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Research Paper

Antimicrobial and Cytotoxic Activities of Colloidal Silver and Titanium Dioxide Nanoparticles Against Food-Borne Bacteria: *Shigella dysenteriae* and *Staphylococcus aureus*

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ABSTRACT

Background: Nowadays, due to the increasing problems of microbial resistance, scientists are searching for the safest and most effective way to fight them. The colloidal silver (Ag) and titanium dioxide (TiO2) nanoparticles can effectively fight against many bacterial microorganisms. Therefore, the purpose of this study was to investigate the antibacterial and cytotoxic properties of Ag and TiO2 nanoparticles, against *Shigella dysenteriae* and *Staphylococcus aureus*.
Methods: In this study, Ag and TiO2 nanoparticles were synthesized, and the minimum inhibitory and bactericidal concentrations (MIC & MBC) were determined. In addition, the cytotoxicity of these agents was evaluated on Hu02 fibroblast cell line.
Results: We found that the MIC and MBC for Ag and TiO2 nanoparticles were similar (12.5 µg/ml) against *S. aureus*, while the MIC's for Ag and TiO2 against *S. dysenteriae* were found to be 12.5 and 25 µg/ml. In addition, the MBC's for Ag and TiO2 against *S. dysenteriae* were 25 and 50 µg/ml. Based on the cytotoxicity tests, the cell viability percentage after 48 hours of exposure to TiO2 was higher than that of Ag (0.025 µg/ml).
Conclusion: The Ag and TiO2 nanoparticles demonstrated good antibacterial properties while they had low toxicity against the Hu02 fibroblast cell line.
Keywords: Ag nanoparticles*; Shigella dysenteriae; Staphylococcus aureus;* TiO2 nanoparticles

Introduction

The growing emergence of resistant pathogens has created major concerns in the treatment of infectious diseases [1, 2]. The rise in antibiotic resistance may lead to greater bacterial resistance and failed treatments [3]. Drug-resistant bacteria have posed major problems in the design and production of antibiotic drugs [4, 5]. Thus, it is important to control antibiotic resistance which is a major approach to preventing infections [2]. Among novel pharmaceutical approaches, nanomaterials have played major roles in clinical medicine and infectious disease control [6-8]. Some nanoparticles have shown efficacy against a variety of infectious diseases and cancers because of their high surface to volume ratio [8-10]. Colloidal silver nanoparticles (cAg) nanoparticles have shown promising effects as antibacterial drugs and gradually emerged as functional biomaterials to prevent the resultant infections. Silver nanoparticles are effective against both gram-positive and -negative bacteria, molds, fungi and eliminate them [11-13]. They have been widely and successfully used in catheters, implants and wound dressings for therapeutic and prophylactic purposes [14].

Titanium dioxide (TiO_2) nanoparticles are effective compounds with a wide range of applications in pharmaceutical industry, such as white pigments for foods, photo catalysts, skin sunscreens, water loss treatment, hydrogen production and antimicrobial activity [13, 15]. The antimicrobial activities of TiO_2 and mixed oxide system (MONs) have interesting properties, which are effective against both gram-negative and -positive bacteria [15]. In addition, TiO_2 has tunable

photocatalytic property, i.e., it can produce reactive oxygen species under ultraviolet light radiation, providing good antimicrobial effect. Also, it kills pathogens by damaging their cell membranes [15]. In addition, TiO2 is extensively used for various purposes, such as strong oxidation, biocompatibility, biochemical and antimicrobial properties [13].

Although there are many reports of antibacterial efficiency of Ag and TiO2 nanoparticles, based on our literature review, no reports was found on the comparison of their efficiency against *S. aureus* and *S. dysenteriae,* and their cytotoxicity evaluation on the Hu02 fibroblast cell line. In addition, *S. dysenteriae* Type-1 is mainly associated with serious life-threatening dysentery in epidemics.

Aim of the Study: This study was planned to search for agents that have antibacterial properties without having toxic effects on the human body cells. We aimed to synthesize Ag and TiO2 nanoparticles, and used a practical method to determine their antibacterial and cytotoxic properties against *Staphylococcus aureus* and *Shigella dysenteriae.* Both of these strains are foodborne bacteria [3, 16]. Also, we investigated the gram-negative *S. dysenteriae* Type-1 because it is one of the main sources of diarrhea in youngsters and elderly people globally, where only a few organisms, i.e., 10-100, are sufficient to cause serious infection [17-19].

Materials and Methods

Materials: The *S. dysenteriae* Type-1 (ATCC-13313) and *S. aureus* (ATCC-25923) were obtained from Pasteur Institute, Tehran, Iran. Streptomycin disks (25µg) and tetracycline (100 µg) were obtained from Mast Dignostica GmbH and Mueller Hinton Agar (Reinfeld, Germany). The AgNO3 solution and NaBH4 were purchased from Sigma-Aldrich (Darmstadt, Germany). The MTT assay for cell line (Hu02 fibroblasts) was obtained from the Iranian Biological Resource Center (Tehran, Iran). The cell culture media were high glucose DMEM, fetal bovine serum (Invitrogen), trypsin (Sigma-Aldrich; EUA), and dimethylsulfoxide (DMSO) (Reinfeld, Germany). Isolates of *Shigella sonnei* resistant to streptomycin and tetracycline, and *S. aureus* were obtained from Milad Hospital, Tehran, Iran.

Synthesis of cAg Nanoparticles: In this study, 0.2 gram of chitosan was dissolved in 10mL of 1% acetic acid. Then, 6mL of the chitosan solution was poured into a beaker covered with aluminum foil and 50µL of AgNO3 solution (20mM) was added, and the beaker was stirred for 30 minutes. The NaBH4 solution was prepared as follows: 0.383g of NaBH4 was weighed and dissolved in 5mL of deionized water. Thirty minutes later, the AgNO3 and chitosan solutions were well-mixed dropwise. Further, 100µL of NaBH4 was added to the beaker and agitated on a stirrer at 700-800 rpm for 5 minutes. About 90 minutes later, the solution's color changed from light to dark yellow [20, 21].

Synthesis of cTiO2 Nanoparticles: The synthesis of colloidal TiO2 nanoparticles was done according to the study of S. Mahshid, *et al.* [22]. Specifically, 5mL of titanium isopropoxide (TTIP) and 15mL isopropanol were mixed. Then, the pH was set to 7.4 by adding HNO_3 or NH_4OH. After vigorous stirring for peptizing process, the mixture was heated at 60-70°C for 18-20 hours. Next, the precipitate was washed with ethanol and dried at 100°C. Finally, a yellow-white powder was obtained and warmed at 200-800°C for 2 hours [22].

Characterization of Nanoparticles: Several techniques were used to characterize the nanoparticles. The UV–Vis spectra were obtained using a JASCO V-630 spectrophotometer (Jasco C-630, Japan) set in the absorbance range of UV-Vis spectra at 200-800 nm. The UV-visible spectra of the solutions containing Ag nanoparticles had a broad band around 206 nm (Figure 1). Also, the absorption band of the solution with TiO2 nanoparticles was 313 nm (Figure 2). A zetasizer nano ZS (Malvern Instruments Co., London, UK) was used to analyze the hydrodynamic average size in percentage, poly-dispersity index (PDI), and zeta potential of the nanoparticles in the media at 30°C. The measurement was made, using a disposable folded capillary zeta cell at 10mm path length. Additionally, the morphology of nanoparticles was investigated, using a scanning electron microscope (FE-SEM TESCAN MIRA3) at 50kx, 100kx, and 200kx magnifications. The size of the nanoparticles, the crystal structure of Ag and TiO2 nanoparticles were determined and characterized by X-ray diffraction method (XRD). This method was employed, using a Philips PW1730 diffractometer to record the X-ray powder diffraction at room temperature, 50 kV, 40 mA and operating CuKα ($\lambda = 1.54060$ Å). The data were recorded at 2θ range (10° to 70°). Further, the fineness size related to the Ag and TiO2 nanoparticles was estimated, using Debye–Scherrer equation 1: $D = (k\lambda)/(\beta c\theta s)$ [23] (1).

In the above formula, D is the diameter of crystallite size, K is a constant, λ is the CuKα wavelength, β is the full width at half maximum (FWHM) of the peak and θ is the Bragg diffraction angle.

Antimicrobial Tests in Colloidal Nanoparticles

(A) Minimum Inhibitory Concentration (MIC): Microdilution was performed according to the CLSI reference method [8, 24]. We used 96-well micro-titer plates. Antimicrobial agents in this experiment included colloidal Ag and TiO2 nanoparticles (100 µg/mL). For each test series, 100µL of Mueller-Hinton broth medium was added to 10 wells in each of the microplates. Then, 100µL antimicrobial agent was added to the first well, and the dilution serial was performed up to the tenth well. Also, wells 11

and 12 were considered as the positive and negative controls. Well-11 contained only the medium while well-12 contained a mixture of the medium and bacterial suspensions. After dilution, the microbial suspension was added to each well and placed in an incubator for 24 hours at 37°C [24]. The first well that prevented the bacterial growth, was considered as the MIC. All tests were done in triplicates and the means were used for the data analyses.

(B) Minimum Bactericidal Concentration (MBC): Based on this method, the well immediately following the first clear well was considered as the MBC, after determining the MIC of the antimicrobial agents. Nevertheless, for more confidence, the MIC well contents and two wells immediately after that were transferred to a dish containing the Muller Hinton Agar medium. Then all plates were incubated for 24 hours and the first dilution that eliminated 99.9% of the bacteria was considered as MBC [25, 26].

Evaluation of Cell Cytotoxicity by MTT Assay: We performed MTT assay based on the depletion of the tetrazolium salt, 3-(4,5-dimethylthiazol-z-yl)-2,5 diphenyl tetrazolium bromide through actively growing cells. This test starts with the breakdown of tetrazolium yellow salt via mitochondrial succinate dehydrogenase enzyme of living cells, and the production of insoluble formazan crystals in purple color. The greater the number of live cells, the more intense would be the color produced. The cytotoxicity of different nanoparticles has been evaluated using Hu02 fibroblast cell line (Biological Resource Center, Tehran, Iran). Briefly, 5×10^3 cells in 180µL high glucose DMEM [10% (v/v) FBS] were seeded in 96-well plates. Next, the cells were incubated high glucose at 37°C, 5% CO_2 overnight. Then, the cells were treated with Ag and TiO2 nanoparticles (100 µg/mL) for 48 or 72 hours, independently, and each test was done in triplicate. Subsequently, 20µL of MTT solution (5 mg/ml) was added to the wells and incubated at 37°C for another 4 hrs. Next, the media in the wells was washed and the remaining blue formazan crystals were solubilized with 150µL of dimethylsulfoxide (DMSO; Merck, Germany). Indeed, MTT was converted to formazan through metabolically viable cells during the next step. The absorbance of the media was read on an ELISA reader at 570nm. The relative cell viability was derived from three independent experiments and expressed as the means of viable cells compared to the untreated cells (100%) [27].

Results

Characterization of Nanoparticles: The absorption spectra of cAg and cTiO2 nanoparticles are shown in Figures 1 and 2. They demonstrated the highest absorption at 313 and 206 nm, respectively. The average size of Ag and TiO2 nanoparticles were 18.2 ± 0.1 and 20.6 ± 0.2 nm, respectively. The morphologies of nanoparticles are shown in Figures 3 and 4. They had spherical shapes at 15.59 to 21.40nm for Ag (Figure 3) and 20.09 to 30.82nm for TiO2 (Figure 4), all of which with smooth surfaces. Also, to study the crystal structure of nanoparticles, XRD was used as shown in Figures 5 and 6. The XRD pattern of pure colloidal Ag and TiO2 nanoparticles showed a sharp characteristic peak at about $2\theta = 24\circ$ for Ag and about $2\theta = 26\circ$ for TiO2, respectively, related with their crystal formations.

Evaluation of MIC and MBC: The MIC and MBC (µg/mL) of the cells in cAg and cTiO2 nanoparticles, respectively, were evaluated for the bacteria, *S. dysenteriae* and *S. aureus,* the results of which are presented in Table 1. We found that the MIC and MBC of Ag and TiO2 nanoparticlas of *S. aureus* were similar (12.5 µg/mL). The MICs for Ag and TiO2 nanoparticles against *S. dysenteriae,* respectively, were 12.5 and 25 µg/mL. Also, the MBC for Ag and TiO2 nanoparticles against *S. dysenteriae*, respectively, were 25 and 50 µg/ml. See Table 1.

Investigation of MTT Assay: For MTT assay, the cytotoxic effects of the agents (Ag and TiO2 nanoparticles) were evaluated on Hu02 cell line. As shown in Table 2, the amounts among the treatment groups of 48 hours had a significant difference based on one-way ANOVA (df: 3, F:16.91, P=0.001). But the amounts found among the 72hrs test groups were not significantly different from each other (df: 3, F: 2.43, P=0.14). In order to determine the groups that were significantly different, a Tukey's post-hoc test was performed. The results indicated that the cell viability percentage after 48 hours of treatment with TiO2 nanoparticles was significantly higher than those of the Ag (0.025).

Statistical Analyses

To analyze the data, the means and standard deviations were plotted using 2019 Excel and SPSS, version 28 software. The means' differences were determined by one-way ANOVA and Tukey's post-hoc test.

Table1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of colloidal Ag and TiO2 nanoparticles.

Bacteria	cAg-NPs (µg/mL)		cTiO2-NPs (µg/mL)	
	MIC	MBC	MIC	MBC
S. dysenteriae ATCC 13313	12.5	25	25	50
S. aureus ATCC 25923	12.5	12.5	12.5	12.5

NPs = Nanoparticles

Table 2. Comparison of mean viability percentage of skin fibroblasts after treatment with various agents at 48 or 72 hours as measured by MTT.

Ag Nanoparticles		TiO2 Nanoparticles	
48 h	72 h	48 h	72 h
65.66±0.57	76±1	87.33±14.1	93.66±3.51

The values were the means ± SDs and the differences in the mean values at $P<0.05$ was considered significant by *t*-test. Ag NPs (48h) with AgNPs (72h); sig: 0. TiO2-NPs (48h) with TiO2-NPs (72h); sig: 0.49. Ag-NPs with TiO2-NPs (48h); sig: 0.05. Ag-NPs with TiO2-NPs (72h); sig: 0.001.

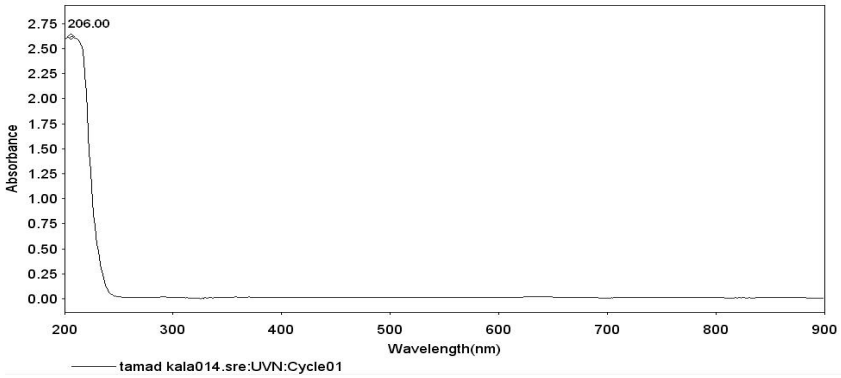


Figure 1. UV-visible absorption spectra of synthesized Ag nanoparticles. The absorption peak was obtained in the visible range at 206nm.

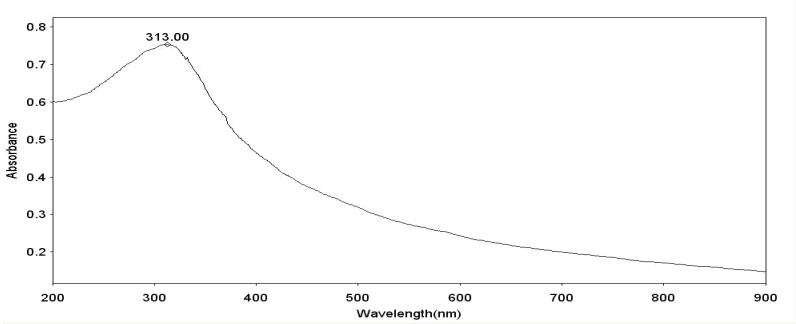
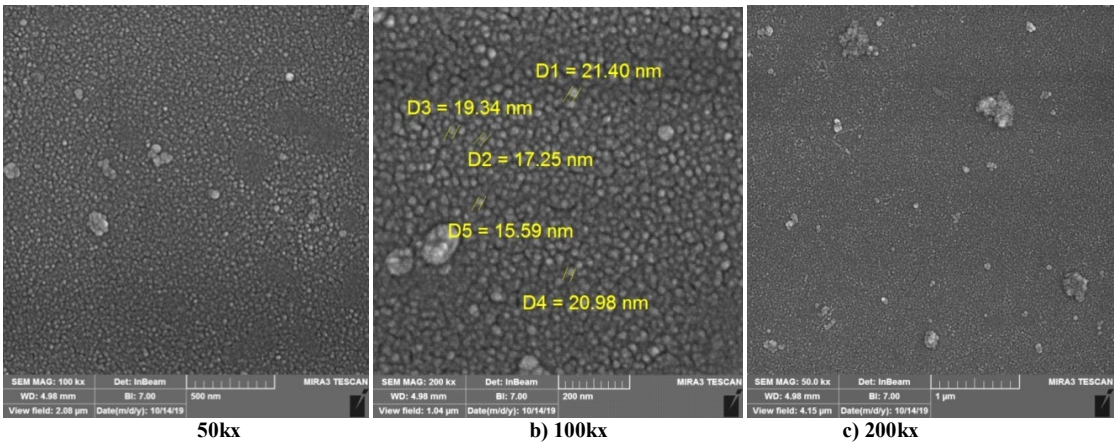
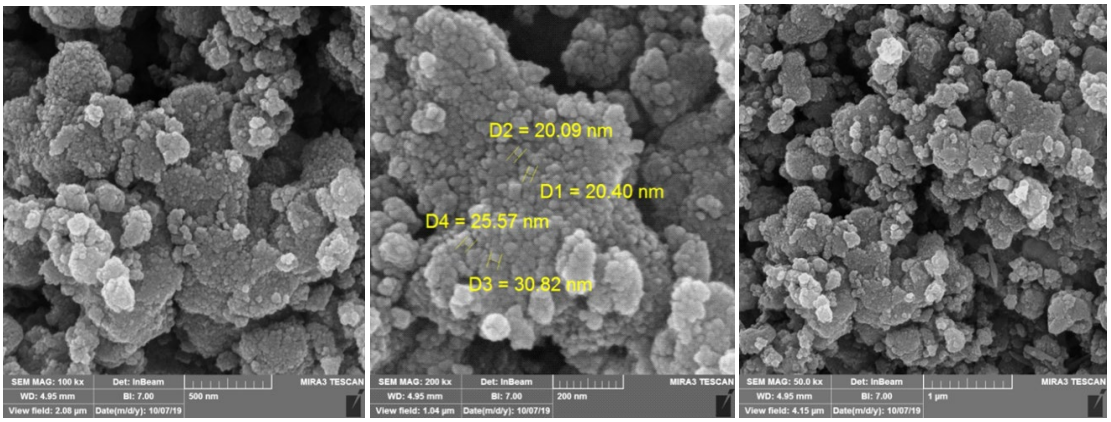


Figure 2. UV-visible absorption spectra of the synthesized TiO2 nanoparticles. The absorption peak was obtained in the visible range at 313nm.



50kx **b) 100kx** **c) 200kx**

Figure 3. Images of colloidal Ag nanoparticles by scanning electron microscopy (SEM).



a) 50 kx **b) 100kx** **c) 200kx**

Figure 4. Images of colloidal TiO2 nanoparticles by scanning electron microscopy (SEM).

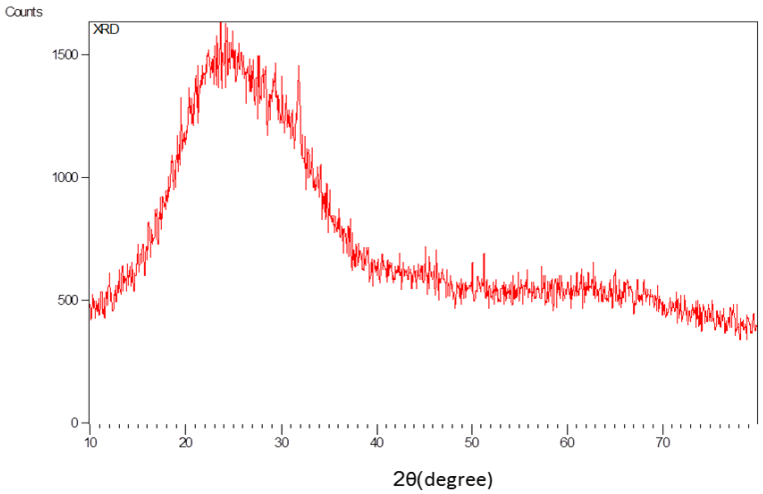


Figure 5. X-ray diffraction analysis of colloidal Ag nanoparticles.

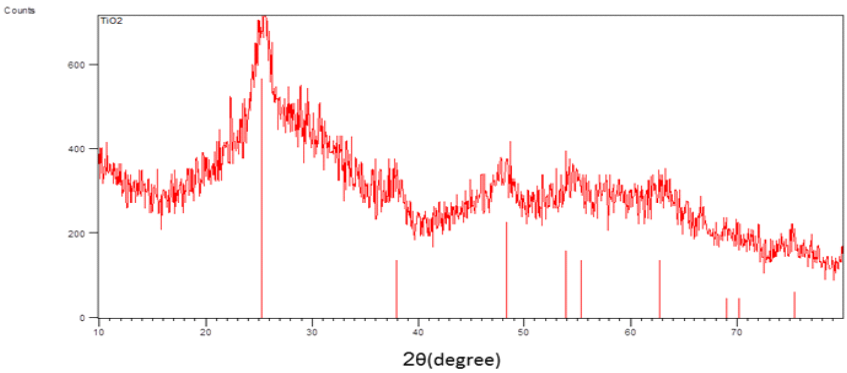


Figure 6. X-ray diffraction analysis of colloidal TiO2 nanoparticles.

Discussion

Characterization of Nanoparticles: The Ag colloid nanoparticles was confirmed by starting from colorless to darkish brown and UV-Vis was set to 200-900 wavelength, in which the 206 nm peak was obtained for Ag nanoparticles (Figure 1). The corresponding value for cTiO2 nanoparticles was 313 nm (Figure 2). The obtained wavelengths indicated the formation of nanoparticles [28]. The scanning electron microscopy (SEM) showed regular shaped spherical particles. The particles' surface was smooth and no beads were observed on the surfaces of the samples. These examinations led to the conclusion that the Ag and TiO2 nanoparticles were in an optimal size range commonly used for drug delivery.

Antibacterial Properties: We assessed the antibacterial properties and cytotoxicity of cAg and TiO2 nanoparticles on clinical samples of *S. sonei* and *S. aureus*. We found that the MIC and MBC values for Ag and TiO2 nanoparticles were similar (12.5 µg/ml) in the case of *S. aureus*. In The MICs for Ag and TiO2 nanoparticles against *S. sonei* were 12.5 and 25 µg/mL, respectively.

Our findings were in agreement with the results of a study conducted by Jayanta Kumar Patra and Kwang-Hyun Baek [29]. They reported that *S. aureus* showed higher sensitivity to Ag nanoparticles compared to the family of *Enterobacteriaceae* (MIC=12.5µg/mL) [29]. On the other hand, some studies have shown that the antimicrobial effect of Ag nanoparticles against gram negative bacteria was more potent than that of gram positive bacteria. An earlier study [30] confirmed the effect of TiO2 nanoparticles in eliminating a series of microorganisms, including *P. aeruginosa, S. aureus*, and *E. coli.* In this regard, another study [31] reported that MIC and MBC of cAg nanoparticles against *S. aureus* were 10 µg/mL. The different findings for gram-positive and -negative bacteria is likely due to the greater thickness of the peptidoglycans in the cell walls of gram-positive bacteria [32]. Also, this contradiction may be due to the size of the nanoparticle or the bacterial species.

The nanoparticles' positive electron charges absorb the negative charges on the plasma membrane of the bacterial cells [33, 34]. Also, the surface adsorption ratio is relatively high because

the surface to volume ratio is also high [35]. Further, the nanoparticles can be connected to sulfhydryl, carboxyl, and phosphate groups, affecting the enzymes and proteins of the bacterial respiratory chain [36]. Our study showed that physicochemical and biological properties, and the permeability of Ag nanoparticles were more than that of pure silver. Silver nanoparticles can be anchored to the bacterial cell membranes and cause alterations such as cellular contents drip, cell lysis and bacterial death [32, 37, 38]. In addition, TiO2 nanoparticles cause peroxidation of the cylindrical phospholipids in the bacterial membranes, disturbing the permeability [30].

Cytotoxic Effect: The current study findings showed that TiO2 nanoparticles had less cytotoxic effect (93.66% live cells) than that of Ag on the fibroblasts cell line (87.33% live cells), respectively, following 48 or 72 hr of incubation. Our findings also showed that the cytotoxicity of Ag and TiO2 nanoparticles decreased after 72hr of incubation. The decline in cytotoxicity after 72 hr was significant for Ag nanoparticles compared to 48hr of incubation. In 2018, another study [39] reported a significant rise in the survival of HCT116 cells line (62-68%) induced by TiO2 nanoparticles after 48hr of incubation. In contradiction to the current study, Huang, *et al*. [40] found that short-term exposure to nanoparticles improved the cell survival in fibroblasts, despite the fact that long-term exposure to TiO2 nanoparticles had no such effects.

In our cytotoxicity assays, the percentage viable fibroblasts was higher after exposure to TiO2 nanoparticles for 48 or 72 hours, compared to a similar treatment with Ag nanoparticles. In this regard, Jin, *et al.* [41] noted that TiO2 nanoparticles had no significant cytotoxicity on mouse fibroblast cell lines. Another study [42] showed that the increase in the concentration of Ag nanoparticles in normal and tumor human fibroblasts and epithelial cells decreased the cell viability and raised the apoptosis and the generation of reactive oxygen species (ROS).

Nevertheless, other studies have demonstrated that nanoparticles promote oxidative stress, which is responsible for mitochondrial injuries [43]. Further, it has been argued that cells exposed to TiO2 are faced with a dramatic reduction in their respiratory chains followed by inactivation of the cell regulation and signal transduction activities [30]. Earlier studies have also shown that Ag nanoparticles toxicity was significantly different versus various cell species [44]. Overall, these conflicting results suggest that the cytotoxicity of various chemicals largely depends on numerous factors, such as cell types, concentration, size, shape, surface areas, purity, crystal structure, surface charges, agglomeration rates, dosage, and exposure time to nanoparticles [39].

Conclusions

Based on the results of the current study, we discovered the various morphological and physicochemical properties of the two nanoparticles of Ag and TiO2. The results were indicative of the formation and production of small spherical particles at nanoscales. Besides, both Ag and TiO2 nanoparticles demonstrated satisfactory antimicrobial properties. In addition, TiO2 nanoparticles had the lowest toxicity level compared to that of Ag. Future studies are warranted on other microbial strains for systematic investigations of the antibacterial properties and cytotoxicity of these novel nanoparticles.

Conflict of Interests

The authors declare that there was no conflict of interests

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Compliance with Ethical Guidelines

The concept and protocol of this study were reviewed and approved by the Institutional Review Board of Iran University of Medical Sciences, Tehran, Iran, prior to its conduction (Ethics review & approval No.: IR.IUMS.REC1394.26604).

Authors' Contributions

Writing the original draft: Somayeh Soleymanzadeh Moghadam; Microbiology laboratory tests: Somayeh Soleymanzadeh Moghadam and Farideh Hajian Hossein Abadi; Nano laboratory tests: Samaneh Mazar Atabaki and Soheil Rahmani Fard; Methodology: Maliheh Nobakht and Zahra Mohammadi; Project Administration: Maliheh Nobakht; Supervision: Pedram Ebrahimnejad. Also, all authors participated in the final evaluation, editing, review and approval of the final draft of the manuscript prior to submission to this journal.

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