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Biological production of silver nanoparticles by soil isolated bacteria and preliminary study of their cytotoxicity and cutaneous wound healing efficiency in rat

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ABSTRACT

Biosynthesis of AgNPs by 37 different bacterial soil isolates was done and confirmed through visible spectrophotometry. Fifteen isolates were identified and two of them with the highest ability of AgNPs production were used for Transmission Electron Microscopy (TEM) and X-ray diffraction (XRD) tests. MTT assay for both of the obtained AgNPs was run and after determination their IC_{50s}, two different toxic and nontoxic doses of each AgNPs solution were chosen for wound healing assay. Forty eight rats were divided into 6 groups; two were the controls, two were administrated by the toxic and two were administrated by the nontoxic doses of AgNPs produced by *Bacillus cereus* and *Escherichia fergusonii*. Administration of the nontoxic doses of AgNPs had better wound healing effect than both of the toxic ones. The control groups had less wound healing properties. In conclusion, biologically produced AgNPs in their nontoxic doses accelerated the collagen formation and the epithelization and decelerated the angiogenesis and duration of completion the epithelization.

1. Introduction

Nanotechnology refers to an interdisciplinary field of applied science and has many applications in the medicinal field. There are various methods for nanoparticles production and two of them are physical and chemical ones [1]. It was shown that physical method for nanoparticles production has some disadvantages such as low rate of production, stability and dispersity [2]. In the chemical technique for nanoparticles synthesis it has been reported that remaining of some toxic reagents on the surfaces of the produced nanoparticles has led to concern about the use of them *in vivo*. Moreover, these two different methods for nanoparticles synthesis usually consume high energies, pressures and temperatures. Also release of the toxic by-products in the environment is another disadvantage of them [3].

Recently the third and new strategy for nanoparticles production is introduced and named the biological technique. This method is known as safe, eco-friendly and cheap. Also it is not time consuming manner and can be easily scaled-up [1]. It has been reported that some microorganisms such as different types of bacteria, fungi and viruses are able to produce metal nanoparticles by the enzymatic and non-enzymatic processes. In the enzymatic reaction, it has been demonstrated that metal nanoparticles are produced even intracellular or extracellular through the enzymes that are responsible for transporting the electrons from especial electron donors such as NADH and $FADH_2$ to the metal ions as the electron acceptors [4].

In the non-enzymatic reduction, certain groups of the polysaccharides or polypeptides such as aldehydes, ketones, carboxyls and amides are responsible for the bio-reduction of the metal ions into the relative nanoparticles [5]. Among different types of metal nanoparticles that are produced by microorganisms are gold [6], silver [7], silica [8], zirconia [9], cadmium [10] and titanium [8] ones. The application of these nanoparticles in the medicinal field is based on the physical and chemical properties of them and are different such as drug delivery [11], antimicrobial agents [12], medical imaging [13], *etc*. It has been reported that silver nanoaprticles (AgNPs) exhibited the best antimicrobial properties so are used widely in the medical field. Moreover, AgNPs have the ability to regulate the cytokines that are involved in wound healing process [14]. Furthermore, there are several available reports about the anti-inflammatory [15], anti-angiogenesis [16] and anti-platelet properties [17] of AgNPs. But almost all of these reports deal with AgNPs that are produced by the chemical and physical methods instead of the biological one.

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So based on the lack of enough knowledge about the cytotoxicity and wound healing properties of the biologically produced AgNPs, recent research was focused on the production of AgNPs by two different divisions of Gram positive and negative bacteria that were isolated from soil samples. Then bacteria and AgNPs were characterized separately. Purification and sterilization of AgNPs was done and their toxicity and wound healing activities were assessed by MTT method and histological studies in rat, respectively. Finally, the ability of production, toxicity and wound healing properties of AgNPs that were produced by Gram positive and negative bacteria were compared with each other.

2. Materials and methods

2.1. Isolation of the bacterial strains

Gram positive and negative bacteria were chosen from Bacillacea and Enterobacteriaceae families, respectively. Both of the bacterial isolates that belong to these two families were isolated from soil samples. For this aim, 10 different soil samples from the plant Rhizosphere of Shahrood agricultural regions were collected and sifted. After that, 1 g of each sample was suspended in 10 mL of ddH_2O. For Bacillacea isolation, soil suspension tubes were exposed to heat shock treatment in a water bath at 80 °C for 10 min [18]. Then 0.1 mL of each suspension was transferred to a flask containing the sterile Nutrient broth (Merck, Germany) medium and placed in a shaker incubator at 37 °C, 150 rpm for 24 h. After observation the turbidity of the medium, a loop full of each suspension was transferred onto the sterile Nutrient agar (Merck, Germany) medium and bacterial single colonies were achieved after incubation at 37 °C for 24 h. For Enterobacteriaceae isolation, 0.1 mL of each soil suspension was transferred to a flask containing the sterile Nutrient broth medium and placed in a shaker incubator at 37 °C, 150 rpm for 24 h. After observation the turbidity of the medium, a loop full of each suspension was transferred onto the sterile MacConkey agar (Merck, Germany) medium and bacterial single colonies were achieved after incubation at 37 °C for 24 h [19].

2.2. Extracellular production of silver nanoparticles

In order to produce AgNPs, a loop full of each bacterial single colony was cultured in a flask containing 50 mL of the sterile Nutrient broth medium and incubated in a shaker incubator at 37 °C, 150 rpm for 24 h. After turbidity achievement, the bacterial cells were harvested by centrifugation (6000 × *g* for 10 min) and the obtained supernatants were used for AgNPs production. For this aim, 20 μL of 1 M silver nitrate (Sigma–Aldrich, USA) solution was added to 20 mL of each bacterial supernatant (at a final concentration of 1 mmol) and the flasks were incubated in a shaker incubator at 37 °C, 200 rpm for 24 h. The negative control flasks were used and contained only the sterile Nutrient broth medium and the sterile Nutrient broth medium plus silver nitrate solution at a final concentration of 1 mmol. After incubation, flasks with the darkest color, which indicates the highest ability of AgNPs production, were chosen for further studies [20].

2.3. Identification of bacteria

2.3.1. Phenotyping tests

Phenotyping identification of bacterial isolates with the ability of AgNPs production was done according to the macroscopic and microscopic properties of them. After that, the characters of the isolates were compared with those listed in Bergey's Manual of Systematic Bacteriology [20].

2.3.2. Genotyping test

DNA extraction was performed using boiling method. For this aim, a loop full of each bacterial single colony was cultured in a flask containing 50 mL of the sterile Nutrient broth medium and incubated in a shaker incubator at 37 °C, 150 rpm for 24 h. Each sample was centrifuged at 14700 × *g* for 30 min and the pellet was washed three times in molecular biology-grade water and centrifuged (14700 × *g* for 30 min). The pellet was re-suspended in 100 μL of molecular biology-grade water and placed in a water bath at 100 °C for 5 min. The suspensions were centrifuged (14700 × *g* for 30 min) and DNA concentration of the samples was determined using Nanodrop spectrophotometer at 260 nm [21]. Partial bacterial 16S rDNA primers for the polymerase chain reaction (PCR) were 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) as the forward and reverse ones, respectively [22]. PCR reaction was performed according to the procedure previously described by Pourali et al. Finally, 5 μL of each PCR product was mixed with 1 μL of loading buffer and electrophoresed through 1% agarose gel that contained 0.5 μg/mL of ethidium bromide (Sigma–Aldrich, USA) in TBE (Tris/Borate/EDTA) buffer. Obtained DNA fragments were purified by the nucleic acid extraction kit (Viventis, USA) and the purified products were sequenced in one direction using 63F primer. Finally data that were obtained from the sequencing process were Blast using BLASTn program available at the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) [23].

2.4. Characterization of silver nanoparticles

2.4.1. Visible spectroscopy

Each of the color changed supernatant was analyzed under Nanodrop spectrophotometer at 300–700 nm. The bacterial supernatant was used as a blank [23].

2.4.2. Transmission electron microscopy (TEM)

Sizes and shapes of the produced AgNPs were obtained using TEM. Two AgNPs samples with the highest absorbance peaks that were obtained from the visible spectroscopy, from both of the bacterial families were chosen and 30 μL of each of them was placed on a carbon-coated copper grid. Excess of the solution was removed after 30 s and the grids were dried. TEM images were obtained by Philips 420T TEM [23].

2.4.3. X-ray diffraction analysis (XRD)

Since each crystal has its own X-ray pattern, the two AgNPs chosen samples that were used for TEM analysis were analyzed under XRD too. Each of the samples was freeze-dried and used for X-ray diffraction analysis by Philips Automatic X-ray Diffractometer. The diffracted intensities were from 30° to 80° 2θ angles [24].

2.4.4. Purification of silver nanoparticles

In order to use the produced AgNPs for *in vitro* and *in vivo* studies, it was important to wash the nanoparticles from the excess of silver nitrate ions, enzymes and other impurities of the bacterial culture supernatants that may interfere with the results. For this aim, 1 mL of each of AgNPs containing supernatant was transferred to a sterile 1.5 mL micro tube and centrifuged under mild conditions (2000 × *g* for 5 min). After that, obtained supernatant was transferred into a sterile 1.5 mL micro tube and centrifuged under strong conditions (14800 × *g* for 30 min). The supernatant was discharged and the pellet was re-suspended in 1 mL of the sterile phosphate-buffered saline (PBS). This step was repeated three times and finally the obtained AgNPs that were suspended in PBS were used for further studies.