatment of CDI.

Abbreviations

CDI, *Clostridium difficile* infection; *C. difficile, Clostridium difficile*; ROS, reactive oxygen species; AuNCs, gold nanoclusters; BHI, brain heart infusion; MHA, mercaptohexanoic acid; TEM, transmission electron microscopy; DLS, dynamic light scattering; UV-vis, ultraviolet-visible; ATCC, American Type Culture Collection; DCFH-DA, 2′,7′-dichlorofluorescein yellow diacetate; DCFH, dichlorodihydrofluorescein diacetate; DCF, 7′-dichlorofluorescein; SEM, scanning electron microscope; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCM 460, human normal colon cells.

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Disclosure

The authors report no conflicts of interest for this work.

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