Mugla Journal of Science and Technology

CYTOTOXICITY AND WOUND HEALING CAPACITY OF BIOLOGICALLY SYNTHESIZED SILVER NANOPARTICLES

Tuba BAYGAR*, Research Laboratories Center, Mugla Sitki Kocman University, Türkiye, tubaygar@mu.edu.tr
(https://orcid.org/ 0000-0002-1238-3227)
Nurdan SARAC, Department of Biology, Faculty of Science, Mugla Sitki Kocman University, Türkiye, sarac_63@hotmail.com
(https://orcid.org/0000-0001-7676-542X)
Aysel UGUR, Section of Medical Microbiology, Department of Basic Sciences, Faculty of Dentistry, Gazi University, Türkiye, ayselugur@hotmail.com
(https://orcid.org/0000-0002-5188-1106)

Received: 14.03.2022, Accepted: 28.05.2022 Research Article
*Corresponding author DOI: 10.22531/muglajsci.1087581

Abstract

Metallic nanoparticles are interesting areas of research due to their unique properties which can be advantageous for producing smart products. Silver nanoparticles (AgNPs) are remarkably used in pharmaceutical industry because of their strong biological activities. The aim of the present study was to investigate the cytotoxicity and wound healing capacity of the biologically prepared silver nanoparticles via green synthesis route. Cytotoxicity of the biogenic AgNPs was determined by MTT (3- [4, 5- dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide) assay against L929 fibroblast cell line. Wound healing properties of the AgNPs were evaluated using in vitro-scratch wound healing assay using 3T3 fibroblast cell line. Biosynthesized AgNPs inhibited the propagation of fibroblasts at a half maximal inhibitory concentration (IC_{50}) of 23.507 µg/mL after 24 h incubation. In vitro wound healing assay also revealed that the biogenic AgNPs stimulated the 3T3 fibroblasts' cell proliferation. It can be suggested that biologically synthesized AgNPs can be used effectively for biomedical applications such as wound dressing materials.

Keywords: silver nanoparticle, green synthesis, cytotoxicity, wound healing

BİYOLOJİK OLARAK SENTEZLENMİŞ GÜMÜŞ NANOPARTİKÜLLERİN SİTOTOKSİSİTESİ VE YARA İYİLEŞTİRME KAPASİTESİ

Özet

Metalik nanopartiküller, akıllı ürünler üretmek için avantajlı olabilecek benzersiz özelliklerinden dolayı ilgi çeken araştırma alanlarıdır. Gümüş nanopartiküller sahip oldukları güçlü biyolojik aktiviteler nedeniyle farmasötik endüstrisinde sıklıkla kullanılmaktadır. Mevcut çalışmanın amacı, yeşil sentez metodu üzerinden biyolojik olarak sentezlenmiş gümüş nanopartiküllerin sitotoksitesini ve yara iyileşme kapasitesini belirlemektir. Gümüş nanopartiküllerin sitotoksisitesi L929 fibroblast hücrelerine karşı MTT (3-[4,5-dimetiltiyazol-2-il]-2,5-difenil-tetrazolyum bromür) analiz yöntemiyle belirlenmiştir. Gümüş nanopartiküllerin yara iyileştirici özellikleri 3T3 fibroblast hücrelerine karşı in vitro çizik testi yöntemi ile belirlenmiştir. Biyosentetik gümüş nanopartiküllere ait 24 saat inkübasyon sonucunda hesaplanan IC_{50} değeri 23.507 µg/mL olarak belirlenmiştir. In vitro yara iyileşme testi sonucuna göre gümüş nanopartiküllerin 3T3 fibroblast hücrelerinin proliferasyonunu uyardığı belirlenmiştir. Çalışma sonucuna göre biyolojik olarak sentezlenen gümüş nanopartiküllerin yara örtüsü gibi biyomedikal uygulamalarda etkin şekilde kullanılabileceği öngörülmektedir.

Anahtar Kelimeler: gümüş nanopartikül, yeşil sentez, sitotoksisite, yara iyileşme

Cite

Baygar, T., Sarac, N., Ugur, A., (2022). "Cytotoxicity and Wound Healing Capacity of Biologically Synthesized Silver Nanoparticles", Mugla Journal of Science and Technology, 8(1), 63-68.

1. Introduction

Pharmaceutical technology has been focusing on developing new agents for diagnostic and therapeutic applications [1]. Nanotechnology and nanoscience are promising technologies to design and invent low-cost and efficient drugs for biomedical sectors. For improving the life quality, biocompatible nanomaterials that have physicochemically engineered properties may provide new strategies [2]. With their unique properties, silver nanoparticles (AgNPs) have been gaining great interest for several industries such as textile, pharmaceutical and food applications. Biological features such as antimicrobial [3], anticancer [4] and antitumor [5] activities of AgNPs have been studied.

There are several chemical and physical methods for nanoparticle synthesis which require toxic and expensive chemicals or high pressure and temperature levels [6]. Ecofriendly methods for nanoparticle synthesis are gaining much importance for saving the environment [7-11]. Microorganisms are recently becoming new tools for biocompatible and efficient metallic nanoparticle synthesis [12-14]. Employing the microorganisms for metallic nanoparticle synthesis is receiving increased attention as a more ecofriendly and simplier approach. When compared to other chemical and physical synthesis techniques, biogenic AgNPs are known to have higher antimicrobial activities with controlled sized and shapes [15]. According to Duran and Seabra [16], the biological caping agent that covers the microbiologically synthesized nanoparticle provides a higher stability as being a protective coating against agglomeration, aggregation and oxidation. Hence, those NPs are considered to be an advantageous option for antibacterial treatments [17]. Microbial NP synthesis is also an attractive route due to high capacity production, easy handling and low toxic resdiues [18]. Microorganisms act as nanofactories and play an important role in the reduction of metal ions and the bioremediation of toxic metals [19]. From this point of view, the *Streptomyces* genus, the largest member of Actinobacteria, emerges as important candidates for the extracellular synthesis of highly stable metallic nanoparticles [20]. The genus *Streptomyces* is an aerobic Gram-positive bacteria that can grow in different environments [21]. Actinobacterial strains, such as *Streptomyces*, are also known to produce important natural bioactive compounds such as antibiotics, antitumors and antimetabolites [22-24]. There are some studies about the biological synthesis and characterization of AgNPs using a Streptomyces strain [25-28]. Antioxidative [28], antimicrobial, antibiofilm [29], *in vitro* mutagenic and antimutagenic [30] properties of the biosynthesized AgNPs using *S. griseorubens* AU2 were also reported by our team with previous reports.

In the present study, the cytotoxicity and wound healing properties of silver nanoparticles biosynthesized by green synthesis method using a microorganism, *S. griseorubens* AU2, have been investigated.

2. Material and Methods

2.1. Biogenic silver nanoparticle synthesis

Silver nanoparticles (AgNPs) were extracellularly biosynthesized via a green synthesis method using *Streptomyces griseorubens* AU2 and extensively characterized in our previous report [28]. Briefly, cell-free *S. griseorubens* AU2 culture (10 mL) and 1 mM $AgNO_3$ (50 mL) were incubated in an orbital shaker at 130 rpm at 28 °C for 24-48h. The colour change of the flasks from opaque white to brownish yellow indicated the presence of AgNPs. The aqueous suspension of AgNPs were centrifugated, washed with distilled water for three times and the pellet was dried. The obtained AgNPs were used for further analysis.

2.2. Cytotoxic activity

2.2.1. Cell line

Mouse fibroblast cells (L929, ATCC CCL-1) that were obtained from American Type Culture Collection (ATCC) were used. Fibroblasts were grown in DMEM (Dulbecco's Modified Eagle Medium), incorporated with FBS (fetal bovine serum) and antibiotics (penicillin and streptomycin). For cellular proliferation, cell cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Cytotoxic activity

2.3.1. Cell line

Mouse fibroblast cells (L929, ATCC CCL-1) that were obtained from American Type Culture Collection (ATCC) were used. Fibroblasts were grown in DMEM (Dulbecco's Modified Eagle Medium), incorporated with FBS (fetal bovine serum) and antibiotics (penicillin and streptomycin). For cellular proliferation, cell cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3.2. MTT assay

Cell viability was examined by MTT (3- [4, 5- dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide) assay in order to investigate the IC_{50} value of the biogenic AgNPs. Briefly, L929 cells seeded in 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h for cell adhesion. Then, fibroblasts were exposed to various biogenic AgNPs concentrations (31.25, 15.63, 7.81, 3.91 and 1.95 µg/mL) and incubated for 24 h and 72 h. MTT (5 mg/mL, prepared in phosphate buffer saline) solution was applied to the wells after incubation periods. Present medium was poured after the incubation periods, 100 µL DMSO was applied to each well and the plates were put in an orbital shaker (Heidolph Unimax 1010, Germany) for 15 min. The absorbances at 540 nm were recorded using a microplate reader (BioTek, HTX Synergy, USA) (n = 8). Cell viability was calculated by the tetrazolium salt conversion to a coloured formazan crystals [31].

2.4. Wound healing capacity

2.4.1. Cell line

3T3 fibroblast cells (NIH/3T3, ATCC CRL-1658) that were obtained from ATCC were used. Fibroblasts were maintained in DMEM supplemented with FBS, antibiotics (penicillin and streptomycin) and L-glutamine. For cellular proliferation, cell cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

2.4.2. *In vitro* scratch wound healing assay

Wound healing assay, which is used to measure the migration rate of a cell population on scratched surfaces, was used to assess the expansion capability of 3T3 fibroblasts after exposing to biogenic AgNPs [32-33]. For determining the wound healing ability, exponentially

growing fibroblast cells were seeded onto a sterile cell culture dish (dimensions 60 × 10 mm) at a density of 75 × 10^4/cm^2 and were maintained for 24 h. The culture medium was changed periodically. After confluence was achieved, a linear area was scraped in the cell monolayer using a sterile 100 µl plastic pipette tip. The dishes were washed with DPBS (Dulbecco's phosphate buffered saline) solutions to remove the cellular debris. Thereafter, cells were treated with fresh media containing 20 µg/mL biogenic AgNP, which is determined below the calculated IC_{50} concentration of 24h, and maintained in culture for 72h. Control group was prepared with basal medium. The scratched areas from each cell culture dish were photographed using a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany) to evaluate the distance between adjacent layers of cells.

3. Results and Discussion

The results of cytotoxicity investigation of biogenic AgNPs on L 929 fibroblast cells can be seen in Figure 1. The % cell viabilities of L929 cells after biogenic AgNP treatment for 24 h and 72 h have been calculated.

The IC_{50} value calculated using the cell viability results of 24 h and 72 h was found to be 23.507 µg/mL and 19.696 µg/mL, respectively (Figure 1).

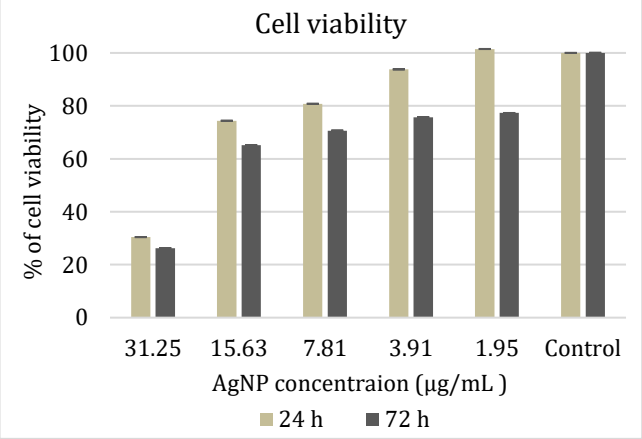

Figure 1. Cytotoxicity evaluation of biogenic AgNPs on L929 cell line.

Biocompatibility is one of the basic features of a biomaterial [34]. As it is given in Figure 1, the MTT analysis results have indicated a dose dependent matter on the viability percentage of L929 fibroblast cells. Składanowski et al. [14] reported that AgNPs biosynthesized from *Streptomyces* sp. NH28 strain did not exhibit any harmful interaction to L929 mouse fibroblast cell line and the IC_{50} value was established as 64.5 µg/mL. Dehghanizade et al. [35] who biologically synthesized AgNPs using *Anthemis atropatana* extract revealed out that the IC_{50} value of the AgNPs was 15.46 µg/ml against L929 cells was at 24 h. It was reported that larger-sized AgNPs exhibit low cytotoxic potential than smaller-sized nanoparticles [36-37]. The green synthesized AgNPs within the present study had average size of 5-20 nm [28]. Chowdhury et al. [38] demonstrated that the AgNPs naturally synthesized using cacao extract were spherical particles ranging in size from 35 to 42.5 nm and they were not cytotoxic to human dermal fibroblasts (HDFs) at concentrations below 32 µg/mL. In a study of Wei et al. [39] , AgNPs that had a size distribution of 50–100 nm, which are quite big than the present study, induced apoptosis and membrane damage in L929 cells and lead to cell death. Jahan et al. [40], who synthesized AgNPs using apple pulp (*Malus domestica*) and cumin (*Cuminum cyminum*) seed extracts, reported that the size distribution range of 5.46–20 nm and 1.84–20.57 nm, respectively. Similar to our study, AgNPs that were synthesized using both extracts were non-toxic against L929 mouse fibroblast cells. There are different factors such as dose, charge, shape and size of nanoparticles that effect the cellular response which is directly related to the cellular cytotoxicity [41]. According to Moadab et al. [42], the cellular toxicity effect of AgNPs is probably due to the interactions of intracellular biomolecules and silver atoms. It has also been recorded that biogenic NPs caused lower cytotoxicity when compared to silver ions or commercial AgNPs [43].

Cell migration is an important stage of the wound healing process and determines whether the applied active agent accelerates the complete closure of the wound [44]. To observe the effect of biogenic AgNPs on fibroblasts' proliferation, *in vitro* wound scratch method was applied on NIH-3T3 fibroblasts and images of the scratched area were taken at regular intervals. AgNP concentration was determined according to the MTT assay. Thus, 20 µg/mL AgNP was applied which is below the IC_{50} value. After AgNP treatment, fibroblasts were stimulated of the cell migration to heal the scar. Similar to the control group, scar on the AgNP-treated plates completely healed after 72 hours (Figure 2).

There are a few studies about the wound healing potential of microbiologically-synthesized AgNPs. Hu et al. [45], who synthesized AgNPs with a size <50 nm using mycelial extract of endophytic fungus Talaromyces purpureogenus, reported that the wound scratch distance of AgNP-treated NIH-3T3 cell group was relatively small when compared to control area. Kumar et al. [46], who biosynthesized AgNPs (at 38±2 nm sizes) using Aloe arborescens, reported promising migration and wound closure results in the wound healing assay of WS1 human skin fibroblast cells. In the present study, the biosynthesized AgNPs with 5–20 nm average size [28] displayed no negative effect on NIH-3T3 fibroblast proliferation but revealed out an increase in cell migration.

Wound healing process involves different steps as coagulation (hemostasis), inflammation, cellular proliferation and remodelling stages [47]. Wound infections that are caused by opportunistic pathogenic microorganims have been presenting a problematic issue for healthcare practices [48-49]. Combining the AgNPs with biomaterials are promising solutions for enhanced wound healing management [50].

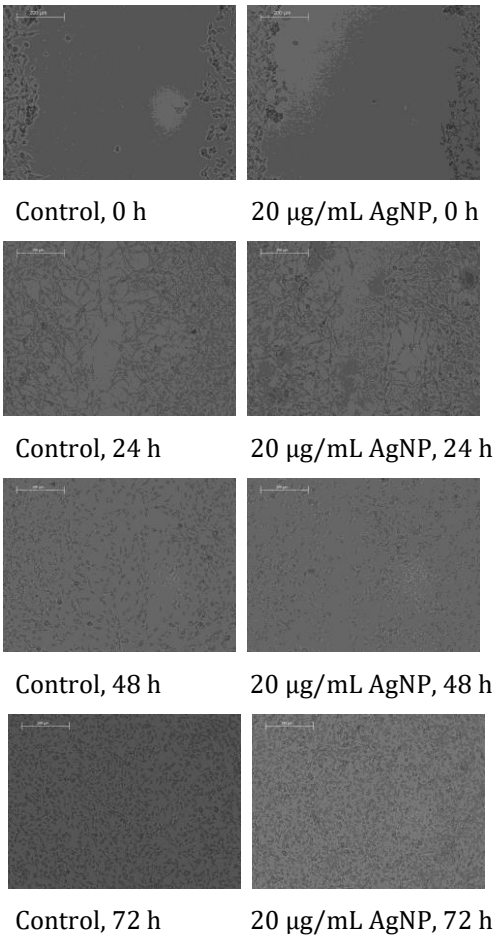

Figure 2. Images of *in vitro* scratch assay for 0., 24., 48. and 72. hours.

4. Conclusion

Green nanotechnology is a new route for inventing multifunctional pharmaceutical nano-agents for medical therapy. The genus *Streptomyces* has been used for bioactive secondary metabolite production for several years. Within the present study, silver nanoparticles that were biosynthesized extracellularly using *S. griseorubens* AU2 have been investigated for their cytotoxic and wound healing properties. A dose dependent cytotoxicity has been exhibited by biosynthesized nanoparticles with the IC_{50} value of 23.507 µg/mL towards L929 normal cell line. Wound healing assay revealed out that the biogenic silver nanoparticles had no negative effect on NIH-3T3 fibroblast proliferation. Besides, silver nanoparticles at a concentration of 20 µg/mL showed no toxicity response to the cells and the migration rate and wound closure were very similar to the control at 48h and 72h. With their wound healing potential, it can be concluded that biogenic silver nanoparticles that synthesized by microorganisms extracellularly might be useful for designing wound dressing materials.

5. Acknowledgment

Cytotoxicity analysis was performed at AUBIBAM (Anadolu University Medicinal Plants, Drugs and Scientific Research Center), Anadolu University, Eskişehir, Türkiye.

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