Iranian Journal of Pharmaceutical Sciences
2021: 17 (4): 15-32
www.ijps.ir

Original Article

Antibacterial Activity of Silver Nanoparticle and L-carnitine Advantages on Mixed Vaginitis Caused by *Candida albicans*/ *Escherichia Coli* in Mice Models: An Experimental Study

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Abstract

Mix vaginitis refers to at least two potential pathogenic microbes in the vagina. Recently, the popularity of nanoparticles is increasing; these materials have been widely used as an antimicrobial agent in the treatment of chronic infections in which silver nanoparticles (AgNPs) are more widely considered. We aimed **to** establish a mixed vaginitis model in adult mice with Candida albicans and Escherichia coli, then evaluated the effect of AgNPs and L. carnitine (LC) to treat the vaginitis. In our study, the microdilution method and minimum biofilm inhibitory concentration were used for the antimicrobial activity of AgNPs. Vaginitis was made by intra-vaginal inoculation of 107 CFU/ml of both *E. coli*/*C. albicans* in adult NMRI mice. Mice were classified into 8 groups: (1) healthy mice without any treatment, (2) mice were infected intravaginally with equal volumes of *C. albicans* and *E. coli* suspensions, (3) healthy mice that received daily intraperitoneal injection of 250 mg/kg LC for two weeks, (4) infected mice that treated with a daily injection of 250 mg/kg LC for two weeks, (5) healthy mice that received daily intravaginal inoculation of 250 ppm of AgNPs for two weeks, (6) infected mice treated with daily intravaginal inoculation of 250 ppm AgNPs for two weeks, (7) healthy mice that received daily intravaginal inoculation of 250 ppm AgNPs and a daily injection of 250 mg/kg LC for two weeks, and (8) mice treated with daily intravaginal inoculation of 250 ppm AgNPs and a daily injection of 250 mg/kg LC for two weeks. All treatments with AgNPs and LC were daily for two weeks. A vaginal smear was taken throughout the experiment, and tissue sections were prepared using the hematoxylin-eosin method. The results showed that the 50% inhibitory concentration (IC-50) of AgNPs for *E. coli*, *C. albicans*, and their mixture was 96.84, 11.23, and 35.67 ppm, respectively, and their IC- 90 values were 201.77, 105.51, and 173.13 ppm, respectively. MBIC-90 % of AgNPs for *E. coli*, *C. albicans*, and the mixture of them were 500, 125, and 250 ppm, respectively. The estrus cycle in treated mice was similar to intact mice**,** and the order of their vaginal tissue sections confirmed the treatment of mixed vaginitis. In conclusion**,** co-administration of AgNPs and LC may eliminate the adverse effect of AgNPs and mixed vaginitis.

Keywords: Ag nanoparticle, Antimicrobial, *C. albicans.*, *E. coli.*, L-carnitine., Mixed Vaginitis.

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Cite this article as Fatahi Dehpahni M, Chehri K, Azadbakht M, Antibacterial Activity of Silver Nanoparticle and L-carnitine Advantages on Mixed Vaginitis Caused by Candida Albicans/ Escherichia coli in Mice Models: An Experimental Study, 2021, 17 (4): 15-32.

1. Introduction

The vagina has a smaller pathogen population, causing vaginitis signs and symptoms, than other anatomical parts of the body. Nevertheless, diagnosis and the establishing of these infections are complex [1, 2]. Mix vaginal infection refers to the presence of at least two potential microbial pathogens in the vagina, each causing signs and symptoms. Given the high rate of occurrence of yeast colonization in the vagina, Candida would likely have the highest prevalence in mixed vaginitis [3]. Fungi and bacteria can exist with each other and lead to mixed vaginitis. This infection rarely occurs (<5%), which is due to the existence of at least two microorganisms [4]. *Candida albicans* is one of the most frequent pathogenic human yeast and mainly inhabits host mucosal and skin [5]. This yeast is an opportunistic microorganism that its proliferation prevents by barriers of the vagina, including *lactobacilli* and their products [6]. The colonized vagina with *Candida* can increase the prevalence of mixed infections. These fungi can form biofilm with other bacteria such as Escherichia coli, which promotes bacterial pathogenicity, protects them from host defense, and increases pathogenicity and drug resistance in fungi [7]. *C. albicans* causes to promote the proliferation and growth of *E. coli* [8]. The mixed vaginal infection needs dual antifungal and antibacterial therapy [4]. On the other hand, the infection caused by biofilm not only are responsible for resisting antimicrobial drugs but also causes chronic and recurrent infections [9].

There are few studies about mixed infections of *E. coli* and *C. albicans*, which have shown that these infections increase antibiotic resistance, and the tolerance pattern of the mixed infection to antimicrobial medicines is completely different from single models of *E. coli* and *C. albicans* [10]. Combination therapy uses for mixed infections. Wherever doctors are unsure of causation and there are severe symptoms, require the administration of two medicines before diagnostic tests are available. For several decades, the Federal Drug Administration in the U.S. has opposed medicines that include more than one antimicrobial agent; that is, combination antimicrobial drugs are not permitted [3]. Therefore, the use of new therapies that can replace antibiotics and reduce their usage is essential for human health. Recently, the popularity of nanoparticles is increasing; these materials have been widely used as an antimicrobial agent in the treatment of chronic infections in which silver nanoparticles (AgNPs) are more widely considered [11]. Many studies have shown that AgNPs have potential antimicrobial properties that strongly

affect ranges of antibiotic-resistant microorganisms such as fungi and bacteria, and not only cause to die planktonic microbes but also play an important role in biofilm destruction [12]. In spite of the wide usage of AgNPs, many studies have shown that exposure to these nanoparticles may have adverse effects on health such as oxidative stress, inflammation stimulation, apoptosis, and so on [13]. *In vitro* and *in vivo* studies have shown that L-carnitine (LC) plays an important role in modulating metabolism-related diseases**,** especially mitochondrial disorders. It plays an important role in the improvement of inflammatory diseases through the modulation and reduction of inflammatory cytokines [14]. LC is a substance that acts like a vitamin that participates in lots of metabolic functions, such as mitochondrial mechanisms, and ketogenesis, so this is an important agent [15]. LC produces Hemeoxygenase and nitric oxide synthase at a high level, which has a protective effect against oxidative stress and improved inflammation. High levels of LC increase antioxidant capacity and reduce free radicals [16].

For the first time, in the present experimental study, we established the mixed vaginal infection model in adult NMRI mice using *C. albicans* ATCC 10321 and *E. coli* ATCC 25922, and we applied AgNPs, as an antimicrobial factor, for inhibiting the combination of *C. albicans* and *E. coli*, and their biofilm in this infection. In our study**,** LC was used for removing tissue damage caused by AgNPs and infection.

2. Material and Methods

2.1. Materials

AgNPs solution stock, with 20 nm size and 2000 ppm concentration, provided from Payam Company of Hamadan, Iran. LC hydrochloride from Sigma Aldrich, RPMI-1640 medium, Loria Bertani (LB), Tryptic Soy Broth (TSB), Mueller Hinton Broth (MHB), and Agar (MHA), provided from Merck Company, Germany. Microbial strains *C. albicans* ATCC 10321 and *E. coli* ATCC 25922 were purchased from the Iranian Scientific and Industrial Research Company (IROST).

2.2. Preparation of Microbial Suspension

Before any experiment, an inoculum of overnight cultures of both *C. albicans* and *E. coli* strains were grown in TSB media at 37° C for 24 h separately. Subsequently, they were harvested with a centrifuge at**,** 3500 rpm for 15 minutes. Each of the tubes containing microbial cells was washed twice by phosphate-buffered saline (PBS) and diluted to a concentration of 10^{7} CFU/ml by spectrophotometer UV-Vis. For the preparation of the mixture of *E. coli* and *C. albicans* suspension, the same volumes of two microbial suspensions were mixed [17, 18].

2.3. Disk Diffusion

The standard microbial suspension of *C. albicans*, *E. coli*, and their combination, as described above, were cultured on the surface of MHA medium. Sterile paper disks were

then impregnated with different concentrations of AgNPs (62.5, 125, 250, and 500 ppm) on a medium surface. Dimethyl sulfoxide was used as the solvent for AgNPs. Plates were incubated for 24 h at 37 ° C. The diameter of the zone inhibition formed around the paper discs was measured and reported in millimeters [19]. All tests were done in triplicates.

2.4. Inhibitory Concentration of Silver Nanoparticles

The MIC of AgNPs against pathogenic *C. albicans*, *E. coli*, and their combination were determined by the microdilution broth method based on the Clinical & Laboratory Standards Institute (CLSI) guideline [19]. Suspension of the *C. albicans*, *E. coli*, and their mixture, as described before, were prepared. At first, 100 µl of MHB medium was poured into wells, and then the highest concentration of AgNPs (500 ppm) was diluted in the first well, and 100 µl from that were transferred into other walls. Serial two-fold dilution of nanoparticles was made in a culture medium with a range of concentration 1.95-250 ppm. Wells 9 and 10 were used as positive and negative controls, respectively. In each well, the final volume was 100 µl, then 50 µl of microbial suspension was added to wells except 10. The microplates were incubated overnight at 37° C. In this study, MIC is determined by visual observation, also the optical density (OD) of 600 nm was measured by ELIZA reader for *C. albicans*, *E. coli*, and their mixture. To determine MBC, two wells before and after the well of MIC were cultured on MHA media and incubated for 24 h. That plates without any microbial contamination were considered as MBC [20]. The 50% and 90% Inhibitory Concentration (IC- 50 and IC- 90) and lethal concentration were calculated by Excel software. All tests were done in triplicates.

2.5. Anti-biofilm Effect of Silver Nanoparticles

Due to the high resistance of biofilm to antimicrobials agents, higher concentrations of AgNPs were used for this test than the Inhibitory concentration. To investigate the anti-biofilm effects of AgNPs against *C. albicans*, 100 µl of yeast suspension containing 107 CFU/ml was added to the RPMI-1640 medium, For *E. coli* biofilm formation, the bacterium was grown into LB broth medium, and for poly-microbial biofilm, 24- h culture of *C. albicans* and *E. coli* were prepared and suspended in RPMI-1640 medium (107 CFU/ml, 1:1). A volume of 100 µl of the suspension was added into wells of a microplate, and it was incubated and shacked **at** 75 rpm for 1.5 h at 37° C. After this adhesion stage, the suspension was removed and wells were washed with PBS. 100 µl fresh media was added, mono and dual-species Biofilm of *C. albicans* and *E. coli* were formed for 24 h at 37° C. For the antimicrobial effect of AgNPs on biofilm, after washing by PBS, a serial dilution of AgNPs (15.62, 31.25, 62.5. 125, 250, and 500 ppm) was prepared in the medium, then poured into the biofilm and kept in an incubator for 24 h at 37° C. subsequently, the medium was removed, washed with PBS, and fixed with ethanol.

Crystal violet measurement was used, microplate wells were washed with PBS and 100 µl of methanol 99% was added to each well. After fixing, this solution was removed, the microplate dried in the air, stained with 1:50 Crystal violet, and incubated at room temperature for 20 minutes. 150 µl of 33 % acetic acid glacial was added to each well, and the absorption was taken by the Eliza Reader at 600 nm [21].

2.6. Mice

Six or seven-week-old female NMRI mice from Pasteur Institute of Iran were used in the experiment. Mice were housed in cages and had free access to water and food. To decide how a great deal dosage of AgNPs in mice, Lethal Dose 50% (LD 50%) should be determined, which is critical at the beginning of the tests. AgNPs were bought as a solution organized at a concentration of 2000 ppm. AgNPs had been diluted in dimethyl sulfoxide to create the doses required to realize the LD 50. Five mice per group were used for this experiment injected with three doses, 1000, 1500, and 2000 ppm of AgNPs intraperitoneally. Mice had been found for physique temperature, body weight, mortality, drowsiness, and diarrhea were monitored for the duration of 14 days. But, our outcomes of this check confirmed that AgNPs solution did not have the LD 50 in this concentration of nanoparticles for two weeks. The murine model of mixed vaginal infection was prepared by intravaginal inoculation of an equal volume of *C. albicans* and *E. coli* as described before. For vaginitis formation in mice, 50 µl of the mixture of suspension was shifted into the vaginal cavity. Two days after the first infection, this model was improved by inoculating repetition [17]. All mice were randomly classified to the 8 groups (n=7 in each group): (1) healthy mice without any treatment, (2) mice were infected intravaginally with the equal volumes of *C. albicans* and *E. coli* suspensions, (3) healthy mice that received daily intraperitoneal injection of 250 mg/kg LC for two weeks, (4) infected mice with equal volumes of *C. albicans* and *E. coli*, that treated with a daily injection of 250 mg/kg LC for two weeks, (5) healthy mice that received daily intravaginal inoculation of 250 ppm of AgNPs for two weeks, (6) infected mice with equal volumes of *C. albicans* and *E. coli*, and treated with daily intravaginal inoculation of 250 ppm AgNPs for two weeks (I + AgNPs), (7) healthy mice that received daily intravaginal inoculation of 250 ppm AgNPs and a daily injection of 250 mg/kg LC for two weeks (AgNPs +LC), and (8) mice in this group were infected with 1:1 rate of *C. albicans* and *E. coli*, and treated with daily intravaginal inoculation of 250 ppm AgNPs and a daily injection of 250 mg/kg LC for two weeks (I+ AgNPs +LC). All tests with animals were agreed upon by the animal ethics committee of Razi University, Iran [22].

2.7. Estrus Cycle Determination

The Estrus cycle of all mice was synchronized with an intramuscular injection of 0.5 mg of estradiol valerate. The Estrus cycle of mice has four stages**,** proestrus, estrus,

metestrus, and diestrus. Stages of the estrous cycle were determined by vaginal smear for each mouse. The presence of epithelial cells during proestrus is an indicator of this stage. In the estrus stage, only horny cells without nuclei are detectable. At the metestrus, epithelial horn cells were observed in vaginal specimens with leukocytes. In diestrus, horn cells may rarely be seen with a large population of leukocyte cells. In summary, 100 µl of the physiological serum was aspirated into the mice's vaginal cavity. The smear was immediately spread on the glass slide, fixed with alcohol, and dried at room temperature. Then, the slides were stained with the Papanicolaou staining method. The stages of the estrous cycle were determined, by the microscope, according to the ratios and morphology of Leukocytes and epithelial cells [23].

2.8. Histological Study

Mice in the infected group with both *C. albicans* and *E. coli* after five days and mice in treated groups after two weeks were sacrificed by cervical dislocation. For the histological study, the vagina was dissected and put in Bouin solution, and follow that dehydration, clarifying, and embedding in paraffin was done. Finally, 5 µm sections of the paraffin block were cut and stained with Hematoxylin and Eosin (H&E) method [24].

2.9. Statistical Analysis

All results were undertaken by SPSS version 26 and Prism, one-way variance (ANOVA), and Tukey. Less than 0.05 P values were considered.

3. Results and Discussion

Mixed vaginitis forms about 32.2% of vaginal infections due to the presence of at least two pathogens in the vaginal area, causing symptoms and complications in the infected women [25]. Due to the frequent colonization of *C. albicans* in the vagina, one of the most common microorganisms involved in this infection is *C. albicans* [3]. On the other hand, *E. coli* is one of the most common bacteria in the female reproductive system that colonizes easily in the vagina due to the anatomical proximity of genital and urinary systems in women [26]. In this study, mixed vaginitis was induced by the simultaneous inoculation of *C. albicans* and *E. coli* in the vaginal cavity of mice. A few studies have shown that gram-negative bacterial endotoxins, such as lipopolysaccharide in the cell wall of *E. coli*, have pro-inflammatory properties, and can regulate *C. albicans* growth and biofilm production [27]. In the past, most of the focus was on mono-biofilms caused by single microbes, but now it has been shown that poly-biofilms are more common and cause more infections. In such infections, interactions between microorganisms affect the pathogenesis and tolerance of drugs [28]. The high antibiotic-resistance exists for the treatment of these co-infections, so it was used to treat AgNPs in this study. Antibiotic-resistant pathogens are currently one of the major problems in global health [29]. The strong antimicrobial activity of AgNPs is due

to their large surface area, which enhances their direct contact with pathogens [30]. Rodziga *et al*. in 2013, investigated the antimicrobial activity of AgNPs on gram-negative bacteria. They showed that these nanoparticles inhibited the biofilm formation of *E. coli* at 4 to 5 μg / ml concentrations, and the ability of *E. coli* biofilm formation at 100 to 150 μg / ml of AgNPs significantly decreased [31]. Chawilbogh *et al*. investigated the effects of different nanoparticles on the pathogen *Candida* fungi, and their studies showed that AgNPs cause serious damage to the fungal structure. AgNPs attach to the cell surface, and this binding causes structural changes and cellular damage in critical cell activity, such as permeability, the effect on respiratory enzyme activity, and ultimately cell death. These nanoparticles completely inhibit yeast growth [32]. To investigate the antimicrobial effect of AgNPs, we used the disk diffusion method and determination of inhibition (IC- 50 and IC- 90). The results of the disk diffusion test showed that the presence or absence of a significant difference between the mean diameters of the zone inhibition at different concentrations of AgNPs could be attributed to the concentration of nanoparticles. The highest zone of inhibition was 20.7±0.34, 22.3±0.21, and 21.7±0.92 mm for *E. coli*, *C. albicans*, and their mixture at 500 ppm of AgNPs, respectively ($p<0.05$) (Figure 1 and Table 1). IC-50 and IC- 90 values are the concentration of AgNPs required for 50% and 90% microbial growth inhibition [24]. According to the IC-50, IC-90, and lethal concentration values are exhibited in Table 2 and Fig. 2. IC- 50 values of the AgNPs for *E. coli*, *C. albicans*, the combination of *E. coli*/ *C. albicans* were 96.84, 11.23, and 35.67 ppm, respectively. The IC- 90 values of the AgNPs for *E. coli*, *C. albicans*, and the mixture of them were 201.77, 105.51, and 173.13 ppm, respectively. This antimicrobial activity of AgNPs is shown significantly more on the *E. coli* model **of** infection than the mixed form and *C. albicans*. The MBC of AgNPs that caused was calculated by culturing the contents of the wells in the MHA and is shown in Fig. 3.

Lara *et al*. investigated the effects of AgNPs on the *C. albicans* biofilm. Their studies showed that AgNPs had significant effects on *Candida* biofilm inhibition. Their results showed that AgNPs cause general loosening of the biofilm structure due to damage to the membrane and cell wall and inhibition of filamentation. As a result of these disorders, the fungus cell wall inhibits its biofilm [33]. Biofilm degradation was occurred by AgNPs at concentrations several times more than the IC. The first step for evaluating the antibiofilm effect of AgNPs against the mixed model of vaginal infection was an assessment of mono- species of biofilm of *E. coli* and *C. albicans* susceptibility to this nanoparticle. Our results presented in Table 3 indicate the minimum biofilm inhibitory concentration (MBIC) of AgNPs for mono and double- species of *E. coli* and *C. albicans*. RPMI1640 culture medium was used for polymicrobial biofilm formation of *C. albicans* / *E. coli*. This biofilm was grown in this medium and the results were calculated by

ELIZA reader with 600 nm optical density (OD). Our results presented in Table 3 indicate that the MBIC-90 % of AgNPs for *E. coli*, *C. albicans*, and the mixture of them were 500, 125, and 250 ppm, respectively.

The strong antimicrobial property of AgNPs has made it a growing material. But these nanoparticles have been shown to have toxic effects [34]. Studies have been carried out to evaluate the toxic effects of AgNPs on blood and blood factors [35]. But research into their effects on vaginal tissue is scarce. According to a study following in vaginal inoculation of AgNPs into the rabbit vagina, these nanoparticles accumulate and cause pathological changes in the vaginal mucus and other surrounding tissues [36]. In our study, tissue sections prepared from vaginal tissue in AgNPs and I + AgNPs groups showed that AgNPs had toxic effects on vaginal tissue and increased leukocyte hyperplasia, increased levels of blood vessels, inflammation, hyperemia, disruption of tissue order and partially epithelial horn. On the other hand, toxins are metals in the reproductive system in women that disrupt the hormonal cycle [37]. Our study also found that the mice, following the introduction of AgNPs into their vagina, were associated with significant changes in the estrous cycle and that all mice receiving AgNPs were mainly in the metestrus and diestrus phases. LC is an endogenous amino acid-like compound in all mammalian species. It plays an important role in regulating and balancing energy in the cell membrane. In this study, administration of LC with silver was used to eliminate the negative effects of AgNPs. Many studies have shown that AgNPs are associated with mitochondrial disorders [38]. In our study, the approximate ratio of a cell type observed in the vaginal smear was considered as an accurate method in determining estrus cycle stages. For the duration of the proestrus cycle, mice in the I, I + LC, and I + LC + AgNPs groups were similar to the control group, but the mice in I + AgNPs had a significant decrease compared to the control group. For the duration of the estrous phase of the cycle, the mice in the I, I + LC, and I + AgNPs groups had a significant decrease compared to the control group. Regarding the duration of the mice in the metestrus phase, the mice in the I, I + LC, I + AgNPs groups had a significant increase compared to the control group. For the duration of the diestrus phase, mice in the I, I + LC, and I + AgNPs groups were similar to the control but I + LC + AgNPs group had a significant decrease compared to the control (Table 4). Our results ON vaginal appearance showed that the use of AgNPs could not treat the infection and made the condition worse. The vaginal discharges compared with the control group, which was normal and odorless, in I and I + AgNPs, I + LC were yellowish and smelly, but in I+ AgNPs + LC group were normal and liked control groups. Vaginal swelling and color in these groups were normal. The results showed that co-administration of these two substances together improved the infections (Table 5). Vaginal tissue histology results were concluded based on microscopic observation

of tissue sections on the slides prepared by H & E staining. In this regard, some features were investigated, including leukocyte levels, hyperplasia, hyperemia, inflammation, keratinization of the epithelium, and order in the connective tissue. In terms of leukocyte levels, compared with control groups (Fig. 4a) and LC (Figure 4b), that were normal, but in AgNPs (Figure 4c) group were abnormal and high, AgNPs +LC (Figure 4d), the group was normal and similar to the control group. In I (Figure 5a), I+LC (Figure 5b), and I+ AgNPs (Figure 5c) groups were abnormal and high, but in I+ AgNPs +LC (Figure 5d) was normal and similar to the control group. In hyperplasia aspect compared with control groups, which did not have this condition, in I, AgNPs, I+ AgNPs, and I+LC groups had hyperplasia, but in LC, AgNPs +LC, and I+ AgNPs +LC groups did not have hyperplasia, were normal, and like the control group. In terms of hyperemia, compared with control groups that did not have this condition, in I, I+ AgNPs groups was very high, and AgNPs, LC, and I+LC groups were high, but in AgNPs +LC, and I+ AgNPs +LC did not have hyperemia, were normal, and similar to the control group. In the inflammation aspect compared with control groups, which did not have it, in I, AgNPs, I+ AgNPs, LC, and I+LC groups had inflammation, but in AgNPs +LC, and I+ AgNPs +LC groups were normal, as the control group and did not have this condition. In terms of keratinization of the epithelium, compared with control groups, that were normal, in I and AgNPs groups were abnormal and increased but in other groups, in I+ AgNPs, LC, and I+LC groups were not seen, but in AgNPs +LC, and I+ AgNPs +LC groups were normal. In terms of the order in the connective tissue, compared with control groups, that were normal, in I, AgNPs, I+ AgNPs, LC, and I+LC groups were abnormal, but in AgNPs +LC, and I+ AgNPs +LC were normal and similar to the control group (Figures 4 and 5). *In vitro* and *in vivo* studies have shown that LC plays an important role in modulating metabolism-related diseases, especially mitochondrial disorders. It plays an important role in improving inflammatory diseases through the modulation and reduction of inflammatory cytokines [14]. In our study, the co-administration of AgNPs and LC showed improvement in the disease. Leukocyte levels normalized, hyperplasia disappeared, inflammation decreased, and blood vessels and tissue order returned to normal. In the group of mice that were treated with both AgNPs and LC, the estrous cycle in mice was normal.

4. Conclusion

Mixed vaginitis formed by *C. albicans* and *E. coli* is a complex type of vaginitis that does not have a specific medical treatment. On the other hand, the fungal and bacterial combination increases the resistance of both strains to antibiotics. In the present study, we used AgNPs with a diameter of 20 nm as a potent antimicrobial agent that was able to remove free microbes and biofilm structure induced by both single and mixed microbes. We also examined the toxic effects of AgNPs

on the vaginal tissue of mice and administration of LC with silver was used to eliminate the negative effects of AgNPs. Histological studies from mice vaginal sections showed the toxicity of AgNPs to the tissues. But co-administration of LC and AgNPs can improve the infection.

Acknowledgements

This paper emanates from MSc thesis of first author submitted to Department of Biology, Faculty of Science, Razi University 67149-67346, Kermanshah, Iran. Authors acknowledge personnel of Laboratory of Microbiology for technical assistance. This study was supported by an intramural fund.

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Tables:

Table 1. Antimicrobial activity. Zone of Inhibition in mm of AgNPs against *E. coli* ATCC 25922, B. *C. albicans* ATCC 10321, and combination of *C. albicans* & *E. coli*.

	Zone of Inhibition (mm)			
AgNPs (ppm)	*E. coli*	*C. albicans*	*E. coli*/ *C. albicans*	P value
62.5	9.98 ± 0.26^{bc}	15.95 ± 0.21^{ac}	11.1 ± 2.85^{ab}	0.038
125	12.41 ± 0.59^{bc}	18.35 ± 0.49^{ac}	16.25 ± 0.49^{ab}	0.005
250	14.32 ± 0.44^{bc}	20.39 ± 0.57^{ac}	16.95 ± 0.21^{ab}	0.009
500	20.7±0.34	22.3±0.21	21.7±0.92	0.060

a) different significant with *E. coli*, b) different significant with *C. albicans*, c) different significant with mixture of *E. coli*/ *C. albicans* ($p<0.05$).

Table 2. IC50 and IC- 90 values of AgNPs of against *E. coli*, *C. albicans*, and Mixture of *E. coli*/ *C. albicans* (ppm).

Microbes	IC- 50	IC- 90	MBC
E. coli	96.84	201.77	500
C. albicans	11.23	105.51	125
Mixture of *E. coli*/ *C. albicans*	35.67	173.12	250

Table 3. The minimal concentration of AgNPs able to reduce mono-species biofilm of *E. coli* ATCC 25922, *C. albicans* ATCC 10321, and their double-species biofilm. Crystal violet method used for mono and dual species biofilms.

AgNPs (ppm)	*E. coli* (%)	*C. albicans* (%)	*E. coli*/ *C. albicans* (%)	P value
15.62	26.23 ± 1.72^{b}	70.56 ± 0.77^{a}	29.71±1.01	0.008
31.25	40.49 ± 2.09^{bc}	79.61 ± 0.85^{ac}	45.55 ± 0.64^{ab}	0.002
62.5	46.76 ± 0.72^{bc}	81.61 ± 0.86^{ac}	67.95 ± 1.34^{ab}	0.001
125	59.36 ± 1.41^{bc}	94.87 ± 1.24^{ac}	83.61 ± 0.86^{ab}	0.001
250	71.01 ± 1.41^{bc}	95.98 ± 1.39^{a}	93.53 ± 0.75^{a}	0.004
500	99.98±0.01	100	100	0.142

a) different significant with *E. coli*, b) different significant with *C. albicans*, c) different significant with mixture of *E. coli*/ *C. albicans* ($p<0.05$).

Table 4. Different stages of estrus cycle in control, LC, AgNPs, LC & AgNPs, I, I + LC, I + AgNPs, and I + LC + AgNPs using crystal violet staining.

	Estrus cycle (Days)				
Groups	Pro-estrus	Estrus	Met-estrus	Di-estrus	P value
Healthy	2.5±0.71 ^{ceg}	3.5±0.71 ^{bcefg}	0.5±0.71^{bc}	3.5±0.71^{cefg}	0.036
LC	1.5±0.71 ^{dh}	1.5±0.71 ^{agh}	4.5±0.71^{acdefgh}	2.5±0.71^{acefg}	0.036
AgNPs	0.5±0.71 ^{adh}	1 ^{adh}	2.5±0.71^{abdh}	6±1.41^{abdgh}	0.010
LC & AgNPs	3.5±0.71^{bcefg}	2.5±0.71 ^{cefg}	1.5±0.71^{b}	2.5±0.71^{cdefg}	0.184
I	0.5±0.71^{adh}	0.5±0.71 ^{adh}	1.5±0.71^{b}	7.5±0.71^{abdh}	0.002
I + LC	1 ^{dh}	0.5±0.71 ^{adh}	1.5±0.71^{b}	7±1.41^{abdh}	0.005
I + AgNPs	0.5±0.71 ^{adh}	0 ^{abdh}	0.5±0.71^{bc}	9^{abdgh}	0.0001
I + LC + AgNPs	3.5±0.71^{bcefg}	3.5±0.71 ^{bcefg}	0.5±0.71^{bc}	2.5±0.71^{cefg}	0.036

a - Significant difference with control group, b - Significant difference with LC group, c - Significant difference with AgNPs group, d - Significant difference with LC & AgNPs group, e - Significant difference with I group, f - Significant difference with I + LC group, g - Significant difference with I + AgNPs group, h - Significant difference with I + LC + AgNPs group ($p<0.05$).

Table 5. Macroscopic signs of mixed vaginitis and their treatment.

Groups	discharge	Swelling of the vagina	Tissue color	Leukocyte count
Healthy [1]	Normal	-	Normal	Normal
I[2]	Yellow and smelly	Low	Red	Moderate
AgNPs[3]	Yellow	High	Red	High
I + AgNPs [4]	Yellow and smelly	Low	Red	Moderate
LC[5]	Normal	Low	Normal	Normal
I + LC[6]	Yellow and smelly	Low	Red	Moderate
AgNPs + LC[7]	Normal	-	Normal	Normal
I + AgNPs + LC[8]	Normal	-	Normal	Normal

[1]: Healthy mice without any treatment

[2]: Mice were infected intravaginally with the equal volumes of *C. albicans* and *E. coli* suspensions

[3]: Healthy mice that received daily intravaginal inoculation of 250 ppm of AgNPs for two weeks

[4]: Infected mice that treated with daily intravaginal inoculation of 250 ppm AgNPs for two weeks

[5]: Healthy mice that received daily intraperitoneal injection of 250 mg/kg LC for two weeks

[6]: Infected mice that treated with a daily injection of 250 mg/kg LC for two weeks

[7]: Healthy mice that received daily intravaginal inoculation of 250 ppm AgNPs and a daily injection of 250 mg/kg LC for two weeks

[8]: Infected mice that treated with daily intravaginal inoculation of 250 ppm AgNPs and a daily injection of 250 mg/kg LC for two weeks

Figures:

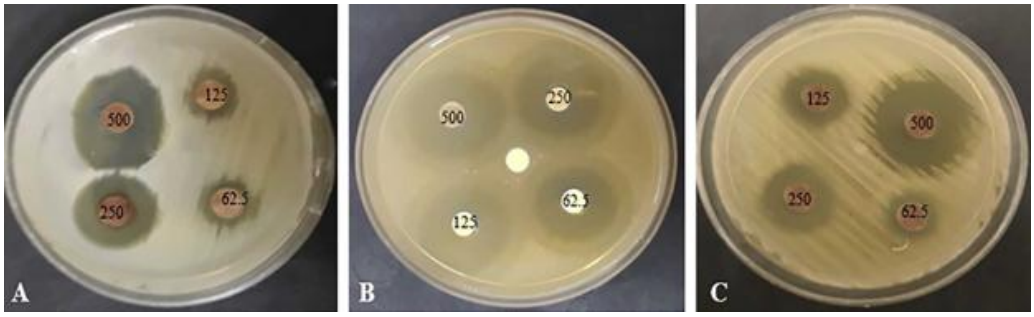
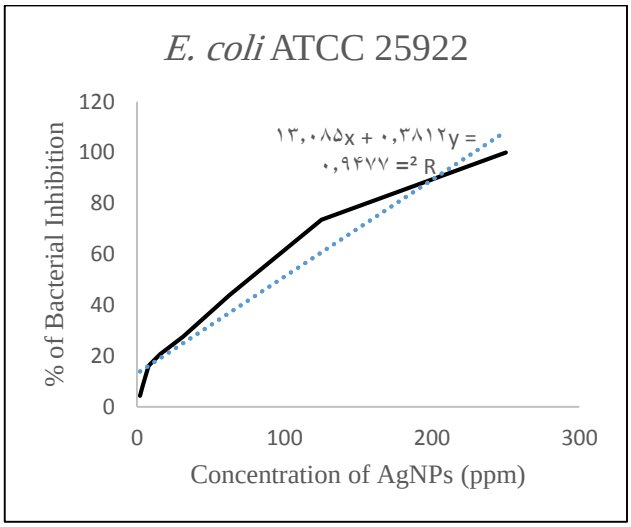
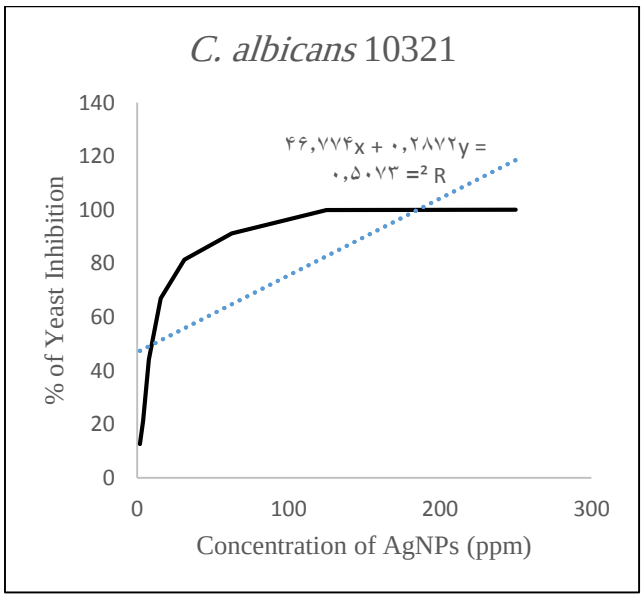


Figure 1. Disk diffusion method. Antifungal and antibacterial effect of four concentrations of silver nanoparticles on A. *E. coli* ATCC 25922, B. *C. albicans* ATCC 10321, and C. combination of *C. albicans* & *E. coli*.





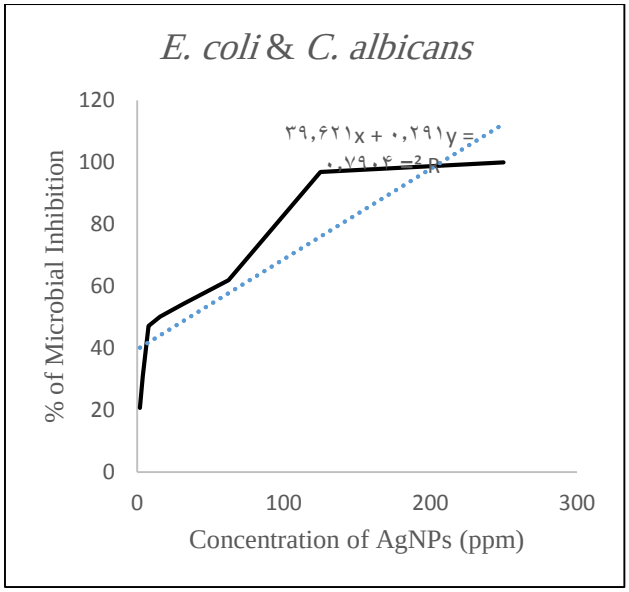


Figure 2. 50 and 90 % Inhibitory Concentration (IC- 50 & IC- 90) of AgNPs in 1.95- 250 ppm concentration against *E. coli* ATCC 25922, *C. albicans* ATCC 10321, and their combination.

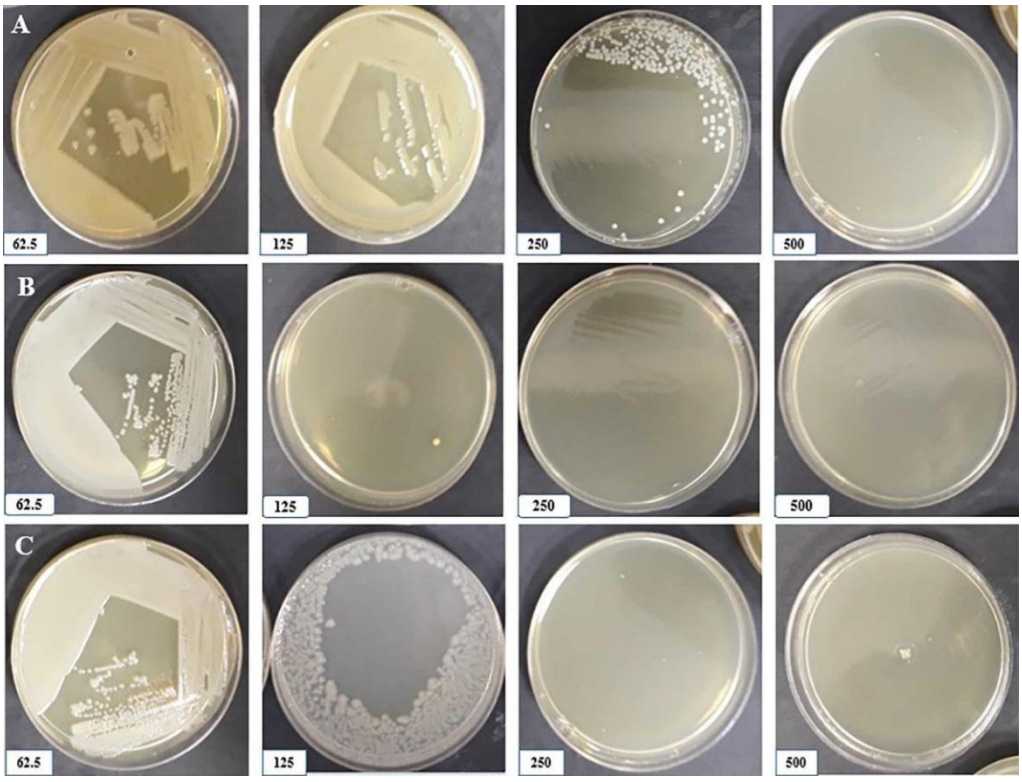


Figure 3. Results of wells cultivation; *E. coli* ATCC 25922 (A), *C. albicans* ATCC 10321 (B), and combination of them (C). To determine the lethal concentration of AgNPs, 50 µl of the contents five wells of each row of microplates wells were cultured on the Müller Hinton agar medium. Plates were incubated for 24 h at 37° C and the bacterial growth was measured.

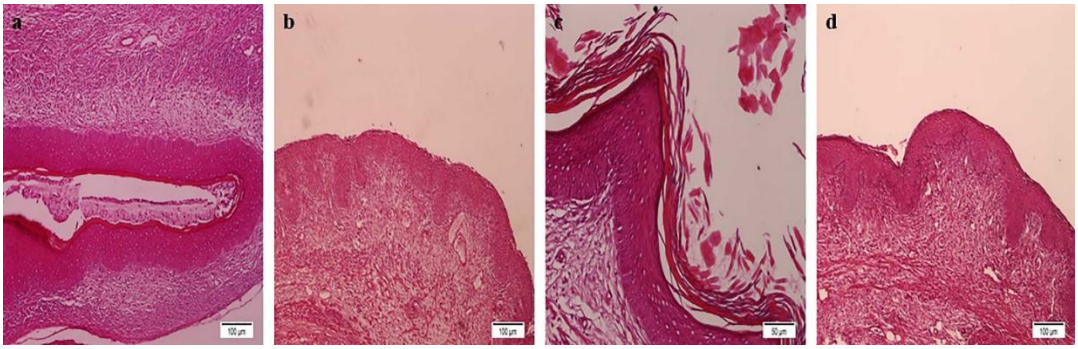


Figure 4. Tissue section examination of mice vagina after Hematoxylin and eosin (H & E) staining; control group (a) was indicated normally blood vessels and level of leukocytes without any bleeding hyperplasia and inflammation. The connective tissue has been regular, LC group (b) was shown that normal leukocyte, increase in blood vessels and there were signs of inflammation, Bleeding, and epithelium keratinization, and irregular connective tissue, AgNPs group (c) was shown that the level increases blood vessels and leukocytes in tissue. Inflammation, hyperplasia, keratinization happened, and connective tissue was very disturbed, and AgNPs + LC group (d) was indicated that the feature of these tissues was normal and like intact mice group.

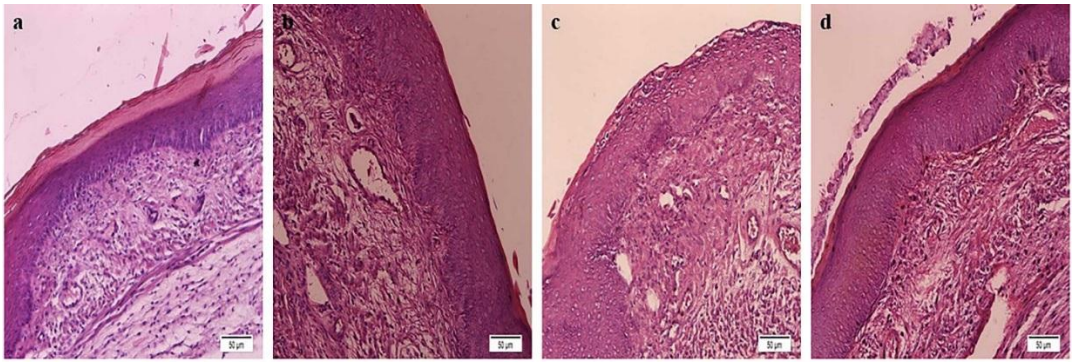


Figure 5. Tissue section examination of mice vagina in treated groups; I group (a) infected mice with the mixture of *E. coli* and *C. albicans* without any treatment, I + LC group (b) Infected mice with the combination of microbes that treated with daily injection 250 mg of LC for two weeks, I + AgNPs group (c), Infected mice with mixed vaginitis that treated with 250 ppm of nanoparticles every day, and I + AgNPs + LC group (d) infected mice that treated with co-administration of 250 ppm AgNPs and 250 mg/kg LC for two weeks. These mice had similar sections of tissue to the intact mice group.

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