Research in Molecular Medicine

Synergism Effects of Vancomycin and Zinc Oxide Nanoparticles on Methicillin Resistance Staphylococcus aureus (MRSA) and Lung Cancer

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Citation Shokrzadeh Lamuki M, Hosseinzadegan M, Hafezi N, Valizadeh H, Mohammadi-Berenjestanak H. Synergism Effects of Vancomycin and Zinc Oxide Nanoparticles on Methicillin Resistance Staphylococcus aureus (MRSA) and Lung Cancer. Research in Molecular Medicine. 2023; 11(3):195-202. https://doi.org/10.32598/rmm.11.3.1328.1

https://doi.org/10.32598/rmm.11.3.1328.1

Article Type:
Research Paper

Article info:
Received: 03 Jun 2024
Revised: 02 Jul 2024
Accepted: 27 Jul 2024

Keywords:
Staphylococcus aureus, Methicillin-resistant *Staphylococcus aureus* (MRSA), Methicillin-susceptible *Staphylococcus aureus* (MSSA), Synergism, Zinc oxide nanoparticles (ZnO-NPs), Vancomycin

ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen and a historically emerging zoonotic pathogen of public health and veterinary importance. It can cause severe chronic infections. The morbidity of MRSA infections has increased worldwide and is of great concern. Nevertheless, a change in treatment strategies, including the use of new antibiotics or combination therapy, is necessary for the treatment of this infection. The research investigated the synergistic effects of vancomycin and zinc oxide on MRSA and the viability of the lung cancer cell line A549 and the normal cell line BEAS.

Materials and Methods: In this study, the minimum inhibitory concentration (MIC) of zinc oxide nanoparticles (ZnO-NPs) and vancomycin was determined using the microdilution method. The fractional inhibitory concentration index (FICI) was calculated using the checkerboard method to evaluate the synergistic effect of ZnO-NPs and vancomycin. The effect of the combination of ZnO-NPs and vancomycin on the viability of lung cancer cell line A549 was also tested by MTT assay.

Results: The MIC values showed that all isolates were sensitive to vancomycin with the exception ofexcept for one isolate with an MIC of ≤2 µg/mL. The synergistic effect of the combination of ZnO NPs and vancomycin was observed in two MRSA isolates and one MSSA strain using the checkerboard methodUsing the checkerboard method, the synergistic effect of the combination of ZnO-NPs and vancomycin was observed in two MRSA isolates and one MSSA strain. The combination of vancomycin and ZnO NPs caused less viability in the A549 lung cancer cell line (25.7%) than in BEAS (90%).

Conclusion: Combining vancomycin and ZnO-NPs at appropriate dosage intervals may be beneficial in treating MRSA. The combination of vancomycin and ZnO-NPs may also play a dual role in lung cancer patients with evidence of resistance to MRSA by reducing cancer cell survival.

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Introduction

Staphylococcus aureus was first identified in the 1880s in purulent fluid from a leg abscess [1]. *S. aureus* is a genus of ubiquitous gram-positive bacteria and is a major cause of endocarditis, bacteremia, osteomyelitis, and skin or soft tissue infections. With the advent of hospital medicine, *S. aureus* quickly became a major cause of healthcare-associated infections [2]. Genomic evidence suggests that methicillin resistance preceded the first clinical use of anti-staphylococcal penicillin [3]. Following the emergence of methicillin-resistant species in the 1960s, the global health community has become increasingly concerned. Methicillin-resistant *S. aureus* (MRSA) was first observed in clinical isolates from hospitalized patients in the 1960s but has spread rapidly in the population since the 1990s [4]. MRSA always shows a multidrug-resistant pattern to penicillin and various antimicrobial classes such as macrolides, fluoroquinolones, aminoglycosides, tetracyclines, and lincosamides. Methicillin resistance is mediated by mecA and acquired by horizontal transfer of a mobile genetic element called staphylococcal cassette chromosome mec (*SCCmec*). This gene encodes a penicillin-binding protein (PBP2a) that reduces the affinity for beta-lactam antibiotics [5]. Today, MRSA strains remain a severe challenge in hospitals. Treatment of patients infected with MRSA has been less effective than those infected with methicillin-susceptible *S. aureus* (MSSA), resulting in higher hospitalization rates and costs.

Vancomycin is one of the oldest antibiotics used clinically for almost 60 years. Vancomycin is effective against gram-positive bacteria such as staphylococci, enterococci, streptococci, pneumococci, and listeria. Recently, vancomycin is usually used for infections caused by MRSA. However, in some cases, vancomycin is ineffective because it penetrates weakly into the tissue, does not act against biofilms, does not inhibit toxin production, and has only a weak inhibitory effect on bacteria. Therefore, there is an urgent need to find effective treatments for these infections. Since developing new antibiotics is difficult, expensive, and time-consuming, researchers focus more on combination therapy as an alternative to treating the infection [6-8]. Nowadays, nanotechnology represents an innovative approach to developing new formulations based on the antimicrobial properties of metallic nanoparticles [9]. Nanoparticles of silver, copper, zinc, iron, titanium, and metal oxide are considered antimicrobial agents against multidrug-resistant bacteria [10-12]. The interaction of antibiotics with nanoparticles is the most common among studies testing the combined effect of nanoparticles with antibiotics. Therefore, nanoparticles could be used as a safe alternative strategy for antibacterial activities. Some studies have found that the efficacy of antimicrobial agents can be enhanced by combining them with nanoparticles against various pathogens, including *S. aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* [9]. Zinc oxide nanoparticles (ZnO-NPs) show potential antibacterial activity in gram-positive and gram-negative bacteria. Treatment of bacterial cells with ZnO-NPs leads to the formation of reactive oxygen species (ROS), lipid peroxidation, and the release ofing bacterial cells with ZnO-NPs leads to the formation of ROS, lipid peroxidation, and releasing reducing sugars, proteins, and DNA from the membrane [13]. The molecular weight of zinc oxide is 81.38 g/mol, and its density is 5.606 g/cm^3 [14]. The exact physical and chemical properties of ZnO-NPs depend on the different methods used to synthesize them. ZnO-NPs have unique physical and chemical properties such as high chemical stability, high electrochemical coupling coefficient, a broad spectrum of radiation absorption, paramagnetic character, and high photostability [15].

Previous studies have also shown improved ZnO nanoparticles' activity when combined with cephalosporins, beta-lactams, and aminoglycosides against various pathogenic microorganisms [9]. Despite the promising results that nanomaterials have achieved regarding their antibacterial effect, some problems still prevent their use on a clinical scale. Knowledge about the interaction of nanomaterials with cells and tissues is still limited. Therefore, before nanomaterials are used as antibacterial drugs, a complete evaluation and risk assessment of their side effects must be done. Recent research shows intravenously injected NPs can accumulate in the bone marrow, liver, lung, colon, and spleen [16]. Some studies also showed the interaction of antibacterial nanomaterials with cells and the generation of intracellular oxidative stress by free radicals, causing hepatotoxicity and pulmonary toxicity. Nanoparticles can cause hemolysis of red blood cells, abnormal sedimentation, hemagglutination, and disruption of chromosome segregation and centrosome proliferation [17]. In general, the toxicity of nanoparticles such as ZnO depends on the size, concentration, and duration of treatment [18].

Today, the effects of nanoparticles on the viability of normal and cancer cells are of great importance. Combining antibacterial drugs and ZnO-NPs can be a promising drug delivery system in inhibiting cancer cells. Since the response of cancer cells to drugs is non-consistent, a quantitative analysis of synergistic, additive, and antagonistic effects is critical before investigating sensitiv-

ity [19]. The human lung adenocarcinoma A549 is well suited as a lung epithelial cell line for in vitro toxicity studies of nanomaterials [20]. When studying the effect of ZnO nanoparticles on cell growth and metabolism, the MTT method can be used to evaluate cell survival [21]. In this study, we investigated the effect of the combination of vancomycin and zinc oxide in inhibiting the cancer cell line at different doses. In addition, the BAES cell line was studied as a normal cell line to control the effect of the combination of vancomycin and zinc oxide.

Materials and Methods

Clinical samples collection and isolation of MRSA and MSSA

Sixteen *S. aureus* isolates, including 14 MRSA and 2 MSSA, were obtained from patients hospitalized at Imam Khomeini Hospital in Sari City, Iran. The samples were collected in sterile bottles and brought to the laboratory to isolate the *S. aureus* bacterial strains. We analyzed the samples to identify the microorganisms and determine their antibiotic susceptibility. Each MRSA isolate was confirmed with cefoxitin using the disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI). In addition, the presence of the mecA gene in each MRSA isolate was verified by polymerase chain reaction (PCR). The MRSA strain COL served as a control for all tests.

Minimum inhibitory concentration (MIC)

The broth microdilution method was used for each bacterial sample to determine the MIC. MIC is defined as the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism after overnight incubation. Diagnostic laboratories mainly use MICs to confirm resistance, but they are most commonly used as a research tool to determine the in vitro activity of new antimicrobials [22]. The antimicrobial activity of vancomycin and ZnO-NPs was evaluated using the broth microdilution method. For this purpose, successive concentrations of zinc oxide 250, 125, 62.5, 32, 16, 8 µg/mL and vancomycin 8, 4, 2, 1, 0.5 µg/mL were diluted in sterile distilled water and added separately to Mueller-Hinton broth (MHB) culture medium. Then, 1.5×10^5 of the MRSA and MSSA isolates were inoculated. At the end, the microplate was evaluated after incubation at 37 °C for 18–24 hours, and the OD was read at 570-630 nm. The MIC is determined as the concentration of the drug that inhibits the visible growth of the bacteria.

Checkerboard method

The checkerboard method is used to evaluate the interaction between the antibiotic of choice and the ZnO-NPs. To determine synergistic effects with combined drugs, we used the checkerboard method containing different ZnO-NPs and vancomycin concentrations. This method added vancomycin at the highest concentration (16 mg/mL) to the first 96-well microplate column, then decreased doublings until the last column. Then, the zinc oxide nanoparticle at the highest concentration (500 mg/mL) was added to the first row, followed by decreasing doublings to the last row, and a combination of the concentrations of the two drugs was added to the remaining wells. The bacterial inoculum was approximately 5×10^5 CFU/mL. Finally, the microplate was incubated at 37 °C for 18-24 hours.

After incubation, the microplate was read at 570-630 nm using an ELISA reader. The fractional inhibitory concentration index (FICI) was calculated for each combination. The mean FICI of all turbidity-free wells along the boundary between turbidity and non-turbidity was then calculated [9]. To determine the correlation between the two drugs, the FICI was calculated using the Equation 1:

1. $FICI=FIC_{Ab}+FIC_{NP}$

where FIC_{Ab}=(MIC of Ab in the presence of NP)/ (MIC of Ab alone)

and FIC_{NP}=(MIC of NP in the presence of Ab)/(MIC of NP alone). Then, the FICI value was interpreted following the interpretation ranges.

If the result of the FICI is less than or equal to 0.5, it is considered a synergy; if it is >4, it is considered an antagonism; if it is between >0.5 and ≤1, it is an additive result; and if it is between >1 and ≤4, it is considered an indifference.

Evaluation of the viability effect of ZnO-NPs and vancomycin at single and combined concentrations on the human lung cancer cell line A549 and the normal human epithelial cell line BEAS by MTT assay

Two different cell lines, including A-549, a lung cancer cell line, and BEAS, a normal human epithelial cell line, were cultured and scattered in 96-well cell culture plates containing DMEM/high glucose (ATOCEL, Austria) with 10% fetal bovine serum (FBS, west, USA) at a concentration of 1×10^5 cells/well. The cells were

incubated at 37 ˚C and 5% CO_2 for 48 hours to reach their logarithmic growth phase. Subsequently, different amounts of vancomycin (8, 4, 2, 1, and 0.5 µg/mL) and zinc nanoparticles (500, 250, 125, 62, and 32 µg/mL) were added separately and combined with triplicate culture cells. A positive control (doxorubicin) and a negative control (culture medium without cells) were also included. After 72 hours of incubation at 37 ˚C, 20 µL MTT solution (5 mg/mL) was added to each well. After incubation for 4 hours at 37 ˚C, the MTT solution was removed, and 200 µL DMSO solution was added. Then, everything was mixed with the pipette and incubated at 37 ˚C for 10 minutes. The viability of the cells was determined in the microplate at 570 nm.

In this study, data analysis was done by Excel and Prism software. In addition, the comparison between the groups was carried out using the t-test.

Results

MIC method

Antimicrobial susceptibility testing of ZnO-NPs and vancomycin against 13 clinical MRSA isolates, the standard MRSA strain, and two clinical MSSA isolates was performed in broth using the standard microdilution method (Table 1). The MIC for zinc oxide was 7.1 of the MRSA isolates at a concentration of 16 and 32 µg/mL and 42.8% and 28.5% of the MRSA isolates at 62.5 and 125 µg/mL, respectively. In addition, the MIC in 50% of the MSSA isolates was 125 or 250. The MIC values for vancomycin at concentrations of 0.5, 1, and 4 µg/mL were 58%, 42.8%, and 7.1% in MRSA isolates. According to the MIC data, all isolates except one were sensitive to vancomycin, with an MIC of ≤2 µg/mL. The MIC of the remaining isolate was 4 µg/mL.

The revised CLSI guidelines define an *S. aureus* strain with an MIC of ≤2 µg/mL as vancomycin-sensitive *S. aureus*. Also, *S. aureus* with an MIC between 4 and 8 µg/mL is considered a vancomycin-intermediately sensitive *S. aureus* strain, and a strain with an MIC of >8 µg/mL is considered a vancomycin-resistant *S. aureus* strain [23].

Checkerboard method

The concentration for the checkerboard test was selected according to the MIC data. This method showed the best synergistic effect when two drugs were combined. The MIC and FIC results are shown in Table 2. The lowest FICI values of the combinations are listed in this Table. These lowest FICI values were determined as a synergistic effect based on the defined standards.

Specifically, the synergistic effect of vancomycin and zinc oxide was observed in two MRSA isolates, 214 (FICI 0.46) and 217 (FICI 0.32), and in one MSSA strain: 232 (FICI 0.31).

MTT assay

According to the results of the checkerboard method, the best concentrations of vancomycin and ZnO-NPs were selected in this step. The viability result is shown in Figure 1.

In brief, according to the results of the MTT test with A-549, viability was shown at the sole concentration of ZnO-NPs (16, 32, 62.5, 125 µg/mL) (100%, 99.73%,

Table 1. MIC results of vancomycin and zinc oxide against 14 MRSA and 2 MSSA strains

Isolated	MIC Concentration Nanoparticle Zinc Oxide (µg/mL)	No. (%) Result	MIC Concentration Antibiotic Vancomycin (µg/mL)	No. (%) Result
MRSA	16	1.14(7.1)	0.5	7.14(58)
	32	1.14(7.1)		
	62.5	6.14(42.8)	1	6.14(42.8)
	125	4.14(28.5)		
	250	1.14(7.1)	4	1.14(7.1)
MSSA	125	1.2(50)	0.5	1.2(50)
	62.5	1.2(50)	1	1.2(50)

Table 2. Result of the checkerboard method

Isolates	MIC_{VAN}	$MIC_{Zno\ NPs}$	FIC_{VAN}	$FIC_{Zno\ NPs}$	$FICI_{VAN+Zno\ NPs}$	Result
S214	0.5	62.5	0.4	0.06	0.46	Synergism
S232	1	62.5	0.25	0.06	0.31	Synergism
S217	1	125	0.2	0.12	0.32	Synergism

Abbreviations: MIC: Minimum inhibitory concentration; FIC: Fractional inhibitory concentration; VAN: Vancomycin; ZnO-NPs: Zinc oxide nanoparticles.

76%, 25.8%) and vancomycin (0.5, 1, 2, 4, 8 µg/mL) (100%, 100%, 99.85%, 99.75%, 99.13%).

The result of the viability of combined concentrations of zinc oxide with vancomycin (62.5+0.5, 62.5+1, 125+1 µg/mL) on the A-549 cell line was (76 %, 76.01%, and 25.7) respectively. The viability of BEAS cells under the influence of various doses of zinc oxide, vancomycin, and a combination of these factors was 90 and above. Additionally, no significant relationship was observed between the two groups (P=0.14).

Vancomycin had no cytotoxic effect on the A-549 and BEAS cell lines. Zinc oxide inhibited cell growth at 125 µg/mL against the A-549 cell line.

Discussion

The infection caused by MRSA is a global public health threat. This infection has a high morbidity and mortality rate and represents a significant financial burden for the patient. Based on a meta-analysis in 2023, the pooled global prevalence of MRSA was 14.69%. Vancomycin remains one of the drugs of first choice for treating MRSA infections. However, in recent years, *S. aureus* isolates with complete resistance to vancomycin have emerged. Nowadays, metallic nanoparticles have shown promising results in the fight against multidrug-resistant bacteria [24-26]. Recent innovations in nanotechnology have led to significant changes in the field of medicine, such as using nanoparticles to treat diseases, e.g. in cancer therapy and combination therapy for resistant bacte-

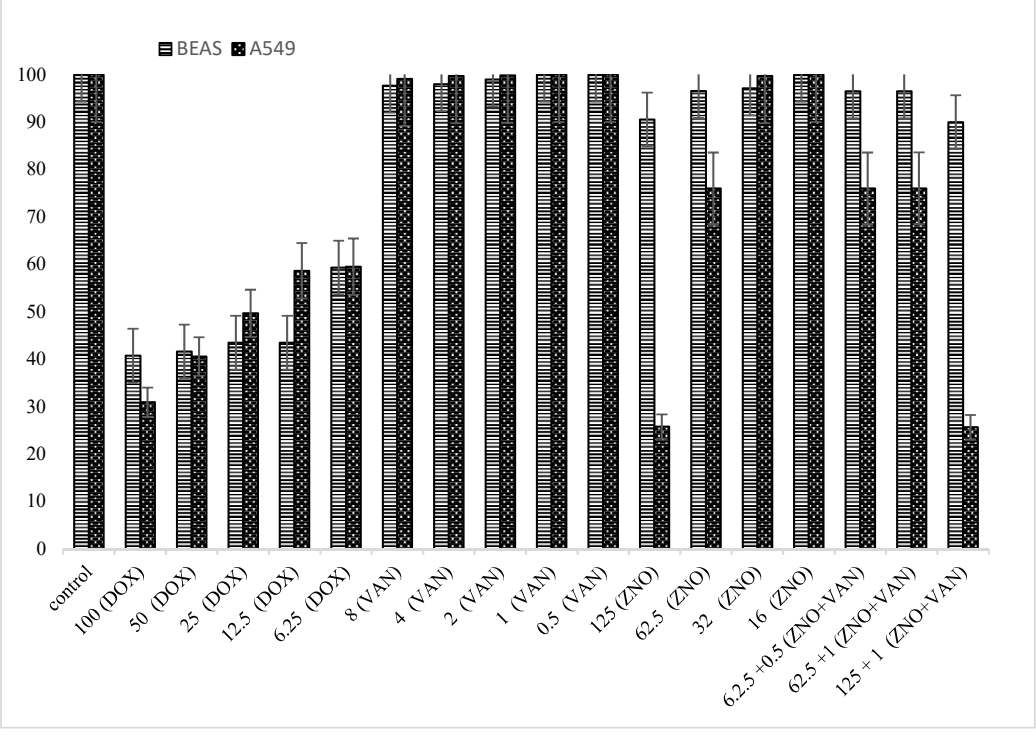

Figure 1. The result of viability with MTT assay

rial infections. Among metal oxide nanoparticles, ZnO nanoparticles have many important properties, such as chemical and physical stability, high catalysis activity, and effective antibacterial action. The antimicrobial effect of ZnO-NPs against MRSA strains, alone or in combination with antibiotics, has already been suggested. Interestingly, ZnO-NPs have been identified by several reports as non-toxic to human cells. Our study focused on developing promising alternative agents for treating these severe infections, such as ZnO-NPs. ZnO-NPs inhibit the growth of bacterial cells through the production of ROS, followed by membrane leakage of proteins, nucleic acids, and lipid peroxidation, and prevent the formation of biofilms. ZnO-NPs should penetrate bacterial cells to develop antibacterial activity. This study investigated the synergistic effect of ZnO-NPs with vancomycin on 13 clinical MRSA and two clinical MSSA isolates [27-30]. Broth dilution can be considered an accurate and confirmatory method to identify the antibacterial activity of ZnO-NPs. Our study has shown that the MICs of ZnO-NPs in *S. aureus* isolates ranged from 16 to 250 µg/mL when using the broth dilution method. Other studies have also reported that the bactericidal effect of ZnO-NPs is concentration-dependent [31]. In a Sharma et al. study, the MIC of ZnO-NP against *S. aureus* was 4000 µg/mL [32]. Jesline et al. reported that ZnO-NPs alone, without antibiotic combination, and at all concentrations significantly inhibited MRSA growth. ZnO-NPs could inhibit bacterial growth and achieve a maximum zone of inhibition of 16 and 17 mm at 500 µg/mL and a minimum zone of inhibition of 12 and 14 mm at 100 µg/mL [33]. Based on the data, all isolates except one were sensitive to vancomycin. In line with other research, it was found that our sensitive MIC values are closely linked to MIC values in the sensitive range. These findings agree with Thamer and Alsammak, who observed that the efficacy of vancomycin was improved in combination with ZnO nanoparticles [34]. Venubabu Thati et al. [35] and Namasivayam et al. [36] also reported that nanoparticles showed enhanced activity with several antibiotics against all tested *S. aureus*. As is well known, ZnO-NPs are antibacterial and can inhibit the growth of microorganisms by penetrating the cell membrane and causing oxidative stress and damage to lipids, carbohydrates, proteins, and DNA. Lipid peroxidation causes changes in the cell membrane that eventually disrupt vital cell functions [37, 38]. In our present study, the effect of ZnO-NPs suspension and vancomycin was evaluated using the checkerboard assay, and the FICI was calculated by evaluating the degree of interaction between ZnO-NPs and vancomycin against 13 clinical MRSA and two clinical MSSA isolates. Vancomycin showed a synergistic interaction with ZnO-NPs against two MRSA isolates, 214 (FICI 0.46) and 217 (FICI 0.32), and in one MSSA strain, 232 (FICI 0.31).

This study investigated the effect of vancomycin and ZnO-NPs on the viability of lung cancer cell lines using the MTT method. In relation to the role of combining vancomycin with ZnO-NPs, previous studies have shown that the drug's efficacy increases when combined with ZnO-NPs. ZnO-NPs have shown significant cytotoxic effects on A549 cells. Studies suggest that ZnO-NPs can induce apoptosis via mechanisms involving increased ROS production and mitochondrial dysfunction, leading to cell death [39]. In addition, previous studies have shown that treatment with ZnO-NPs and vancomycin leads to significant activation of pre-apoptotic signaling pathways, including increased activity of caspases, which are very important for the execution of apoptosis in cancer cells [40]. The synergistic use of vancomycin and ZnO-NPs could provide a dual approach against lung cancer cells and bacterial infections. Studies show that ZnO-NPs inhibit the growth of bacteria and increase the efficacy of antibiotics such as vancomycin against resistant strains. This dual action could be particularly beneficial for lung cancer patients at risk for infections due to compromised immune systems from chemotherapy [41].

It has been previously described that ZnO-NPs decreased the viability of colon cancer cells. The cytotoxic mechanisms of ZnO-NPs are attributed to increased production of ROS levels and decreased mitochondrial membrane potential [42]. According to Heng et al., more than 99% of human bronchial epithelial cells remained viable up to 10 µg/mL of ZnO-NPs but considerably decreased at concentrations below 10 µg/mL [43]. Similar findings were reported in mouse embryo fibroblast cells, which at 10 and 20 µg/mL ZnO-NPs, the viability of cells was nearly 80% and 20%, respectively [44].

In conclusion, integrating ZnO nanoparticles with vancomycin offers a promising strategy for improving treatment protocols against MRSA infections in lung cancer patients. This approach targets the malignancy and addresses potential secondary infections, enhancing overall treatment efficacy and patient safety. Further research is warranted to explore this combination treatment's full therapeutic potential and mechanisms.

Ethical Considerations

Compliance with ethical guidelines

This study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Mazandaran University of Medical Sciences, Sari Iran (Code: IR.MAZUMS.REC.1399.7102).

Funding

This study was funded by Mazandaran University of Medical Sciences, Sari Iran (Grant No.: 7102).

Authors contribution's

Methodology and investigations: Hamideh Mohammadi-Berenjestanaki; Data collection: Nasim Hafezi, Hanieh Valizadeh and Motahare Hosseinzadegan; Experiments: Motahare Hosseinzadegan; Writing the original draft: Nasim Hafezi and Hanieh Valizadeh; Data analysis, review and editing: Mohammad Shokrzadeh Lamuki; Conceptualization, study design and final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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