Gold Nanoclusters Exert Bactericidal Activity and Enhance Phagocytosis of Macrophage Mediated Killing of Fusobacterium nucleatum

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Fusobacterium nucleatum is a typical periodontal opportunistic pathogen that contributes to inflammatory diseases at oral or extra-oral sites. The emergence of antibiotics resistance calls for a substitute therapeutic strategy to control bacterial infections effectively. In addition, macrophage is an essential part of host defense against microorganism invasion in vivo. Herein, we synthesized gold nanoclusters (AuNCs) as potent antibacterial material to kill F. nucleatum through bactericidal behavior and pro-phagocytic activity. Firstly, we evaluated the bactericidal effect of AuNCs on F. nucleatum by the means of plate counting, growth curve measurement, bacterial viability analysis, and live/dead fluorescent imaging. Morphological changes and the intracellular reactive oxygen species levels of bacteria were further analyzed to explore the possible mechanisms. Additionally, the impact of AuNCs on the ability of macrophages to uptake bacteria under normal and inflammatory circumstance was also evaluated by fluorescent imaging. We found that AuNCs exhibited superior antibacterial performance on F. nucleatum in vitro by a combination of membrane disruption and oxidative stress. Furthermore, in the presence of AuNCs, macrophages displayed enhanced phagocytosis against F. nucleatum without inducing any significant cytotoxicity. Therefore, AuNCs are providing a new platform for the prevention and treatment of F. nucleatum-associated diseases.

Keywords: gold nanoclusters, Fusobacterium nucleatum, antibacterial activity, reactive oxygen species, phagocytosis

INTRODUCTION

Fusobacterium nucleatum, a Gram-negative anaerobic microorganism, is frequently detected in the oral microbiota of healthy or diseased humans (Brennan and Garrett, 2019). An abnormal quantity of F. nucleatum in oral cavities has long been known to cause dysbiotic microbial communities and immune response disorder, subsequently inducing infectious diseases (Hajishengallis and Lamont, 2012; Hajishengallis, 2015). In the formation of subgingival plaque, F. nucleatum functions as an important bridging microorganism that connects primary colonizers with secondary colonizers to mediate periodontal health and disease (Kolenbrander et al., 2010). Moreover, the potential relationship of F. nucleatum with systemic conditions, such as adverse pregnancy outcomes, acute appendicitis, brain abscesses, osteomyelitis, pericarditis, and gastrointestinal disease has been widely reported (Brennan and Garrett, 2019). Specifically, F. nucleatum has recently
garnered attention due to its robust association with colorectal cancer and poor prognosis, although the exact mechanisms remain unclear (Brennan and Garrett, 2019; Kitamoto et al., 2020). Therefore, *F. nucleatum* has come to the forefront of scientific research, which may provide a target for the treatment of related diseases.

Most drugs used to treat bacterial infections are chemical antibiotics. However, the emergence of multi-drug-resistant (MDR), even extremely drug resistant (XDR), bacteria because of the overuse and misuse of traditional antibacterial drugs poses a great threat to our health (Rather et al., 2017). A novel and efficacious strategy to achieve greater antibacterial effects is urgently required. Advances in engineered nanomaterials and nanotechnologies have provided new platforms for antibacterial infections (Pelgrift and Friedman, 2013). Previous studies reported that when the size of nanoparticles (NPs) decreases to less than 2 nm, called “ultrasmall metal nanoclusters (NCs)”, they act as broad-spectrum potent killers of Gram-positive and Gram-negative bacteria (Wang et al., 2015; Chang et al., 2019; Yougbare et al., 2019; Tang et al., 2020). In particular, gold nanoclusters (AuNCs) such as Au25(SR)18 (SR denotes thiolate ligands) have sparked increasing interest due to their excellent biocompatibility, facile preparation and functionalization (Song et al., 2016). Practical applications of AuNCs and related nanomaterials have been researched extensively in many areas such as drug delivery, biological imaging, biosensing, and antibacterial agents (Zhang and Wang, 2014; Zhang et al., 2015; Zheng and Xie, 2020). In 2017, Zheng et al. demonstrated that AuNCs exerted higher antimicrobial activity on *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* than larger-sized gold nanoparticles via attacking membrane disruption and interfering metabolism (Zheng et al., 2017). Another study reported that AuNCs functionalized with quaternary ammonium (QA) salts exhibited antibacterial therapeutic effects against MDR bacteria *in vivo* without any drug resistance or detectable toxicity (Xie et al., 2018). Furthermore, AuNC-constructed metal-organic network films had strong and broad antibacterial performance against methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamases *Escherichia coli* (ESBL *E. coli*) for combating implant-associated infections (Chu et al., 2020).

Numerous studies have linked the antibacterial behaviour of AuNCs with the production of reactive oxygen species (ROS), which can cause oxidative stress to lipid, DNA, and proteins, resulting in significant damage to cell structures, leakage of intracellular contents and cell death (Ahmed et al., 2018; Tian et al., 2020; Zheng et al., 2021). However, the antibacterial effect of AuNCs on *F. nucleatum* has not yet been well explored.

To survive in the host environment, a key strategy for pathogens is to interfere with normal cell signaling to defuse the defenses that are aimed at controlling and eliminating incoming pathogens (Rosenberger and Finlay, 2003). As the front line of immune defenses, macrophages release various pro-inflammatory cytokines, reactive oxygen and nitrogen mediators to combat invading pathogens, and recruit other immune cells to the affected area (Garlet, 2010). In particular, at the early stage of an infection, macrophages are critical participant in phagocytosing microbes from the blood stream and tissue by ligand-receptor combination and downstream signals trigger (Weiss and Schaible, 2015). Reduced phagocytic ability and impaired cytokine production of macrophages often result in compromised host defenses and prolonged infections (Kodali et al., 2013). Some studies have indicated nanomedicine may impair macrophages response to bacteria and result in bacterial infections. For instance, Aluminum NPs were found to impair phagocytosis function and repress immune response to respiratory pathogens (Braydich-Stolle et al., 2010). Increased susceptibility to lung infection was associated with exposure to nanoparticles via macrophages cytotoxicity, diminished phagocytic function and damaged innate immune responses (Kodali et al., 2013). RAW 264.7 cells pre-exposed to 10 nm SiO2 NPs showed a dramatic reduction of phagocytosis against *E. coli in vitro* (Bancos et al., 2015). Therefore, except for direct antibacterial efficiency, the impact of AuNCs on macrophage function is a key issue that should also be anticipated before their biological applications. However, previous researches usually focus on the interaction between AuNCs and bacteria, and the role of AuNCs in macrophages’ behavior has not been reported at present.

In our study, we first determined the antibacterial effect of AuNCs on *F. nucleatum in vitro* and investigated the possible mechanisms. And then the impact of AuNCs on the phagocytic ability of macrophages of *F. nucleatum* was further evaluated to determine the potential therapeutic applications of AuNCs in the bacterial infections.

### MATERIALS AND METHODS

#### Materials and Chemicals

All chemicals used for the synthesis of AuNCs were obtained from Sigma-Aldrich (St. Louis, MO, United States). The bacteria *F. nucleatum* was purchased from the Guangdong Microbial Culture Collection Center (GDMCC, 1.1290). Human periodontal ligament cells (PDLCs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, United States) and RAW264.7 macrophage cells were obtained from the American Type Culture Collection.

The LIVE/DEAD BacLight Bacterial Viability Kit was obtained from Invitrogen (Carlsbad, CA, United States), and the Alamar Blue Cell Viability Kit was purchased from KeyGEN BioTECH (Nanjing, Jiangsu, China). Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, United States). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Tokyo, Japan). The Reactive Oxygen Species Assay Kit, CFDA–SE (carboxyfluorescein diacetate, succinimidyl ester) fluorescent probes, and DAPI staining solution were purchased from Beyotime Biotechnology (Shanghai, China).

#### Synthesis and Characterization of AuNCs

AuNCs were synthesized by the sodium borohydride (NaBH4) reduction method as described elsewhere (Wang et al., 2021).
Transmission electron microscopy (TEM, JEM-2100; JEOL Ltd., Tokyo, Japan), high-resolution transmission electron microscopy (HR–TEM, Tecnai G2 F20 S-TWIN; FEI Company, Hillsboro, OR, United States), ultraviolet–visible (UV–Vis) spectrophotometry (CARY 300 Bio UV–Vis Spectrophotometer; Agilent Technologies, Santa Clara, CA, United States), and dynamic light scattering (DLS, Malvern Zetasizer Nano ZS90; Malvern Panalytical, Malvern, United Kingdom) were used to characterize the AuNCs.

Bacterial Identification and Culture

F. nucleatum was cultivated on Columbia blood agar plates supplemented with hemin (5 mg L\(^{-1}\)), yeast extract (5 g L\(^{-1}\)), menadione (1 mg L\(^{-1}\)) and 5% sterilized defibrinated sheep blood at 37°C in an anaerobic incubator (N2, 80%; H2, 10%; CO\(_2\), 10%). After identified using 16S ribosomal RNA gene sequencing (Novogen, Tianjin, China), a single colony was inoculated in brain heart infusion broth (BHI broth) and continuously cultured in the same condition above. During the exponential growth phase, the bacterial suspension was centrifuged at 8,000 rpm and 4°C for 5 min, washed three times with PBS and resuspended in buffer for subsequent use.

Spread Plate Method

Bacterial suspensions at concentrations of \(2.5 \times 10^6\) CFU mL\(^{-1}\) were treated with AuNCs (0.1, 0.2, and 0.4 mM) or PBS and placed in a rotary shaker set at 200 rpm and 37°C for 6 h. Then the F. nucleatum suspension was centrifuged at 8,000 rpm for 5 min and washed three times with PBS to remove AuNCs that had not been internalized. Then the treated bacteria were resuspended in PBS and diluted, aliquots of 10 \(\mu\)L were plated on Columbia blood agar plates and cultured in anaerobic conditions for 48 h to allow the formation of bacterial colonies. Finally, the bacterial colonies were counted and analyzed to evaluate the killing efficiency of AuNCs.

Growth Curve Analysis

Bacteria were treated with AuNCs for 6 h as described above. Then they were resuspended in BHI broth, seeded in 96-well microtitre plates and cultured at 37°C for 30 h. The absorbance of the suspension at 600 nm was recorded every 6 h by a SpectraMax M3 (Molecular Devices, San Jose, CA, United States) to obtain the growth curves of the bacteria.

Alamar Blue Cell Viability Assay

Alamar Blue is a resazurin-based solution that indicates cell health conditions by employing the reducing power of living cells. F. nucleatum was treated with AuNCs at 37°C as described above for 6 h; then the absorbance of a solution containing Alamar Blue was measured at 570 and 600 nm. Finally, the reduction rate was calculated according to the formula:

\[
\text{Ro} = \frac{A_{570}/A_{600}}{A_{570}} \times 100 \quad (1)
\]

where \(A_{570}\) and \(A_{600}\) refer to the absorbances of all sample groups after subtraction of \(A_{570}\) and \(A_{600}\) from the control group. Higher absorbance values represent a higher bacterial metabolic activity.

Live/Dead Bacterial Imaging

SYTO9 dye was used to label all bacteria with green fluorescence, while propidium iodide (PI), a cell-impermeable, red fluororescent dye, was employed to stain only dead cells with permeable membranes. The excitation/emission (Ex/Em) maximum of SYTO9 was 480/500 nm and the excitation/emission (Ex/Em) maximum of PI was 490/635 nm. Bacteria were treated with AuNCs at 37°C for 6 h, centrifuged at 8,000 rpm for 5 min, and incubated with SYTO9/PI dyes in the dark for 15 min. Finally, 5–10 \(\mu\)L of the bacterial suspension was dropped on the bottom dishes and images were captured by confocal fluorescent microscopy at magnifications of 60× (NikonA1; Nikon Inc., Tokyo, Japan). The fluorescent intensity was calculated using Image J to determine the percentage of dead F. nucleatum after being treated with AuNCs for 6 h.

Morphological Analysis of the Bacteria

F. nucleatum was treated with the AuNCs (0.2 mM) for 6 h in the same way, completely immobilized with 2.5% glutaraldehyde at 4°C overnight and dehydrated with a graded ethanol series (50, 70, 80, 90, 95, and 100%) for 15 min in each solution. Following dehydration, the bacteria were placed on cell slides, freeze-dried and observed by field emission–scanning electron microscopy (FE–SEM, S-4700; Hitachi, Tokyo, Japan). Meanwhile, another batch of samples treated in the same process were embedded in epoxy resin, cut into ultrathin sections by a microtome, and observed at 80 kV by TEM (HT7800; Hitachi) to analyze ultrastructural changes.

Levels of Intracellular ROS

DCFH-DA (6-carboxy-2′,7′-dichlorodihydrofluorescein; Molecular Probes, Eugene, OR, United States) dye was applied to test intracellular ROS levels according to the manufacturer’s instructions. Briefly, a bacterial suspension at a concentration of \(10^6\) CFU mL\(^{-1}\) was incubated with DCFH-DA probes for 30 min at 37°C. Then the cells were treated with the AuNCs (0.2 mM) for 2 h, with PBS as the control. Finally, the fluorescence intensity of all samples was observed by confocal fluorescence microscopy and a microplate reader under Ex/Em wavelengths of 488/525 nm.

Phagocytic Activity of Macrophages

F. nucleatum was inoculated in BHI medium and grown at 37°C overnight before incubation with CFDA–SE for 30 min to obtain fluorescent labeling F. nucleatum. Lipopolysaccharides (LPS) of E. coli at 20 ng·mL\(^{-1}\) was used as a proinflammatory stimulus to induce macrophages into activated condition. Macrophages were seeded at densities of \(5 \times 10^5\) cells mL\(^{-1}\) in glass bottom cell culture dishes and treated with AuNCs (0.2 mM), LPS (20 ng·mL\(^{-1}\)), or LPS (20 ng·mL\(^{-1}\)) + AuNCs (0.2 mM) for 24 h, with medium as the control group. Subsequently, fluorescently-tagged F. nucleatum were added to the RAW264.7 macrophages at a ratio of 10:1 (bacteria to macrophages) for 2 h to allow cellular uptake to occur. Finally, samples were washed with PBS, fixed with 4% paraformaldehyde, stained with DAPI, and observed by confocal laser scanning microscopy to evaluate the efficiency of phagocytosis.
The Cytotoxicity Assessment of AuNCs

The CCK-8 assay was performed to investigate the cytotoxicity of the AuNCs toward PDLCs and RAW264.7 macrophage cells. Briefly, cells were seeded at densities of 10,000 per well in a 96-well plate and cultured in DMEM medium supplemented with heat-inactivated FBS (10%) and penicillin/streptomycin (1%) at 37°C and 5% CO2 overnight. Then AuNCs were added to the cells at final concentrations of 0.1, 0.2, and 0.4 mM and cultured for 24 h to perform the CCK-8 assay with untreated cells as the control.

All data were presented as means ± standard deviations of triplicate samples. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, United States) using the two-tailed unpaired Student’s t test, and statistical significance was set as P < 0.05.

RESULTS AND DISCUSSION

Characterization of AuNCs

AuNCs appeared as a reddish-brown solution (Figure 1A). The morphology and diameters of the AuNCs were analyzed by TEM and HR-TEM. AuNCs were uniformly dispersed and approximately 2–3 nm in size, as shown in Figure 1B. The UV-Vis absorption spectrum of AuNCs indicated distinct absorption peaks at 400, 450, 670, and 770 nm (Figure 1C), which was in concordance with previous research (Wang et al., 2021). The hydrodynamic size distribution of the AuNCs was approximately 7 nm confirmed by DLS (Figure 1D). These results confirmed the successful synthesis of AuNCs for the subsequent experiments.

Antibacterial Activity of AuNCs on F. Nucleatum

The antibacterial activity of AuNCs on F. nucleatum was firstly investigated by Spread Plate Method. Compared with the control, the antibacterial efficiency of AuNCs was dose-dependent with reduction rates of 91.98 ± 0.05%, 55.67 ± 0.03%, 4 ± 0.001% at concentrations of 0.1, 0.2, and 0.4 mM, respectively (Figure 2A). Bacterial growth curves showed that at the 6 h time point, bacteria reached their exponential growth phase and AuNCs’ antibacterial activity was time- and dose-dependent (Figure 2B). When the concentration was 0.2 mM, they showed remarkable antibacterial ability, especially at 6 h. The results of the Alamar Blue metabolic assay indicated that the activity of F. nucleatum was as low as 68.52 ± 10.39% and 36.60 ± 6.9% of the control at concentrations of 0.2 and 0.4 mM, respectively (Figure 2C), which was consistent with the above results. Finally, bacterial live/dead fluorescent staining assays were performed to determine the killing efficiency of AuNCs via confocal laser scanning microscopy. Figure 3A showed that bacteria treated with AuNCs exhibited higher red fluorescence intensity than that of the control group, and ~40% F. nucleatum population membranes were effectively destroyed as illustrated in Figure 3B, indicating AuNCs exhibited potential bactericidal effect on F. nucleatum.
Together, we observed that when the concentration of AuNCs was 0.2 mM, bacterial viability and growth was notably affected in time-dependent manner. Especially the inhibitory effect on bacterial growth was obvious when the exponential growth phase was reached at 6 h, which may be contributed to the unbalanced metabolism induced by nanomaterials treatment. Energy metabolism is necessary to the growth and survival of microorganisms. Under low metabolic activity, the bacteria are injured and their growing rate will reduce significantly, sometimes even stopping growing. Recently, Jiang and his coworkers recently found AuNCs at low concentration (100–150 μM) exhibited a potent killing activity for Gram-negative bacteria E. coli within
30–60 min (Wang et al., 2021). In a similar study, AuNCs showed half-maximal inhibitory concentrations of about 2.6 and 3.2 μM for Gram-positive (S. aureus, S. epidermidis, and B. subtilis) and Gram-negative bacteria (E. coli and P. aeruginosa), respectively, suggesting that AuNCs have a broad antibacterial spectrum (Zheng and Xie, 2020). The different results of bacterial sensitivity toward AuNCs may ascribe to their difference in structure rigid, membrane composition and metabolism activity. Compared with Gram-positive bacteria, Gram-negative bacteria exhibited higher binding efficiency with ultra-small AuNCs (Wang et al., 2021). However, Gram-negative strains F. nucleatum have shown more resistant to antibiotics or NPs than gram-positive bacteria because of the presence of outer membrane proteins (OMPs), which serve as a solid barrier to protect them from chemical drugs and harmful irritation (Bolstad et al., 1996). Furthermore, anaerobes like F. nucleatum in lower oxygen environment use other reduction-potential substrates like nitrogen as acceptor instead of oxygen, resulting in lower respiratory metabolism efficiency and less sensitivity to antibiotics than aerobic organisms (Jabłońska and Tawfiq, 2019). Therefore, AuNCs may provide a new platform for the treatment of F. nucleatum associated infections.

**Mechanism of Antibacterial Activity**

To clarify the underlying mechanism of the AuNCs' antibacterial activity on F. nucleatum, SEM and TEM were firstly utilized to observe the morphological changes and microstructural disruption of cells. Normally, F. nucleatum was a spindle-rod shape with an intact cell membrane and a distinct profile (indicated by a black arrow), as shown in Figure 4A. In the presence of AuNCs, the bacterial cell became obviously wrinkled and developed nano-sized pores on the membrane, suggesting increased membrane permeability (indicated by a white arrow). Moreover, the intracellular density of treated F. nucleatum was also decreased because of broken membranes and leakage of intracellular contents, as displayed by TEM in Figure 4B. In addition, intracellular ROS generation was further tested using DCFH-DA that could be oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) by oxidants. Figure 5A indicated the fluorescence intensity in the bacteria following AuNCs treatment was much higher than that of control, which was 2–2.5 folds when quantified by microreader at 488/525 nm (Figure 5B).

Based on the results, it was concluded that significant ROS augmentation may oxidize the bacterial membrane lipid efficiently and result in the large holes on the membrane, which could facilitate the further internalization and accumulation of the AuNCs inside bacteria to enhance bacteria killing efficacy. Many previous studies have explored that AuNCs can promote pro-oxidative gene expression (e.g., dmpI, narJ, and nark) and inhibit pro-reduction gene expression (e.g., sod) to cause redox imbalance (Tian et al., 2020). As a result, genes related to cell structures (e.g., DEGs), energy metabolism (e.g., glycolysis and TCA cycle) and DNA repair were significantly inhibited following AuNCs treatment (Wang et al., 2021). In a biological context, ROS represent metabolic byproducts that play an important role in cellular homeostasis. However, excess ROS generation is responsible for lipid peroxidation, protein denaturation, DNA damage, and eventually, bacterial death, which has become a strategy for treating chronic infections with heavy bacterial loads (Dryden et al., 2017). Based on this, we focused on the ROS-induction ability of AuNCs to determine their antimicrobial activity. Compared with nanoparticle, atomically precise AuNCs with more active sites also possess efficient catalytic activity that elicits excessive ROS generation for enhanced antibacterial performance (Zhang et al., 2018). Therefore, AuNCs were efficient at killing F. nucleatum by destroying bacterial
membranes and enhancing ROS oxidative stress. However, details on the ROS-generating process are scarce and require further investigation.

**Impact of AuNCs on the Phagocytic Ability of Macrophages**

As professional phagocytes, macrophages are critical immune cells of first line of defense that respond to many different infections, including bacterial and fungal pathogens (Rosowski, 2020). A canonical pathway for macrophages to control pathogens is promoting killing by phagocytosis, which involves selective macrophages receptor recognizes pathogens-associated surface molecular patterns and host-derived inflammatory mediators release (Masud et al., 2019). When microorganisms are engulfed by macrophages, they are exposed to a series of defense reactions such as protease, acidification within the phagosome and reactive oxygen stress to trigger intracellular signaling pathways and host defense (Weiss and Schaible, 2015). Phagocytosis deficiency, especially in the early stage of infectious disease, will subsequently leads to a high bacterial burden and chronic infectious diseases (Amin Yavari et al., 2020). Therefore, macrophages have represented a promising target of nanoparticle-based therapeutic interventions to successfully promote pathogen control at a time of rapidly emerging multiple drug resistance of bacteria (Amin Yavari et al., 2020). However, the impact of nanomedicines on macrophage behavior reported in many studies was contradictory. For example, Yao et al. reported CuInS2 and CdTe nanoparticles could inhibit phagocytosis of *S. aureus* by THP-1 and showed an immunosuppressive effect in *vitro* (Yao et al., 2019). In contrast, ZnO-nano films also were found to play a dual role in antimicrobial and reinforced innate defense on implant surfaced for implant-associated infections therapy (Wang et al., 2017). Furthermore, Tian and his coworkers found ZnO-nano reservoirs could enhance the anti-infective efficacy of macrophages through a combination of intrinsic antibacterial properties and reinforced host defenses (Tian et al., 2020). Iron oxide nanoparticles (IONPs) have been shown to improved macrophages-mediated bactericidal effect against *Staphylococcus aureus* by triggering a Fenton reaction (Yu et al., 2020). Differences in the nanomaterial (topography, charges, size, and internal architecture), experimental conditions (exposure time, concentration), cell types and solution chemistry could significantly affect their results (Rayahin and Gemeinhart, 2017; Petithory et al., 2021).

In our study, we found after AuNCs treatment, the trend of bacterial uptake ability was as follows: control<AuNCs<LPS<LPS + AuNCs, suggesting AuNCs treatment could enhance macrophages’ phagocytic ability, in particular when they were activated by LPS (Figure 6). Moderate stimuli such as LPS activate macrophages with potential damaging ability against invading pathogens (Jiang et al., 2017). In condition of low inflammation, macrophages are found to undergo necrotic death with defective bacterial killing responses. While in high inflammatory condition, macrophages are actively induced into highly phagocytic activity in ROS-mediated response (Rosowski, 2020). Insufficient ROS concentration in macrophages would contribute to a failure of bacterial clearance (Fang, 2011). Two new controllers of ROS production have been identified to targeting of pathogen by macrophages (Torrace et al., 2014). Bernut et al. recently found reduced ROS production by macrophages could inhibit their ability to control bacteria and subsequently lead to their death (Bernut et al., 2019). In contrast, Mg (OH)2 nanoparticles have been implicated in enhanced antibacterial activities of macrophages by activating the reactive oxygen species (Zhu et al., 2021). Therefore, a strategy to enhance ROS generation of macrophages and promote immunoregulatory response is a potential therapy to combat bacterial pathogens and control infections. Small-sized AuNCs have been found to possess unique properties, such as discrete energy, catalytic properties and quantum size effects (Zhang and Wang, 2014; Zhang et al., 2018). In catalytic reactions, energy transfer to oxygen molecules can induce the production of ROS, including peroxides, superoxides, hydroxyl radicals, and singlet oxygen (Li et al., 2012). And the presence of AuNCs could promote intracellular ROS induction to a higher but within normal level, subsequently enhanced the ROS-mediated anti-infective performance of macrophages (Nishimura et al., 2016; Jiang et al., 2017). However, the exact mechanisms underlying the

![Figure 5](image1.png)

**Figure 5** | ROS levels of *F. nucleatum* exposed to AuNCs (0.2 mM) for 2 h. (A) Visualization of ROS concentration in *F. nucleatum* exposed to AuNCs by confocal microscope. (B) Quantification of ROS fluorescence intensities in *F. nucleatum* at OD488/525 nm. (**P < 0.001).
enhanced phagocytic activity of macrophages is incompletely understood and required further research.

Biocompatibility is an important factor that must be taken into consideration for the biomedical application of nanomaterials. Periodontal ligament cells are a population of heterogeneous cells that characterized by collagen production and osteoblastic features, playing an important role in the health of periodontal ligament tissues. Furthermore, they are in close proximity to bacterial invasion and antibacterial treatment (Jönsson et al., 2011). Therefore, in this study, the viability of macrophages and PDLCs after treatment with AuNCs at concentrations of 0.1,
0.2, and 0.4 mM for 24 h was evaluated. Results displayed in Figure 7A suggested that AuNCs did not negatively impact the viability of macrophages but had a slightly positive effect on the proliferation of PDLCs, although the differences were not statistically significant (Figure 7B). Previous researchers found that AuNCs could enter cells by pinocytosis, be transported to endosomes, and disposed of in lysosomes (Tian et al., 2020). However, these organelles are absent in bacteria; therefore, ultra-small nanomaterials could readily enter through physical pores by simple diffusion, eventually reaching lethal concentrations in the bacteria (Tian et al., 2020). Compared with bacteria, cellular balanced redox homeostasis is ensured by more complicated ROS generator and antioxidants system, which is of great significance in reducing the incidence of pathological dysfunction and diseases (Yang et al., 2019). Furthermore, the presence of cholesterol and the self-repair program that restores damaged structures in mammalian cells could make them more resistant to external stimuli than bacteria (Tang and Marshall, 2017; Tian et al., 2020). Therefore, AuNCs exhibited less side effect on cells than bacteria.

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