Evaluation of the antibacterial activities of Ag/ZnO nanoparticles against *Streptococcus agalactiae*, *Staphylococcus aureus* and *Escherichia coli* isolated from infected bovine mammary glands

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ABSTRACT

The objective of this study was to evaluate the antibacterial activity of Ag/ZnO nanoparticles against *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus agalactiae* isolated from bovine mastitis in dairy cows, both in vivo and in vitro. Ag/ZnO nanoparticles were synthesized by means of the thermal decomposition of oxalate precursors. SEM images revealed that the nanoparticles had a size range of 10-50 nm. A toxicity assessment of nanoparticles conducted using the *Artemia salina* model indicated that the nanoparticles were not toxic at high concentrations (LD50>6384 μg/ml). A total of 138 mastitic milk samples were cultured to assess the antimicrobial effects of Ag/ZnO NPs. The antimicrobial susceptibility of the isolated bacteria was assessed by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill test. To evaluate the efficacy of the treatment, the somatic cell count (SCC), California mastitis test (CMT), and histological tests were employed to assess the cow udders with mastitis that had been treated with Ag/ZnO NPs. The most frequently isolated pathogen was *S. agalactiae* (50.72%), which demonstrated the highest susceptibility to Ag/ZnO NPs *in vitro* and *in vivo* tests.

Key words: mastitis; antimicrobial effect; dairy cow; silver-zinc oxide nanoparticles (Ag/ZnO NPs)

Introduction

Mastitis is a mammary gland tissue inflammation that occurs following infection by a wide range of microbial species, especially Gram-positive and Gram-negative bacteria. This disease imposes significant financial losses on the animal husbandry and milk production industries (SCHWARZ et al., 2019). In the veterinary business landscape, mastitis has emerged as a significant and costly challenge for the livestock industry (GUIMARAES et al., 2017). The major pathogens causing masti-

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tis are *Staphylococcus*, *Streptococcus*, *E. coli* and *Klebsiella pneumoniae*, according to epidemiological data (COBRIKA et al., 2020).

The most common antibiotics for the treatment of mastitis are oxytetracycline, neomycin, novobiocin, penicillins, cephalosporins, and sulfonamides (KROMKER and LEIMBACH, 2017). Although these antibiotics are effective in treating these infections, there is a growing prevalence of antibiotic resistance in microbial strains isolated from mastitis (ROYSTER and WAGNER, 2015). The prevention and treatment of mastitis have been shown to reduce the risk of other diseases (MEKONNEN et al., 2017). Today, metal nanoparticles are used as disinfectants which also have great potential for the prevention or treatment of the bacterial pathogens causing mastitis, without major concerns such as the presence of antibiotic residues in milk and meat (KALINSKA et al., 2019, NIRALA et al., 2019). In this study, we investigated the antibacterial effects of combined silver and zinc oxide nanoparticles (Ag/ZnO NPs) on bacteria isolated from mastitic milk samples of dairy cows.

Materials and methods

Synthesis of Ag/ZnO NPs. $AgNO_3$ (0.3 M) and $ZnCl_2$ (0.3 M) were added to 50 ml of ethanol in a two-neck flask to form a creamy white solution. The solution temperature was then raised to 50°C, and it was stirred for 30 minutes. Oxalic acid (0.6 M) was subsequently added to the solution and refluxed for 120 minutes. The resulting white gel-like substance was subsequently oven-dried at 80°C and then calcined at 550°C for 120 minutes in a furnace to obtain AgO/ZnO nanoparticles (KAUR, 2013; JAFARI et al., 2018).

The structural properties of the nanoparticles were characterized by XRD (Bruker D8-Advance diffractometer using CuKα radiation), FTIR (spectrum RXI, Perkin–Elmer), and SEM (Cam Scan MV2300) at 20 kV accelerating voltage.

Toxicity assessment of Ag/ZnO NPs by Artemia salina. The toxicity of different concentrations of Ag/ZnO NPs (256-16384 µg/ml) was evaluated using the brine shrimp (*Artemia salina*) lethality test. Artificial seawater was prepared by the addition of 23 g of sodium chloride, 11 g of magnesium chloride, 2 g of sodium sulfate, 1.3 g of calcium chloride, and 0.1 g of potassium chloride to 1000 ml of deionized water. Subsequently, 5 ml of the NPs was added to a tube containing 5 ml of the seawater and 10 brine shrimp larvae, resulting in concentrations of 256, 512, 1024, 8192, and 16384 µg/ml of the NPs. The tubes were maintained in a Bain Marie water bath at 31°C and under continuous illumination for a period of 24 hours. Artificial seawater containing 10 brine shrimp larvae was employed as a negative control. The number of viable larvae was enumerated via the use of a magnifying glass (AKHBARI et al., 2014).

Isolation and identification of bacterial pathogens. One hundred and thirty-eight Holstein-Friesian cows on five farms in Tehran, Iran, with an age range of 2-8 years, and with symptoms of bovine mastitis were investigated. Routine milking took place three times per day. Milk samples were grown on blood agar and McConkey agar, followed by Gram-staining. Biochemical and microbiological tests, including catalase, oxidase, coagulase, DNase, hemolysis types and sugar fermentation, were conducted to identify Gram-positive bacteria. Additionally, biochemical and microbial tests, such as citrate and malonate consumption, motility, indole production, MR-VP, and sugar consumption, were performed in order to identify Gram-negative bacteria (MCVEY et al., 2013).

The most frequently isolated bacteria were confirmed by PCR tests. Genomic DNA extraction was performed using a CinnaPure DNA kit (Cinacloon, Iran). The oligonucleotide primers used in the PCR tests are presented in Table 1. The total volume of PCR mixture (25 µl) contained 5 µl of DNA template, 18 µl of master mix (1x), 1 µl (10 pmol) of forward and reverse primer, and 2 U of Taq DNA polymerase (Fermentas Inc, Canada). The PCR amplification conditions for *S.agalactiae* were as follows: initial denaturation at 95˚C for 2 min; 35 cycles of 95˚C for 30 s, 52.8˚C for 30 s, 72˚C for 30 s; and a final extension at 72˚C for 5min. The PCR amplification conditions for *E. coli* were as follows: initial denaturation at 95˚C for 2

Table 1. Primer sequences used in this study

Target gene/ bacterium	Sequences of primers	Annealing temperature (^{0}C)	Amplicon size (bp)	Reference
16S rRNA/ *S.agalactiae*	5′-TTTGGTGTTTACACTAGACTG-3′ 5′-TGTGTTAATTACTCTTATGCG-3′	52°C	120	(KARSIDANI et al., 2010)
universal stress protein A/ *E. coli*	5′-CCGATACGCTGCCAATCAGT-3′ 5′-ACGCAGACCGTAGGCCAGAT-3′	60°C	883	(YANG et al., 2015)
16S rRNA/ *S. aureus*	5′-AACTCTGTTATTAGGGAAGAA CA-3′ 5′-TTCCTCCGGTTTGTCACC-3′	55°C	751	(CIFTCI et al., 2009)

min; 33 cycles of 95˚C for 30 s, 60˚C for 30 s, 72˚C for 90 s; and a final extension at 72˚C for 5min. The PCR amplification conditions for *S.aureus* were as follows: initial denaturation at 95˚C for 2 min; 33 cycles of 95˚C for 30 s, 55˚C for 30 s, 72˚C for 80 s; and a final extension at 72˚C for 5 min. PCR products were visualized by electrophoresis in a 1.5% agarose gel under UV light. *S.agalactiae* ATCC12386, *S.aureus* ATCC-12228 and *E. coli* ATCC 25527 were used as positive controls.

MIC and MBC determination. The MIC of Ag/ZnO NPs was determined for *E. coli*, *S. aureus* and *S. agalactiae* using the microbroth dilution method in 96-well plates. A fresh culture of the bacteria was grown in Mueller-Hinton broth (MHB), then a bacterial suspension to a final concentration of 1.5×10^9 CFU/ml and different concentrations of Ag/ZnO NPs were added to the wells. The plates were incubated for 24h at 35°C, then 2,3,5-triphenyl-tetrazolium chloride was added to the plates to detect bacterial growth. The lowest concentration of NPs that inhibited visible bacterial growth was defined as the MIC.

To determine MBC, 5 µl of the wells without visible bacterial growth were plated on Mueller Hinton Agar (MHA). The plates were incubated at 35°C for 24h. The lowest concentration of Ag/ZnO NPs showing no colonies on MHA plates was determined as the MBC (JOHNSON and CASE, 2004; JAFARI et al., 2011).

Agar well diffusion assay. The agar well diffusion test was done after determination of the MIC. Fresh cultures of the bacteria were grown in MHB, and then a bacterial suspension to a final concentration of 1.5×10^9 CFU/ml was prepared and spread on the MHA plate using a cotton swab. Five mm diameter wells were made on the MHA plate using a sterile cutter, then Ag/ZnO NPs at concentrations corresponding to the MIC and LD50 were added to the wells and incubated at 37°C for 24h. The average diameter of the growth inhibition zone around each well was then measured. Each experiment was performed in triplicate (CHARANNYA, 2018).

The growth rate of bacterial cells exposed to Ag/ZnO NPs. The time-kill test was conducted to evaluate the growth rate of bacterial cells exposed to Ag/ZnO NPs. A fresh culture of the bacterium was prepared in MHB, and a bacterial suspension in a final concentration of 10^6 CFU/ml was added to the Ag/ZnO NPs to yield a final concentration equivalent to the MIC, 1/2 MIC, and 2× MIC for each bacterium. The final concentrations were as follows: 64, 128, and 256 µg/ml for *E. coli* and *S. aureus*; 32, 64, and 128 µg/ml for *S. agalactiae*. Subsequently, the bacterial suspensions were incubated at 37°C in a shaker incubator. At 1.30, 3, 6, 12, and 24 hours, a sample (100 µl) was removed and serially diluted before plating onto MHA. The resulting bacterial colonies were then counted. (CARSON et al., 2002).

Somatic cell count test (SCC) and California mastitis test (CMT). The somatic cell count in the milk was quantified using a Fossomatic instrument (Foss Electric, Hillerød, Denmark) (BERRY et al., 2006). The California mastitis test (CMT) was

employed to identify subclinical cases of mastitis. The kit elements for the CMT contained reagents that reacted with nucleic acids present in cells in the milk at a specific pH, with an indicator that produced a purple-blue color when the reaction occurred (MUHAMMAD et al., 2010).

Animal treatment. A total of 30 Holstein-Friesian cows, aged between two and eight years, with positive CMT and obvious bacterial infection caused by *S. aureus*, *S. aglactiae*, or *E. coli*, were selected for treatment. The cows were assigned to six groups (n=5 per group) in relation to individual bacterial infections, and received either a high or low dose of Ag/ZnO NPs. The udders of the subjects were washed with warm clean water using disposable gloves, and then disinfected with 70% ethanol. The cows were subjected to treatment through an intramammary infusion of 10× MBC of Ag/ZnO NPs (as a low dose) and 15× MBC of Ag/ZnO NPs (as a low dose) into the teat canals, at 8-hour intervals for 72 hours, immediately after routine milking (three times per day). Milk samples were collected before the initiation of the treatment, and examined using the SCC and CMT (YANG et al., 2019).

Determination of Ag and Zn residues in milk. The samples from routine milking after 24h, 48h, and 72h of the treatment were collected for determination of Ag and Zn residues in the milk. After milking, the samples were incubated at room temperature for 48h. Next, 5 ml of the aqueous phase of the samples was mixed with 5 ml of nitric acid and kept at 85°C for 2h. After dissolution, the samples were analyzed using the ICP-MS instrument (ELAN DRC-e, PerkinElmer, USA) to detect Ag and Zn (BIRGHILA et al., 2008; FABRICIUS et al., 2014).

Histological examination. Cows with long-term mastitis caused by *S. agalactiae*, that had had significantly decreased milk yield for at least 4 months were slaughtered and three udders were collected for histological examinations.

Two cows from the treatment group that had mastitis caused by *S. agalactiae* and were treated with high doses of Ag/ZnO NPs (as previously described in the “animal treatment” section), were selected and slaughtered, five days after the final treatment. Then, the treated udders were collected for histological examination. The udders were fixed in 10% buffered formalin for 72h, then dehydrated through a graded series of ethanol, cleared in xylene, and infiltrated with molten paraffin before preparation of paraffin blocks. Sections five microns thick were obtained using a rotary microtome. The sections were then stained with the standard haematoxylin and eosin (H&E) dyes (RESTUCCI et al., 2019).

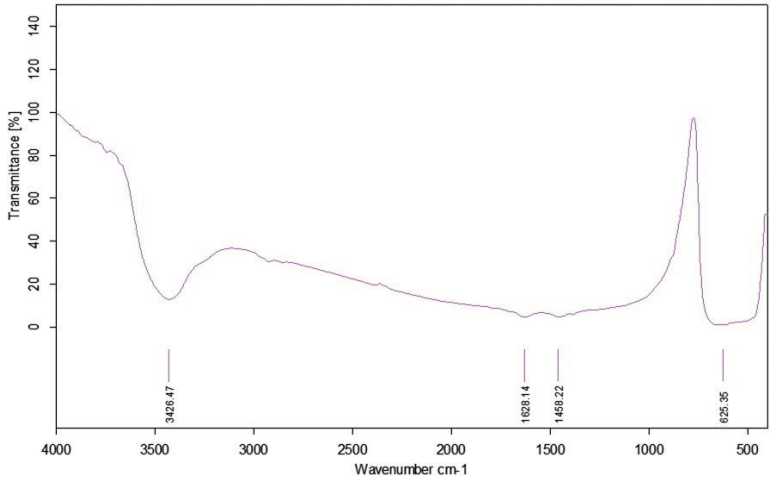
Statistical analysis. The results were statistically analyzed using the paired t-test. A P value of ≤ 0.05 was considered statistically significant.

Results

Physicochemical characterization of Ag/ZnO NPs. Fig. 1a shows the FT-IR spectra of Ag/ZnO NPs. The broad adsorption peak at 3426.47 cm^{-1} is related to the stretching mode of OH groups of adsorbed water and surface hydroxyl groups involved in H-bonding. The adsorption peak at 1628.14 cm^{-1} corresponds to the bending vibration of the OH groups. The peak at 1458.22 cm^{-1} is attributed to the bending vibrations of C-H in the methyl group. The significant bond at 625.36 cm^{-1} is assigned to the stretching mode of Zn-O (KADAM et al., 2018). The XRD pattern of Ag/ZnO NPs (Fig. 1b) was compared and interpreted with standard data from the International Centre of Diffraction Data (ICDD). The average crystallite size of Ag/ZnO NPs was 12.15 nm, calculated using the Debye-Scherer equation from the major diffraction peaks. Fig. 1c shows the SEM image of Ag/ZnO NPs. According to the SEM image, the nanoparticles have mostly spherical shapes (10-50 nm).

Toxicity assessment of Ag/ZnO NPs by Artemia salina. At all Ag/ZnO NPs concentrations tested (256 to 8192 μg/ml), all larvae survived at the end of the experiments. However, 20% of the larvae died at the highest concentration tested (16384 μg/ml). Therefore, the LD50 was determined to be above 16384 μg/ml.

Bacterial isolates. From 138 bovine mastitis milk samples identified by phenotypic tests,



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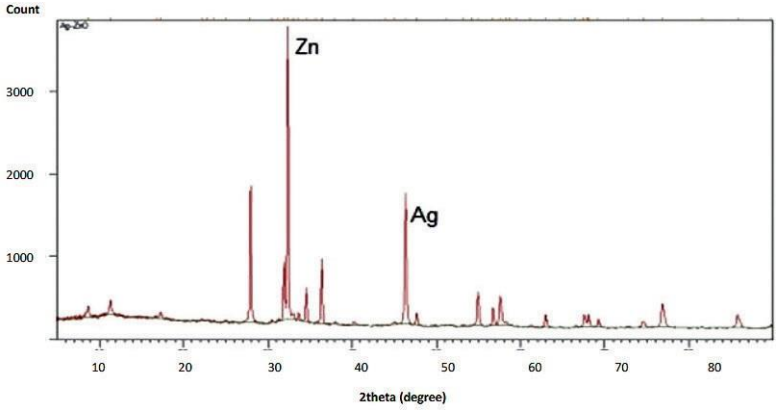

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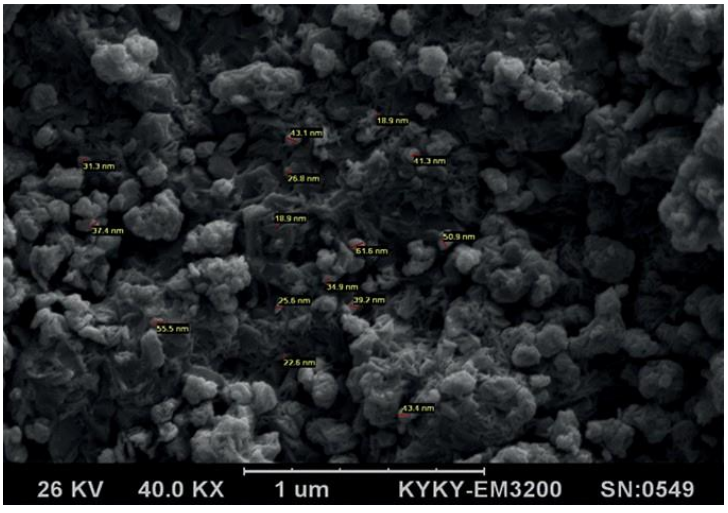

C

Fig. 1. Properties of Ag/ZnO NPs. (a)FT-IR, (b) XRD, and (c) SEM image

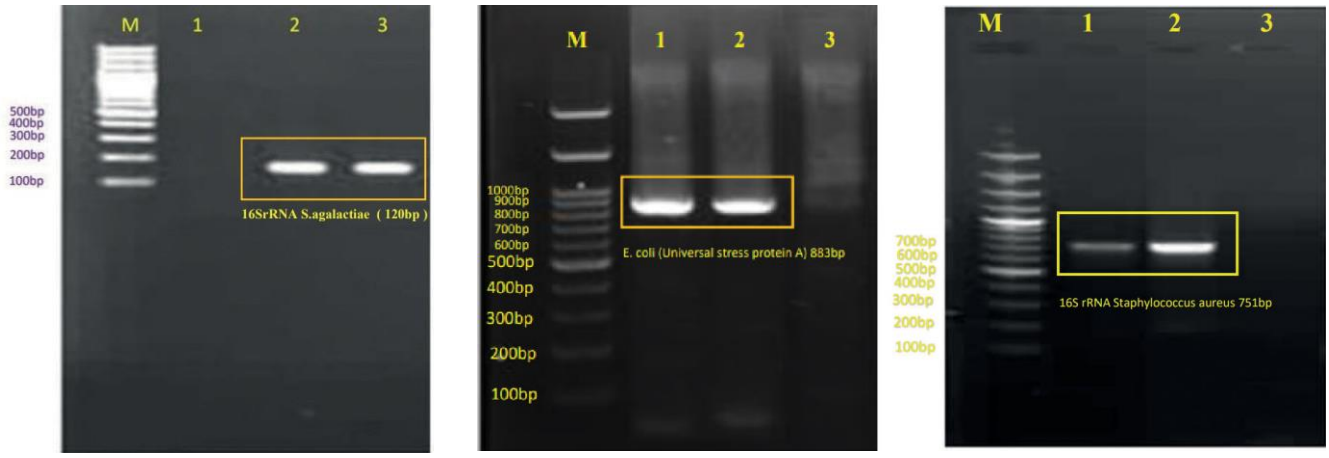

Fig. 2. Gel electrophoresis of PCR products

(A) *S. agalactiae*; Lane M: marker 100 bp, Lane 1: negative control, Lane 2: isolated bacterium, Lane 3: positive control. (B) *E. coli*; Lane M: marker 100 bp, , Lane1: isolated bacterium, Lane 2: positive control, Lane 3: negative control. (C) *S. aureus*; Lane M: marker 100 bp, Lane 1: isolated bacterium, Lane 2: positive control, Lane 3: negative control

the bacteria isolated were *S. agalactiae* (n=70) 50.72%, *E. coli* (n=35) 25.36%, *S. aureus* (n=33) 23.91%, *Corynebacterium bovis* (n=8) 5.79%, *Streptococcus dysgalactiae* (n=6) 4.34% and *Klebsiella* spp. (n=3) 2.17%. Identification of *S. agalactiae*, *S. aureus* and *E. coli* was also confirmed by PCR tests (Fig. 2). The PCR amplicon size for *S. agalactiae*, *E. coli* and *S. aureus* was 120, 883 and 751 bp, respectively.

MIC, MBC and agar well diffusion test. The MIC and MBC of the Ag/ZnO NPs against *S. aureus, S.agalactia*, and *E. coli* are presented in Table 2. The results of the agar well diffusion test at the MIC concentrations (64 µg/ml for *S.agalactia*, 128 µg/ml for *S. aureus* and *E. coli*) and concentrations around the LD50 value (16384 µg/ml) are listed in Table 3.

The growth rate of bacterial cells exposed to Ag/ZnO NPs. The treatment of *S. aureus* with Ag/ZnO NPs at the MIC concentration resulted in a complete reduction in viability after six hours of exposure. A similar reduction was observed for *S. agalactiae* and *E. coli*, occurring after 3 hours. Treatment of *S. aureus* with a concentration of 2× MIC resulted in zero viability over a period of three hours. The results for *S. agalactiae* and *E. coli* were comparable, with a reduction in viability observed after 1.5 hours (Fig. 3).

Animal treatment. SCC and CMT were performed on the milk samples taken from the animals in groups, before and 72h after the final infusion of Ag/ZnO NPs into the udders. As shown in Table 4, in the group with *S. aureus* infection in their udders, administration of a low dose (10×MBC=5120 µg/ml) and a high dose of Ag/ZnO NPs (15×MBC=7680 µg/ml) resulted in a significant reduction (P=0.0007 and P<0.0001) in SCC. Two other groups with *S. agalactiae* and *E. coli* infections had comparable outcomes. Furthermore, the CMT score demonstrated a notable alleviation in mastitis following treatment.

Determination of Ag and Zn residues in milk. Table 5 shows the Ag and Zn levels in the milk samples after the treatment. The milk samples after 24h of treatment (which was after the 3rd infusion of Ag/ZnO NPs into the udders) had 54 ppb of Ag and 1800 ppb of Zn. Forty-eight and 72 hours after the treatment (which were after the 6th and 9th infusion of Ag/ZnO NPs into the udders), the Ag

Table 2. MIC and MBC of Ag/ZnO NPs

Bacteria strains	MIC (µg/ml)	MBC (µg/ml)
S. aureus	128	512
S. agalactiae	64	256
E. coli	128	256

Table 3. Results of antimicrobial activity by the agar well diffusion method

Bacteria strains	Inhibition zone diameter (mm) (Concentrations equal to MIC)	Inhibition zone diameter (mm) (concentration=16384 µg/ml)
S. aureus	8± 2	13±1
S. agalactiae	14±3	16±2
E. coli	10±2	15±2

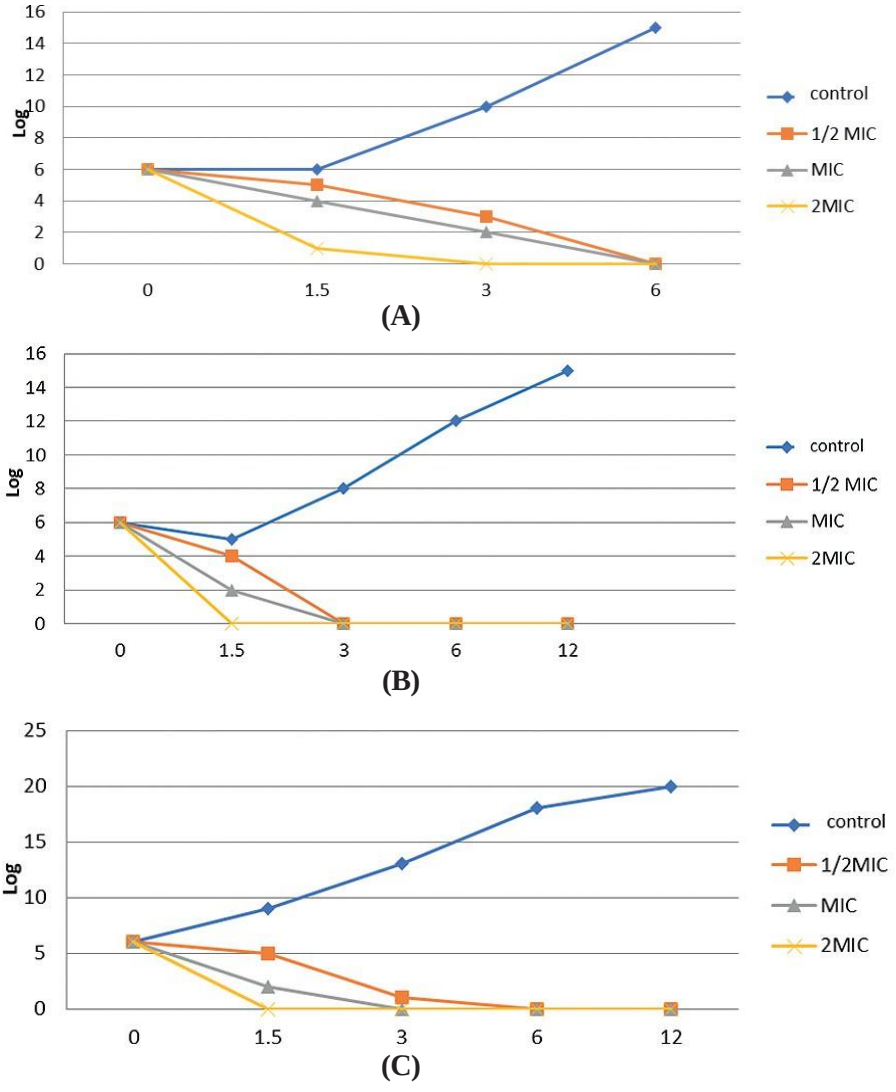

Fig. 3. The bacterial growth curve at 1/2 MIC, MIC, and 2MIC
(A): *S. aureus*, (B): *S. agalactiae*, and (C): *E. coli*

Table 4. SCC and CMT results in different groups

	***S. aureus* group**		***S. agalactiae* group**		***E. coli* group**	
Treatment dose*	Low dose (*n*=5)	High dose (*n*=5)	Low dose (*n*=5)	High dose (*n*=5)	Low dose (*n*=5)	High dose (*n*=5)
SCC×10^3 (cells/ml) pre-treatment	2200±206.5	1400±42.7	7922 ±351.8	8033±305.9	4684 ±206.5	2750 ±47.7
SCC×10^3 (cells/ml)	1050 ±42.5	478 ±19.5	4967 ±394.2	728 ±18.6	810 ±22.3	384 ±18.3
P- value	0.0007	<0.0001	0.0006	<0.0001	<0.0001	<0.0001
CMT pre-treatment	+2	+1	+3	+3	+2	+1
CMT post- treatment	+1	Trace	+2	+1	+1	Trace

*: for *S. aureus*: low dose of Ag/ZnO NPs= 10×MBC= 5120 μg/ml; high dose of Ag/ZnO NPs= 15×MBC= 7680 μg/ml. For *S. agalactiae* and *E. coli:* low dose of Ag/ZnO NPs= 10×MBC= 2560 μg/ml; high dose of Ag/ZnO NPs= 15×MBC= 3840 μg/ml.

Table 5. Ag and Zn levels in milk samples after treatment

Element	After 24h	After 48h	After 72h	Unit
Ag	54	18	12	ppb
Zn	1800	1000	900	ppb

levels in the milk samples were 18 and 12, and Zn levels were 1000 and 900 ppb, respectively.

Histological examination. Figure 4 presents histological images of the mammary gland with clinical mastitis. As illustrated in Fig. 4a, the infiltration of inflammatory cells within the interstitial tissue of the mammary gland of the cow is indicative of mastitis in the tissue. In the vicinity of the glands, the interstitial tissue of the lobule is transparent, and the proliferation of collagen fibers surrounding the mammary alveoli (tubular acinar glands) has been caused by chronic mastitis. Figure 4b illustrates the presence of single-nucleus inflammatory leukocytes, including lymphocytes and monocytes, within the interstitial tissue between tubule-acinar glands. The expansion of connective tissue is indicative of chronic mastitis. Figure 4c illustrates the presence of inflammatory cells and the expansion of connective tissue between tubule-acinar glands, which are contributing factors to the development of chronic mastitis. Figure 4d indicates mastitis with a severe accumulation of leukocytes, particularly neutrophils, between tubule-acinar glands and secretory parts, accompanied by the destruction of interstitial tissue, which suggests a chronic disease process.

Fig. 5. Presents histological images of the mammary gland and skin layers of a cow treated with Ag/ZnO NPs. As illustrated in Figure 5, a lactiferous duct is observed, and no atrophy or inflammation is evident. In the hypodermis, mammary glands are observed in their unaltered state. The epidermis displays acanthosis, mild spongiosis, and hyperpigmentation in the basal layer, which is indicative of skin alterations resulting from previous chronic mastitis.

Discussion

The objective of this study was to present a novel approach to the treatment of mastitis using Ag/ZnO NPs. Mastitis is a significant disease in mammals, particularly dairy cows. It is caused by a diverse range of Gram-negative and Gram-positive bacteria (ESPECHE et al., 2012; KEMPER et al.,

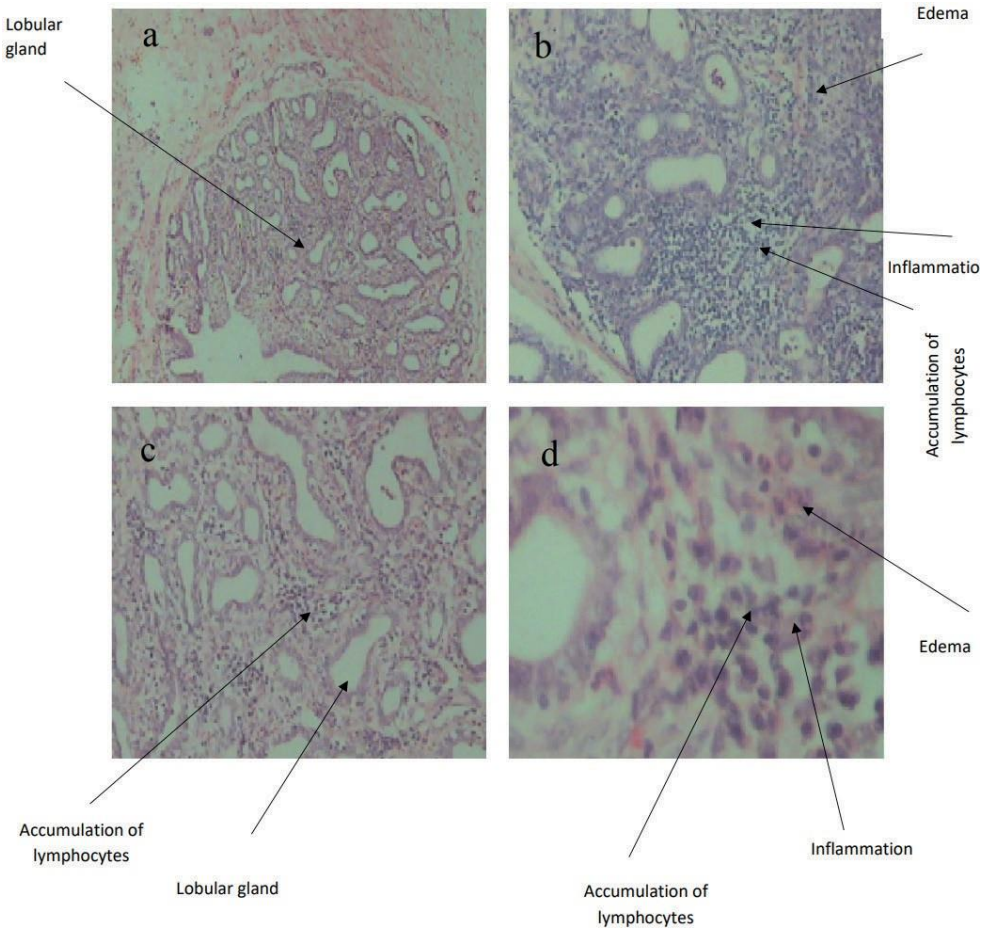

Fig. 4. Histological analysis of mammary glands before treatment

4a: the expansion of the connective tissue around the lobular glands (H&E. x40). 4b: The presence of mononuclear leukocytes, including lymphocytes and monocytes, is indicated in the interstitial tissue between the tubule-acinar glands (H&E. x100). 4c: An increase in lymphocytes in the interstitial tissue between the tubular-acinar glands (H&E.x100). 4d: The presence of inflammatory cells, including neutrophils and mononuclear leukocytes, along with edema of the interstitial tissue (H&E.x400).

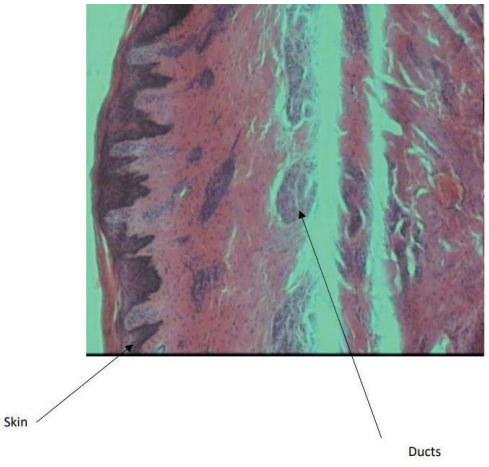

Fig. 5. Mammary glands after treatment with Ag/ZnO NPs

2013). In our study, 138 milk samples from mastitic cows were analyzed. The most frequently isolated pathogen was *S. agalactiae* (50.7%), followed by *E. coli* (25.3%) and *S. aureus* (23.9%). These three bacteria have been identified as significant pathogens associated with mastitis on the basis of data from other studies (HOLKO et al., 2019; COBIRKA et al., 2020; DUSE et al., 2021). A high prevalence of *S. agalactiae* is typically observed in regions with suboptimal hygiene and milking conditions, as well as in herds that are not adequately managed (KABELITZ et al., 2021).

The results of the micro broth dilution, agar well diffusion, and time-kill tests demonstrated that *S. agalactiae* exhibited the greatest susceptibility to Ag/ZnO NPs, in comparison to *S. aureus* and *E. coli*. Nevertheless, at the MIC concentration in the time-kill test, it was observed that all three bacteria were killed within three to six hours. In a study by Cuadra et al., ZnO/Ag nanocomposites prepared by the sol-gel method demonstrated a reduction in the percentage of live *S. aureus* ATCC 29213 and *E. coli* NCIMB 9484 of over 90%. A significant reduction in live bacteria was observed after three hours of treatment (CUADRA et al., 2022). In a study by Mtavangu et al., Ag/ZnO NPs prepared by green synthesis demonstrated antibacterial activity against *S. aureus* ATCC 6538P and *E. coli* ATCC 9677, as evidenced by the disk diffusion method (MTAVANGU et al., 2022). The same findings were reported in a study that investigated the antibacterial effects of Ag/ZnO, Ag, and Zn NPs against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. The findings indicated that Ag NPs and Zn NPs did not impede visible bacterial growth, whereas Ag/ZnO NPs did (ZHANG et al., 2014). The inhibitory effect of Ag/ZnO NPs on the growth of both gram-negative and gram-positive bacteria represents a significant advantage, rendering them promising material for the design of effective compounds for management of bacterial growth in a variety of systems. The inhibitory effect of the NPs is attributed to the combined actions of ZnO and Ag NPs, which facilitate the destruction of the cell wall and/or membrane, the generation of intracellular reactive oxygen species (ROS), the disruption of protein synthesis and related function (ZHANG et al., 2014; PANCHAL et al., 2020; CUADRA et al., 2022; MTAVANGU et al., 2022).

The results of a toxicity assay conducted using *Artemia salina* larvae indicate that Ag/ZnO NPs exhibit minimal toxicity at high doses (LD50>16384 µg/ml). The significant reduction in somatic cell count, mastitis score, and notable improvement observed in the histology images of udder tissue following treatment with varying doses of Ag/ZnO NPs in all six groups of cows in the short-term (72-hour) treatment period provides evidence that these NPs are an efficacious agent in the treatment of mastitis in vivo. Moreover, the low residual concentrations of silver and zinc observed in milk samples following treatment are indicative of the superiority of Ag/ZnO NPs over other antimicrobials. These findings collectively indicate that Ag/ZnO NPs are a promising candidate for use at high doses and for short periods for the treatment or alleviation of mastitis. Nevertheless, further studies are necessary to assess the safety and efficacy of these nanoparticles in long-term exposure in different formulations.

Conclusions

The findings of this study indicate that Ag/ZnO NPs exhibit significant antibacterial effects, both in vitro and in vivo, in mastitis treatment. Additionally, these NPs exhibit low toxicity, making them promising agents for the treatment and/or preparation of disinfectants used in the milking process of dairy cows. Nevertheless, further studies are necessary to investigate the effects of different types of NPs produced by various methods, subjected to prolonged exposure, and formulated in diverse ways.

Ethical approval

Ethical approval for our study was granted by the Ethics Committee of Islamic Azad University, Qom branch(IR.IAU.QOM.REC.1397.015).

Author's contributions

Pegah Shakib and Zeinab Sharafi: Conceptualization, Original draft, Methodology, Editing.

Shahrdad Arastoo: Investigation, Methodology. Mohammad Reza Zolfaghari: Visualization, Conceptualization, Writing and editing. The authors of the article declare that they have no conflict of interest in the publication of the article and are fully aware of the details of the article.

Declaration of competing interest

The authors have declared that no competing interests exist.

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ARASTOO, S., Z. SHARAFI, P. SHAKIB, B. MOHAMMADIAN, M. R. ZOLFAGHARI: Procjena antibakterijske aktivnosti nanočestica Ag/ZnO protiv bakterija *Streptococcus agalactiae, Staphylococcus aureus* i *Escherichia coli* izoliranih iz zaraženih mliječnih žlijezda krava. Vet. arhiv 95, 121-134, 2025.

SAŽETAK

Cilj istraživanja bio je *in vivo* i *in vitro* procijeniti antibakterijsku aktivnost nanočestica Ag/ZnO protiv bakterija *Staphylococcus aureus, Escherichia coli* i *Streptococcus agalactiae* izoliranih iz mliječnih žlijezda krava s mastitisom. Nanočestice Ag/ZnO sintetizirane su termičkom razgradnjom oksalatnih prekursora. SEM snimke pokazale su da je raspon nanočestica od 10 do 50 nm. Procjena toksičnosti nanočestica, provedena upotrebom modela *Artemia salina*, pokazala je da nanočestice nisu toksične pri visokim koncentracijama (LD50>16384 μg/mL). Ukupno je 138 uzoraka mlijeka krava s mastitisom uzgojeno u kulturi kako bi se procijenili antimikrobni učinci nanočestica Ag/ZnO. Antimikrobna osjetljivost uzgojenih bakterija procijenjena je određivanjem minimalne inhibicijske koncentracije (MIC), minimalne baktericidne koncentracije (MBC) i *time-kill* testom. Za procjenu učinkovitosti liječenja krava tretiranih nanočesticama Ag/ZnO upotrijebljeni su broj somatskih stanica (SCC), California mastitis test (CMT) i histološki testovi. Najčešće izoliran patogen bio je *S. agalactiae* (50,72 %), koji je pokazao najveću osjetljivost na nanočestice Ag/ZnO u testovima *in vitro* i *in vivo*.

Ključne riječi: mastitis; antimikrobni učinak; mliječne krave; nanočestice srebro-cink oksida (Ag/ZnO NPs)