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Original Article

تاثیر نانوذرات Ag، Cu و ZnO بر بیان *TNF-α* در موش‌های Balb/C آلوده به *استافیلوکوکوس اورئوس*

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چکیده

سابقه و هدف: مقاومت به آنتی‌بیوتیک‌ها، به‌ویژه در *استافیلوکوکوس اورئوس* مقاوم به متی‌سیلین (MRSA)، یک چالش جهانی است. نانوذرات (NPs) مانند نقره (Ag)، مس (Cu) و اکسید روی (ZnO) پتانسیل ضدباکتریایی دارند. این مطالعه اثربخشی این نانوذرات را علیه MRSA و تأثیرشان بر بیان ژن *TNF-α* در موش‌های Balb/c بررسی کرد.

مواد و روش‌ها: ۶۳ موش به ۹ گروه (۷تایی) تقسیم شدند. گروه‌های ۵ تا ۹ با MRSA آلوده و سپس با نانوذرات Ag (15.625 mg/L)، Cu (62.5 mg/L)، Zno وانکومایسین یا محلول نمکی درمان شدند. پس از ۱ و ۵ روز، بیان *TNF-α* در طحال با Real-Time PCR سنجش شد. همچنین MIC و MBC نانوذرات تعیین گردید.

یافته‌ها: نانوذرات Ag و Cu اثرات بازدارندگی و باکتریکشی داشتند، درحالی‌که ZnO بی‌اثر بود. در روز ۵، بیان *TNF-α* در گروه وانکومایسین به‌طور معنی‌داری افزایش یافت و پس از آن نانوذرات Ag، Cu و ZnO بیشترین اثر را نشان دادند. در روز ۱، تنها وانکومایسین، Ag و Cu بیان *TNF-α* را افزایش دادند.

نتیجه‌گیری: نانوذرات Ag بیشترین تأثیر را بر بیان *TNF-α* داشتند، که نشان‌دهنده پتانسیل ضدباکتریایی برتر آن‌هاست. این نتایج از کاربرد نانوذرات به‌عنوان جایگزین یا مکمل آنتی‌بیوتیک‌ها در عفونت‌های MRSA حمایت می‌کند.

واژگان کلیدی: *استافیلوکوکوس اورئوس*، MRSA، *TNF-α*، نانوذرات، MIC، MBC.

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Original Article

The Effect of Ag, Cu, and ZnO Nanoparticles on *TNF-α* Expression in *Staphylococcus aureus*-Infected Balb/C Mice

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Abstract

Background & Objectives: Antibiotic resistance, especially in methicillin-resistant *Staphylococcus aureus* (MRSA), is a global challenge. Nanoparticles (NPs) such as silver (Ag), copper (Cu), and zinc oxide (ZnO) have shown antibacterial potential. This study aimed to evaluate the effectiveness of these nanoparticles against MRSA and their impact on *TNF-α* gene expression in Balb/c mice.

Materials & Methods: Sixty-three mice were divided into nine groups of seven. Groups 5 to 9 were infected with MRSA and then treated with Ag (15.625 mg/L), Cu (62.5 mg/L), ZnO nanoparticles, vancomycin, or saline solution. *TNF-α* expression in the spleen was measured by Real-Time PCR on days 1 and 5. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the nanoparticles were also determined.

Results: Ag and Cu nanoparticles showed inhibitory and bactericidal effects, while ZnO was ineffective. On day 5, *TNF-α* expression significantly increased in the vancomycin group, followed by Ag, Cu, and ZnO groups. On day 1, only vancomycin, Ag, and Cu nanoparticles increased *TNF-α* expression.

Conclusion: These findings indicate that Ag NPs, through modulation of *TNF-α* expression in Balb/c mice, may serve as promising antibacterial agents warranting further translational studies.

Keywords: *Staphylococcus aureus*, MRSA, *TNF-α*, Nanoparticles, MIC, MBC.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen responsible for both hospital-acquired and community-acquired infections. Its ability to cause diverse infections stems from its wide array of virulence factors. This pathogen is considered one of the most undeniable health problems in the world due to its potential pathogenicity and increasing resistance to antimicrobial drugs (1). Methicillin resistance is caused by *SCCmec* chromosomal segment, which contains the *mecA* gene. This gene encodes a protein called *PBP2a*

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(penicillin-binding protein), which is involved in bacterial cell wall synthesis and has a lower affinity for methicillin than other penicillin-binding proteins (2). Generally, conventional antibiotics are natural metabolites produced by a wide range of fungal and bacterial strains against other microorganisms. In recent decades, synthetic antibiotics have been developed to treat infectious diseases caused by bacterial pathogens in humans and animals. Many strategies like chemical modifications in natural antibiotics have boosted the efficiency through increasing solubility and pharmacokinetics. Structural modifications and changes in target binding sites are two important strategies to enhance antibiotic function, like modifications in sulfanilamide, which have brought many achievements to β-lactams. Despite successes in the design and production of new antibiotics, it seems that the current antibiotics are losing their bactericidal or bacteriostatic properties as microbial pathogens continuously neutralize their effects. Bacteria become resistant to antibiotics due to chromosomal changes and genetic mutations caused by plasmids and transposons (3). Today, antibiotic resistance in bacteria is one of the human concerns, which is the substantial cause of patient treatment failure and increased mortality. Given the rapid emergence of antibiotic resistance, there is an urgent need to develop novel therapeutic strategies (4).

Nanotechnology has opened new avenues for combating antibiotic resistance by enabling the development of nanoparticle (NP)-based antimicrobial agents, including silver (Ag), gold (Au), copper (Cu), zinc oxide (ZnO), and other metallic NPs (5). Due to their unique characteristics, like increased surface-to-volume ratio, nanoscale size, specific physical structure, and strong interaction with bacterial cell wall, NPs have many capabilities in diagnosis, treatment, drug delivery systems, and gene therapy (1,6). NPs cause immediate cell damage by destroying the integrity and continuity of the cell membrane, oxidative stress, and apoptosis in the treated cells. Some NPs, like Ag NPs, kill the bacteria through DNA fracture, increasing lactate dehydrogenase leakage, activating reactive oxygen species, and regulating different signaling molecules. The difference between the negative charge of the microorganism and the positive charge of the NPs acts as an absorbing electromagnet, which lets the NPs bind to the cell surface and causes cell death. The ions released from NPs interact with thiol groups of bacterial surface proteins and delay bacterial cell adhesion and biofilm formation (1), which prevents *S. aureus* growth, stabilization, and reproduction (7).

Tumor necrosis factor-α (*TNF-α*) is a pro-inflammatory cytokine produced by activated macrophages, B lymphocytes, and T lymphocytes. It is one of the main inflammatory cytokines and plays a central role in host defense, inflammation, and immune system function, which is related to the pathogenesis, development, and progression of various infections, autoimmune diseases, and malignant diseases. Bacterial lipopolysaccharide (LPS) is the most important inducer of *TNF-α* production in the immune system (8). Continuation of the immune reaction due to inappropriate and excessive production of *TNF-α* cause some inflammatory or autoimmune diseases, so neutralizing and blocking its receptors is the main strategy in the treatment of these diseases. Many studies have investigated the effects of NPs on the production of cytokines, and their toxic effects on the proliferation of peripheral blood mononuclear cells (PBMCs) (9). For

example, in one study, the protein levels of interleukin-5 (IL-5), interferon-γ (IFN-γ), and *TNF-α* were measured to determine the activation of PBMCs. The findings revealed that at high levels of 15 ppm, Ag NPs showed a significant toxicity effect in PBMCs, and cytokine products caused by phytohemagglutinin were significantly inhibited by Ag NPs (IL-5: 10 ppm, INF-γ, and TNF-α: 3 ppm) (10). Although Ag NPs have a cytotoxic effect in high concentrations, the production of cytokines with Ag NPs is concentration-dependent (11).

In this study, we aimed to evaluate the antibacterial effects of silver (Ag), copper (Cu), and zinc oxide (ZnO) nanoparticles against MRSA (*Staphylococcus aureus* ATCC 33591, American Type Culture Collection) by determining their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values using disk diffusion, agar dilution, and microdilution assays. Additionally, we investigated the modulatory effects of these NPs on *TNF-α* gene expression of *S. aureus* infected Balb/C mice model.

Materials and methods

A. Bacterial preparation and cultivation: MRSA (*Staphylococcus aureus* ATCC 33591, American Type Culture Collection) was obtained from the Molecular Medicine Department, Research Institute for Science and Industry, Iran. Before use, the bacterial strain was sub-cultured in Mueller Hinton Broth (MHB; Merck, Germany) and incubated at 37 °C for 18–24 h to revive and ensure the purity of the culture. Subsequently, the cells were streaked on Mueller Hinton Agar (MHA; Merck, Germany) for isolation and maintained at 4 °C for further use (12).

B. Determination of the MICs and MBCs of NPs against S. aureus: To obtain the MICs for Ag, Cu, and ZnO NPs, an overnight culture was prepared in the Mueller Hinton Agar (MHA; Merck, Germany) medium, and then the broth dilution method was performed in a 96-wells microplate. For this purpose, 100 µL to the rows of negative control (Blank) and 95 µL of Mueller Hinton Broth (MHB) medium to other wells were added, respectively. Then, in all the wells of the first column, 100 µL of prepared target NPs were added. After combining the medium and NPs, 100 µL from the first well was taken and added to the second well, and this action continued until one well was left, and the 100 µL was discarded from the last well (Positive control and without NPs). This action was repeated for the next rows in the same way. The concentration of NPs in the wells were 1000, 500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.90625, 1.953125 mg/L, respectively. Subsequently, 5 µL of the 24h suspension of the *S. aureus* with 1.5×10^8 dilution (0.5 McFarland) was added to each well except for blank wells (Negative controls). Next, the microplates were incubated for 24 h at 37 °C in a shaker incubator. The first well that inhibited the growth of bacteria was considered the MIC of target NPs.

For calculating the MBCs values, 10 µL of target wells were added to the MHA medium (Wells belong to MIC of each NP in the previous test, and two after and before them), and plates were incubated for 24 h at 37 °C in the incubator. For interpretation of the results, the plates were checked carefully after incubation time (13).

C. Determination of the S. aureus sensitivity to antibiotics and nanoparticles: The antibiotic susceptibility of MRSA (ATCC 33591) was evaluated using the disk diffusion method according to the Clinical and Laboratory

Standards Institute (CLSI, 2023) guidelines. Briefly, the bacterial strain was cultured on Mueller Hinton Agar (MHA; Merck, Germany), and a bacterial suspension equivalent to 0.5 McFarland standard was prepared using sterile physiological saline. One milliliter of the bacterial suspension was poured onto MHA plates and uniformly spread with a sterile cotton swab. The antibiotic discs used in the test included vancomycin (30 µg), tetracycline (30 µg), oxacillin (1 µg), penicillin (10 µg), rifampin (5 µg), and linezolid (30 µg) (Padtan Teb Co., Iran) (14). In addition to antibiotic discs, sterile paper discs (6 mm in diameter) impregnated with serial dilutions of silver (Ag), copper (Cu), and zinc oxide (ZnO) nanoparticles were placed on the inoculated MHA plates, ensuring a minimum distance of 24 mm between the centers of the discs to avoid overlapping inhibition zones (14). Plates were incubated at 37 °C for 24 h, and the diameters of inhibition zones were measured in millimeters.

The agar dilution method was also performed to evaluate the effect of Ag, Cu, and ZnO nanoparticles on MRSA growth. Twelve tubes containing 25 mL of molten MHA were prepared, and nanoparticles were added to each tube at different concentrations as listed in Table 2, except for the control tube, which contained no NPs. After vortexing to ensure homogeneity, the agar mixtures were poured into sterile Petri dishes and allowed to solidify. Then, 100 µL of the MRSA suspension (0.5 McFarland) was inoculated onto the surface of each plate. The plates were incubated at 37 °C for 24 h, and colony counts were determined using a digital colony counter (WTW, Germany) (15). All tests were performed in triplicate to ensure reproducibility.

D .Antimicrobial properties of NPs and vancomycin against S. aureus in an animal model: To investigate the antimicrobial effect of Ag, Cu, and ZnO NPs in an animal model, male Balb/C mice, 6–7 weeks old, weighing 22±5 g, were purchased from Royan Institute for Biotechnology in Tehran. The animals were housed under standard laboratory conditions with a temperature of 22±2 °C, relative humidity of 55±5%, a 12:12 h light/dark cycle, and free access to food and water. An overnight suspension of *S. aureus* bacteria equivalent to 6×10^7 CFU/ml (optical density: 0.480) was prepared for infecting the mice (16). The next day, 63 male Balb/C mice were randomly divided into nine groups of seven each as follows: the first group (Control) contained no NPs and no microbial strain. The second, third, and fourth groups served as NP controls for Ag, Cu, and ZnO, respectively. The fifth group was infected only with *S. aureus* without any treatment. The sixth, seventh, eighth, and ninth groups were all infected with *S. aureus* and treated with Ag, Cu, ZnO NPs, and vancomycin (20 mg/kg, intraperitoneal; Sigma-Aldrich, USA), respectively (17). On the first day in the morning, 100 µL of physiological serum was injected intraperitoneally into the control and NP control groups to ensure consistent handling stress across all groups. The fifth to ninth groups received 200 µL of *S. aureus* suspension (6×10^7 CFU/ml) intraperitoneally. Three hours after bacterial injection, the first group received 100 µL of physiological serum. The second, third, and fourth groups received 100 µL of Ag NPs (15.625 mg/L), Cu NPs (62.5 mg/L), and ZnO NPs (62.5 mg/L), respectively. The fifth group was treated with 100 µL of physiological serum, while the sixth, seventh, and eighth groups were treated with 100 µL of Ag NPs (15.625 mg/L), Cu NPs (62.5 mg/L), and ZnO NPs (62.5 mg/L),

respectively. The ninth group received 100 µL of vancomycin. Treatments were administered once daily for five consecutive days (17). In the second stage, three mice from each group were randomly selected for autopsy. The mice were anesthetized using a combination of 2 ml ketamine (100 mg/mL), 1 ml xylazine (20 mg/mL), and 9 ml normal saline, prepared as a cocktail and administered intraperitoneally based on each animal's body weight (18).

E. RNA extraction and preparation of Cdna: An amount of 40 to 50 mg of fresh spleen tissue was taken, and the tissues were pounded until they were completely homogenized by adding 800-1000 µL of RNSol to petri dish plates. RNA extracts were obtained using RNJia Kit (ROJE Technology Co, Iran) according to a specific protocol. Left sediments of the last step were dissolved in 20-30 µL diethylpyrocarbonate (DEPC) water, and the microtubes were transferred to the refrigerator at -80 °C. Examination of the purity and quality of the RNA was performed by NanoDrop One C device (Thermo Scientific Co, US). RNA OD in 280 nm should be in the range of 1.8-2.2 when treated with DEPC water to demonstrate the suitable quality of RNA. A cDNA synthesis kit was used (Vivantis Tech Co, Malaysia) to synthesize cDNA from the extracted RNAs according to the following protocol. A total volume of 20 µL was prepared including; 4 µL buffer 5X, 1 µL dNTPs, 1 µL RT enzyme, 1 µL Random Hexamer (RH) primer, 10 µL extracted RNA, and 3 µL DEPC water. The PCR was run according to the thermal and time cycles, and the final product was stored at -20 °C (19).

F. Assessment of TNF-α gene expression change using Real-Time PCR: For Real-time PCR, cDNA synthesized from extracted RNA, SYBR Green Master Mix, and designed primers of *β-actin* and *TNF-α* were used. SYBR Green is a DNA-binding dye that intercalates into the minor groove of double-stranded DNA. The main advantages of this method are its low cost and high sensitivity, whereas its limitation lies in binding to non-specific products such as primer dimers, which may generate non-specific amplification signals. The specificity of amplification was confirmed by analyzing melting curves to detect non-specific bands or primer-dimer formations.

Before Real-time PCR, the cDNA concentrations of all samples were equalized using a NanoDrop One C spectrophotometer. The PCR reactions were performed using a Rotor-Gene Q device (QIAGEN, Germany) in a final volume of 20 µL containing 7.5 µL of DEPC-treated water, 10 µL of 2× SYBR Green Master Mix, 1 µL of cDNA template, and 1.5 µL of primers. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 52 °C for 15 s, and extension at 72 °C for 20 s. The melting curve analysis was performed from 55 °C to 94 °C with 1 °C increments per second (20). In this study, *β-actin* was used as the internal reference gene due to its stable expression. The forward and reverse primer sequences of *β-actin* and *TNF-α* genes used in this study are presented in Table 1. Each primer pair was designed using NCBI Primer-BLAST and Gene Runner software (version 6.5) to ensure sequence accuracy and specificity. The sequences are shown in the 5′→3′ direction along with their expected product sizes.

G. The statistical analysis of Real-time PCR results: Real-Time PCR data analysis was performed according to threshold cycle comparison method. The statistical calculation of this study was performed using SPSS 16 and

Graphpad prism 6 software, and the results were analyzed with one way ANOVA. Also, changes in target gene expression between the control and treated samples were obtained by Tukey's HSD post-hoc test ($P<0.05$). The differences between the threshold cycles obtained from the tested samples (Mice treated with *S. aureus* infected with NPs and antibiotic) and the control samples were calculated. With the ΔΔCt formula, the ratio of the target gene to the reference gene (*β-actin*) was calculated through $2^{-\Delta\Delta Ct}$, whose calculation formula is as follows. After the reaction, the Ct value was calculated for all samples using Rest 2009 software.

ΔCt = Ct target - Ct reference

ΔΔCt = ΔCt test sample – ΔCt control sample

Relative expression = $2^{-\Delta\Delta Ct}$

H. Ethical Considerations: All animal experiments were conducted in compliance with the ethical principles for laboratory animal care and were approved by the Ethics Committee of Islamic Azad University Varamin (IR.IAU.VARAMIN.REC.1402.007), Tehran, Iran. The study followed the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (8th Edition, National Academies Press, 2011) and adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines to ensure ethical and transparent reporting of animal research.

Results

A. The MICs and MBCs of NPs: Bacterial growth was observed to increase as the NP concentrations decreased. The MICs for Ag and Cu NPs were determined to be 15.625 mg/L and 62.5 mg/L, respectively. None of the tested ZnO NP concentrations were able to inhibit bacterial growth, so disk diffusion and agar dilution tests were used to further evaluate its antimicrobial activity. MBC results showed that Ag NPs at 250 mg/L and Cu NPs at 500 mg/L exhibited bactericidal activity against *S. aureus* (Figure 1a, 1b). All experiments were performed in triplicate.

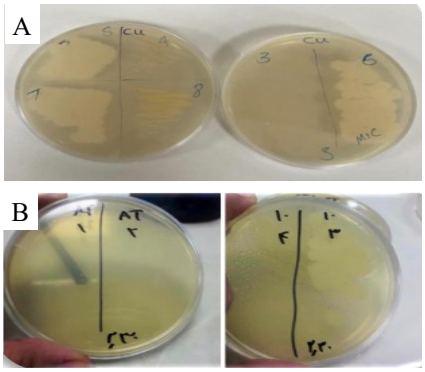

Fig 1. The MBCs results of (a) Cu NPs and (b) Ag NPs against *S. aureus* by microdilution method.

B. Sensitivity of S. aureus to Antibiotics and ZnO NPs by Disk Diffusion and Agar Dilution: The disk diffusion assay showed resistance to tetracycline, oxacillin, penicillin, and ZnO NPs, indicated by the absence of inhibition zones. Whilst, rifampin, linezolid, and vancomycin showed strong antibacterial effects with inhibition zones of 34, 31, and 16 mm, respectively (Figure 2a). Notably, colony counts in eleven test plates were comparable to the control plate (plate 12), confirming *S. aureus* resistance to ZnO NPs (Figure 2b). Several factors such as antibiotic diffusion rate, bacterial load, drug concentration, and agar properties influence halo diameter and susceptibility results. Table 1 summarizes the concentrations of ZnO NPs used in the agar dilution assay.

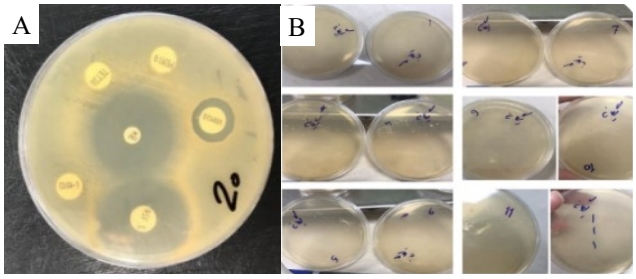

Fig 2. Susceptibility of *S. aureus* by (a) Antibiotic disk diffusion test (b) Agar dilution test.

Table 1. Sequence of primers used in Real-time PCR.

Gene	Sequence (5' to 3')	Type
TNF-α	CCAGGAGAAAGTCAGCCTCCT	F
	TCATACCAGGGCTTGAGCTCA	R
β-actin	AGAGCTATGAGCTGCCTGACG	F
	CTGCATCCGGTCAGCGATAC	R

Table 2. Volume and concentration of antimicrobial agent used in agar dilution test.

Concentration of antimicrobial agent (µg/ml)	Antimicrobial agent volume (µL)	Final concentration when added to 25 mL of agar (21)
10	320	128
10	160	64
10	80	32
10	40	16
1	200	8
1	100	4
1	50	2
0.1	250	1
0.1	125	0.5
0.1	62.5	0.25
0.1	31.25	0.125

C. TNF-α gene expression changes in nine groups of treatment: After RNA extraction from spleen tissues, *TNF-α* gene expression levels were analyzed on day 1 and day 5 post-treatment (Figures 3 and 4). On day 5, the lowest *TNF-α* expression level was observed in the healthy control group (1.389). The infected group without treatment showed a slight increase (1.541), which was not statistically significant compared to the control. Vancomycin treatment resulted in the highest gene expression level (4.144), followed by Ag NPs (3.597), Cu NPs (2.521), and ZnO NPs (2.029). These increases were statistically significant ($P < 0.05$). Among the tested nanoparticles, Ag NPs induced the highest expression, suggesting a stronger immunostimulatory effect. The results also indicate that vancomycin significantly elevated *TNF-α* levels, which may reflect an enhanced inflammatory response.

On day 1, vancomycin again led to the highest *TNF-α* gene expression level (3.182). Ag and Cu NPs also significantly increased gene expression to 2.440 and 2.078, respectively, compared to the control. These results demonstrate that both vancomycin and metal nanoparticles, particularly Ag NPs, can stimulate *TNF-α* expression early during the infection response.

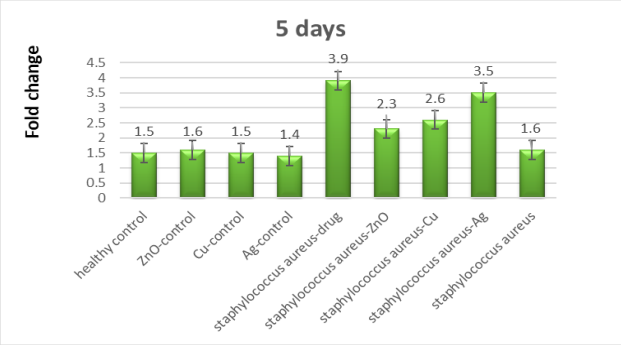

Fig 3. *TNF-α* gene expression changes (Fold change) within five days of treatment.

Legend: The stars on the graphs show P-Value, a statistical parameter. The vertical column indicates the level of *TNF-α* gene expression, and the horizontal column indicates the groups.

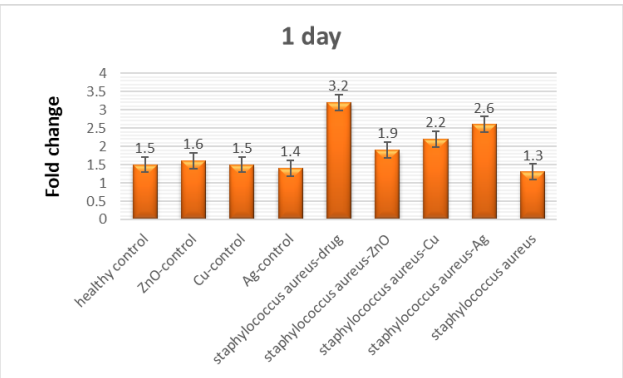

Fig 4. *TNF-α* gene expression changes (Fold change) within one day of treatment.

Legend: Asterisks indicate statistically significant differences between groups (*p* < 0.05, **p** < 0.01). The vertical axis represents the relative expression level of the *TNF-α* gene, and the horizontal axis indicates the experimental groups.

Discussion

This study aimed to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of silver (Ag), copper (Cu), and zinc oxide (ZnO)

nanoparticles against methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591). The antibacterial activity of antibiotics and nanoparticles (NPs) is commonly assessed by agar diffusion, disk diffusion, and MIC assays, and comparable results have been reported by Hashemi and Shokri (2022) when evaluating the effects of silver and copper nanoparticles (22). The serial two-fold dilution method used for MIC determination identifies the lowest concentration of an antimicrobial agent that inhibits bacterial growth. S. *aureus*, a facultative anaerobic Gram-positive coccus, has drawn great scientific attention due to its ability to cause a wide range of infections and its increasing resistance to antimicrobial agents. Several mechanisms contribute to this resistance, including metabolic pathway alterations, mutations in chromosomal DNA gyrase and topoisomerase IV genes, ATP dependent efflux pump activity, and modification of antibiotic target sites such as ribosomal subunits and penicillin-binding proteins (PBPs). Furthermore, *S. aureus* can neutralize antibiotic effects by producing enzymes such as β-lactamases, chloramphenicol acetyltransferase, and aminoglycoside-modifying enzymes. The antibacterial properties of nanoparticles are mainly attributed to the continuous release of metal ions in aqueous environments. The large surface area of NPs, facilitates their interaction with bacterial membranes, where they bind to sulfhydryl (–SH) groups and disrupt membrane integrity. In addition, NPs generate reactive oxygen species (ROS) and other free radicals that interfere with bacterial respiration, damage cell walls, and inhibit DNA replication (23). Altogether, these mechanisms provide nanoparticles with a potent and broad-spectrum bactericidal activity, often surpassing that of conventional antibiotics.

In the last few years, reports have been published based on the effect of the various NPs on Gram-positive and Gram-negative bacteria and fungi like *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus epidermidis* (24). In the reports, Gram-negative bacteria like *E. coli* showed more resistance to NPs than Gram-positive bacteria like *S. aureus* owing to cell physiology, and the availability of an outer membrane containing lipopolysaccharide (LPS), and different metabolisms (25). Although the antibacterial effects of Ag NPs have not been extensively studied in animal models, in the present study, the results of the antibacterial effects of Ag NPs in Balb/C mice showed that these particles maintain their antibacterial effects in animal models. These results are consistent with the antibacterial properties of Ag and Cu NPs on the gram-positive bacteria *S. aureus*, that previously have been reported. Several studies have demonstrated that silver nanoparticles exhibit strong antibacterial effects against *Staphylococcus aureus* at low concentrations (e.g., 5 µg/mL) (26). However, due to variations in production methods, nanoparticle size, and shape, results across studies may differ significantly (27). Some reports indicate that silver nitrate is effective against *S. aureus* within the range of 8-80 µg/mL, while others, including Dehkordi et al., reported a lower effective range of 1.25-10 µg/mL (28). These discrepancies highlight the importance of the need for in vivo validation. In our study, we observed effective antibacterial activity at concentrations as low as 5 µg/mL against Staphylococcus aureus, which is consistent with the range reported in the above studies.

In 2008, Ruparelia et al. (29) reported that bacterial susceptibility to NPs was found to

vary depending on the microbial species. Disk diffusion studies with *Bacillus. Subtilis*, *E. coli*, and *S. aureus* indicated greater effectiveness of the Ag NPs compared to the Cu NPs. They also highlighted that *B. subtilis* was more adversely affected by the Cu NPs, and showed the highest susceptibility to NPs than other strains. We also used disk diffusion, antibiogram, and serial dilutions methods, and our findings align with their results, demonstrating the greater impact of Ag NPs compared to Cu NPs. The findings obtained by Gouyau et al. (2021) were relatively different from ours and mentioned studies (30). They argued that Ag and Cu NPs had no activity on *S. aureus* but revealed a high antibacterial activity against *E. coli*, with a MIC of 128 µmol/L. In addition, they concluded that Au NPs had a weak antibacterial activity (i.e., slight inhibition of bacterial growth) against the two bacteria tested (30). In our research regarding the antibacterial property of ZnO NPs *in vitro* conditions, the result was in contrast to Mirhosseini's research, when the *S. aureus* was resistant to ZnO NPs. In other words, no measurable MIC value could be determined under the tested conditions. Mirhosseini (31) reported that ZnO NPs had a favorable ability to stop the growth of *E. coli* and *S. aureus* in milk. These oxide NPs disrupt the lipid and protein components of the bacterial cell membrane, leading to leakage of intracellular contents and ultimately resulting in bacterial cell death. In addition, the production of hydrogen peroxide and Zn^{+2} ions has been suggested as the key mechanism of the antibacterial action of ZnO NPs. Antibacterial effects, MICs, and MBCs of the NPs vary according to solvents used as a chemical reducer, bacteria type, the applied dose, and sustainability of NP, inherent power of the NP, and environmental conditions (32).

The effect of vancomycin and three NPs on *TNF-α* gene expression at 1 and 5 days post-treatment was investigated in this research, and results showed that the application of NPs had a significant effect on gene expression and, as a result, the inflammatory response in mice. Therefore, these findings highlight the potential of NPs as alternatives to conventional antibiotics against the mentioned bacteria. Infection and inflammation increase the expression of TNF-α, IL-2, and IL-6, which then regulate the expression of CYP450s, the enzymes responsible for the metabolism of substances in the liver. MRSA produces IL-6, TNF-α, MCP-1, and IL-10 IFN-γ and causes liver damage in mice, Jiang et al. reported (33). During this study, by infecting mice with MRSA bacteria, the level of *TNF-α* expression increased compared to the control sample, which was consistent with Jiang's study. MRSA not only causes inflammation but also releases various toxins that damage the hosts, and inhibit the CYP450 enzyme. Many studies previously reported the superparamagnetic iron oxide NPs (SPIO NPs), silica NPs (Si NPs), Cu NPs, Ag NPs, and dioxide NPs (TiO2-NPs) induced more inflammatory reactions through the production of cytokines like *TNF-α* and activating ROS in animal models. Studies conducted on the effect of ZnO NPs on cells indicate the production of oxygen free radicals followed by oxidative stress and increased inflammatory response of cytokines such as *TNF-α* and these findings are consistent with our results. Increases in the expression of *TNF-α* and *NF-kB* genes treated with Ag NPs after infection with *Campylobacter jejuni* in broiler chickens have been proposed by Vadalasetty et al. (34). However, the plasma concentrations of IgG and IgM were lower in chickens that received Ag NPs compared to the

non-supplemented control group. Shang et al. (2016) argued bacterial pathogens that induced intestinal inflammation increased levels of cytokines, including a wide range of interleukins, *IFN-γ*, and *TNF-α* gene expression in the rabbits. In the acute phase, the bacteria induced a significant increase in the expression of the most pro-inflammatory genes. These results confirm the pro-inflammatory activity of Ag NPs, which has been previously reported in chickens and mice (35). The absorption, excretion, distribution, metabolism, and toxicity of NPs are largely associated with their physicochemical characteristics and environmental conditions. Nevertheless, the exact molecular mechanisms by which NPs contribute to pro-inflammatory responses remain unclear in this study and warrant further investigation.

Conclusion

It is possible to understand the value and importance of nanoparticles as an alternative to antibiotics against the bacteria. The toxicity of NPs and their effect on the increase of the inflammatory cytokines such as *TNF-α* due to oxidative stress cannot be ignored. The release of cytokines with NPs is concentration-dependent. In other words, they prevent the production of inflammatory cytokines at safe concentrations. NPs may serve as potential therapeutic agents in infectious diseases, yet further studies are needed before clinical use.

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Conflict of Interest

The authors declare no financial or non-financial competing interests that could affect the objectivity, integrity, or reporting of this study.

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