*Research Article*

# Therapeutic Effects of Co-administration of Silver Nanoparticles and Vitamin C on Vaginal Infection Caused by Group B *Streptococcus*

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## Abstract

*Background:* Group B *Streptococcus* (GBS) is a bacterium commonly isolated from the vagina. Silver nanoparticles (SNPs) are potential antibacterial agents, and studies have shown their toxic effects. Vitamin C (VC) is an essential vitamin with a protective role against toxicological conditions. We aimed to the evaluation therapeutic effects of the co-administration of SNPs and VC on vaginal infection caused by GBS in mice models.

*Methods:* Vaginitis model was established by intravaginal inoculation of GBS. The Co-administration of SNPs and VC was used to treat the infections. The antibacterial activity of SNPs was determined by the minimum inhibitory concentration. The toxicity of nanoparticles was measured by MTT assay. The microbial load and estrous cycle of mice during treatment were evaluated. Finally, blood samples and vaginal tissue sections were isolated and analyzed.

*Results:* The results showed that SNPs have excellent effects on GBS, and the MIC was 512 ppm. Cell viability after exposure at 512 ppm of SNPs was 32.11% but after treatment with VC increased viability at 512 ppm of nanoparticles to 65.32%. In mice that received SNPs and VC at the same time, the bacteria were completely removed from the vagina, and estrus cycle returned to normal cycle. Analysis of the prepared blood samples and microscopic examination of the vaginal sections confirmed the results.

*Conclusion:* SNPs have a potential antibacterial effect on GBS. But nanoparticles have toxic effects on mammalian cells. The simultaneous use of VC, as a powerful antioxidant, can completely eliminate this toxic effect of nanoparticles.

## Introduction

Vaginal infections are usual gynecologic issues affecting women of different ages.[1] Group B *streptococcus* (GBS) is an asymptomatic bacterium isolated from the reproductive tract system of women. These opportunistic bacteria can create an invasive infection in the vagina using colonization, host cell attachment, progression of the disease, and invasion.[2,3] The major virulence factors of GBS include capsule, β-hemolysin, pilus proteins, surface glycoproteins, surface adhesins, and so on.[2] GBS infection and colonization of vaginal tissue need the ability of the bacteria to attach and persist to vaginal mucosal surfaces. GBS can form biofilm communities, which facilitate proliferation and bacterial survival by increasing resistance to nutrient deprivation, antibiotics, and host defenses.[3] Vaginal infection is an inflammatory disease that is related to severe complications, including urinary tract infections, sexually transmitted diseases, preterm labor, pelvic inflammatory disease, and infertility. So, it is necessary to diagnose this infection properly and use an appropriate curative method.[4] Infected women are usually administered ampicillin and penicillin as first-line antibiotics, but in conditions of resistance and allergy, are instead cured with erythromycin, clindamycin, and vancomycin.[2]

While antibiotics are commonly used for the treatment of GBS vaginal infection, the resistance to several types of antibiotics has increased during recent years.[5,6] Antibiotic resistance in bacteria could be stemmed from the overuse of antibiotics, interfering with the therapeutic method of infection. As a result, these infections require stronger antibacterial agents and more costs of therapy.[6] Currently, studies about the development of new agents with anti-microbial effects are very hard processes that need years to research the safety and efficacy of the materials, while multi-drug resistant pathogens keep growing worldwide. Nanoparticles have been developed in the post-antibiotic durations as new factors that help to fight microbial pathogens.[7,8] Among several types of nanomaterials, silver nanoparticles (SNPs) introduce as a great alternative to antibiotics because they can be used to prevent microbial infections.[9-11] From ancient, silver has been applied as an

excellent antimicrobial substance.[10] SNPs are introduced as a material with a diameter of 1–100 nm.[12] The antimicrobial effects of SNPs are due to releasing Ag ions[11]. The potential of SNPs as antibiotic agents is linked to their different mechanisms and functions. They attack bacterial cells in many structures and kill them.[10]

Because of the development of the nanomaterial market and the huge offer of products containing them, studies of their safety have become important in the environment and for humans. The major factor considered is nanoparticle toxicity. SNPs can cross the barriers of defense in organisms and produce mild to severe toxic effects.[10] SNPs have toxic properties in various systems like mammalian cells, burn wounds, human hepatoma cells, alveolar macrophages, fibroblasts, cultured keratinocytes, and so on. SNPs possess extremely toxic effects because of their biological reactivity. Actually, releasing Ag ions is responsible for this toxicity.[13-15] Studies that provide a mechanism of SNPs toxicity are few, although their applications in many fields such as industry and medicine are growing.[16] So, assessment of their toxic effects becomes an urgent duty.[9] In this study, we used vitamin C (VC) to eliminate the cytotoxic effect of SNPs.

VC is a necessary water-soluble substance in biology. This is a widely affordable and available antioxidant agent that has been applied to curtail oxidative damage.[17] VC entrance into cells is necessary for its function.[18] VC simply clears physiological reactive oxygen species such as hydroxyl, superoxide, and peroxyl radicals. In some research, this ability of VC was used to the reduction in nanomaterial toxicity following its supplementation with nanoparticles studied.[19] There is a widespread notion that VC supports the functions of the immune system.[20] On the other side, it has been demonstrated that VC is related to human fertility and has evolutionary importance, but its accurate physiological function in reproduction metabolism has been unclear.[21] The aim of our study was to establish the murine model of vaginal infection by intravaginal inoculation of GBS. For the first time, we applied the combination of SNPs and VC for vaginitis treatment in infected mice. We measured the antibacterial effect of SNPs on planktonic and biofilm forms of GBS. To eliminate the toxic effect of nanoparticles, we used VC. We evaluated the protective effect of VC on cell viability and hematological parameters induced exposure of SNPs and vaginal GBS infection in NMRI mice.

## Methods
### Materials
The SNPs were purchased as a brown powder with a particle diameter of 20 nm from Sigma Aldrich Company. The nanoparticles stock solution was prepared using 5% dimethyl sulfoxide (DMSO) at a concentration of 2048 ppm and stored in dark containers at room temperature. VC was purchased from Alborz Pharmaceutical Company. In this study, penicillin,[2] as a routine first-line treatment, was purchased from Caspian Pharmaceutical Company and used to control the therapeutic effect of SNPs and

VC in mice vaginal infection. The culture medium used in this experiment, Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), Muller Hinton Agar (MHA), Muller Hinton Broth (MHB), Todd Hewitt Agar (THA), DNase agar, Bile Esculin agar, and Mannitol salt agar, purchased from Merck Company.

### Preparation of GBS
*Streptococcus agalactiae* ATCC 13813 (Iranian Research Organization for Science and Technology) bacteria were cultured in TSA including 5% blood of sheep (TSA- blood) for 24 hours at 37 °C. To prepare the microbial suspension, three GBS colonies were transferred to TSB and incubated for 37 to 24 hours at 37 °C. After the incubation, the bacterial cells were centrifuged (12000 rpm, at 25 °C for 3 minutes) and transferred to sterile normal saline solution and the turbidity was examined using a spectrophotometer. The turbidity required for microbial testing should be equal to the standard 0.5 McFarland, which is approximately $1\times 10^8$ CFU / ml. GBS strain was prepared in TSB containing 5% sheep blood and 20% glycerol at -80 °C and stored for long-term use.[22]

### Investigation of antibacterial effect of silver nanoparticles
In this study, SNPs were used as an antibacterial agent to control mice vaginal infection caused by GBS. In order to evaluate the antibacterial activity of nanoparticles against planktonic and biofilm forms of GBS, three methods were applied including disk diffusion, minimum inhibitory concentration, and minimum biofilm inhibitory concentration (MBIC).

### Disk diffusion method
24-hour cultures of GBS were used for this experiment. Briefly, the bacteria were cultured on an MHA medium. Disks impregnated with SNPs in different concentrations of 128, 256, 512, and 1024 ppm were put on the inoculated plates. In addition to nanoparticles, disks containing P, VC, and DMSO were applied, and the plates were incubated for 24 hours at 37 °C. After overnight incubation, the zone of inhibition around each disk was measured by a ruler. This test was performed in three replications.[2]

### Minimal inhibitory and bactericidal concentrations (MIC and MBC)
MICs of SNPs and P against GBS were evaluated by microdilution method in 96 microtiter plates based on the CLSI (Clinical and Laboratory Standard Institute 2022) guidelines. One hundred μL of MHB medium was added to the wells of the plate. The broth media was inoculated with twofold serial dilutions of antibacterial agents (8, 16, 32, 64, 128, 256, 512, and 1024 ppm). The final volume of each well, containing MHB and antibacterial agents, was 100 μL. 10 μL of GBS bacterial suspension ($1\times 10^8$ CFU/mL) was added to each well and the microplate was incubated 24 h at 37 °C. Two wells were considered as growth control (containing MHB and GBS) and sterility

control (containing MHB plus antibacterial agents without GBS). The MICs of SNPs and P against GBS were performed after 24 hours of incubation at 37 °C by reading the turbidity due to bacterial growth (by ELISA reader at 600 nm, Biotek/ synergy TH), at different concentrations of nanoparticles, and visual determination of a well in which no turbidity was observed. To determine the MBCs, 10 μl of wells contents that had no turbidity were cultured on MHA plates. After 24 h of incubation at 37 °C, MBCs were defined as the lowest concentration that resulted in no growth of treated bacteria compared to untreated bacteria. Assays were performed in triplication.[22]

### Minimum biofilm inhibition concentration (MBIC)
GBS strain biofilm was examined on polystyrene 96-well microtiter plates (Maxwell) with a flat bottom. In summary, to produce GBS biofilm, 180 μl of TSB medium was added to each well. In the next step, 20 μl of bacterial suspension was transferred to the wells containing 180 μl of culture medium, and the microplates were incubated for 24 hours at 37 °C. After incubation, the liquid culture medium was removed and the planktonic cells were washed with phosphate buffer solution (PBS). After the removal of planktonic cells, 200 μl of fresh TSB containing different concentrations of SNPs (8, 16, 32, 64, 128, 256, 512, and 1024 ppm) was added. The microplates were incubated for 24 hours at 37 °C. Two wells, one without nanoparticles and one well without biofilm, were included as positive and negative controls. To evaluate the MBIC, a staining method of 0.1% crystal violet was applied. After incubation, unconnected cells were removed by turning the microplate plate and shaking the liquid. The plates were washed three times with PBS, which removed the unbound cells, culture medium, and nanoparticle. In the next step, 200 μl of crystal violet solution was added to each well. The microplate was incubated at room temperature for 10-15 minutes. The plates were washed three times with PBS to remove the dye. After drying, 200 μl of 30% acetic acid was added to each well of the microplate plate to dissolve the crystal violet. The microplate was incubated at room temperature for 10-15 minutes. Finally, the content of the wells was transferred to a new microplate and optical density was determined using an ELISA reader at 600 nm.[22]

### Cytotoxicity effect of SNPs and protective effect of VC
The cytotoxic effect of SNPs and the cytoprotective effect of VC on normal L-929 fibroblast cells were investigated using an MTT assay. The base of this test was the measurement of the decrease of the yellow stain of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple dye formazan crystal, using the activity of the cytochrome oxidase and succinate dehydrogenase of mitochondria in living fibroblast cells. Cells of fibroblast were cultured in DMEM (Dulbecco Modified Eagle Medium) with 10% of fetal bovine serum (FBS), streptomycin /penicillin (1%), and incubated overnight at 37 °C under $CO_2$ atmosphere (5%). For this test, the supernatant culture medium in the

flask was emptied and the cells were washed using PBS. In order to separate the attached cells, 1.5 ml of trypsin solution was added to the flasks of the culture medium and kept in an incubator at 37 °C for five minutes. To neutralize trypsin, a fresh culture medium containing FBS was added to the cells and transferred to a new falcon. The cells were then centrifuged at 1200 rpm for five minutes and added to microplate wells. After 24 hours of incubation at 37 °C, the culture medium in the wells was replaced with a new culture medium containing different concentrations of SNPs (8, 16, 32, 64, 128, 256, 512, and 1024 ppm) and kept in 37 °C under 5% $CO_2$. Finally, 20 μl of MTT was added to each well under dark conditions and incubated for four hours. After incubation, the contents of the wells were emptied and 100 μl of DMSO was added to each well and placed on a shaker for 15 minutes to dissolve the Formazan crystals produced during the MTT reduction processes. Finally, the optical density at 570 nm was read by ELISA reader and the cell survival percentage was calculated according to the following formula:[23] The percentage of cell viability= (OD in sample well/ OD in control well) × 100

### Animals
Animals were kept in the laboratory, and animal room, under standard status including suitable ventilation, 12:12 h light-dark cycle, free access to food and water, and a constant temperature of 20-22 °C. 7-8 weeks-old female NMRI mice were purchased from Pasteur Institute in Tehran, Iran. The mice used in this experiment were kept under standard conditions including free access to water and food, 12-hour cycle of light/darkness and a temperature of 25 °C and were divided accidentally into 10 groups (n=7): (1) control group: healthy and intact mice that received only water and pellets diet and were not treated, (2) SNPs group: healthy mice that received intra-vaginal inoculation of MBIC-90% (512 ppm) concentration of SNPs once a day for two weeks, (3) VC group: healthy mice that received intraperitoneal daily injection of 250 mg/ kg VC for two weeks, (4) P group: healthy mice that received intraperitoneal daily injection of MBIC-90% antibiotic for two weeks, (5) VC+SNPs group: healthy mice that received both intraperitoneal injection of 250 mg/ kg VC and intra-vaginal inoculation of MBIC-90% concentration of SNPs once a day for two weeks, (6) I group: the animals that were infected by intra-vaginal inoculation of 1×10⁸ CFU/ ml of GBS (7) I+SNPs group: infected mice that received intra-vaginal inoculation of MBIC-90% concentration of SNPs once a day for two weeks, (8) I+VC group: infected mice that received intraperitoneal daily injection of 250 mg/ kg VC for two weeks, (9) I+P group: infected mice that received intraperitoneal daily injection of MBIC-90% concentration of antibiotic for two weeks, and (10) I+ SNPs+ VC: infected mice that received co-administration of intraperitoneal injection of 250 mg/ kg VC and intra-vaginal inoculation of MBIC-90% concentration of SNPs once a day for two weeks.

### Murine vaginal infection model

Before infection in mice, a sample of vaginal swabs was first prepared and cultured to ensure that the mice were not infected. To initiate the infection process in healthy mice, the estrus cycle was standardized by intraperitoneal injection of 0.5 mg of 17-β estradiol valerate dissolved in 0.1 ml of sterile sesame oil (Barij essence company, Iran) 48 hours before inoculation of GBS and day of infection. Mice were infected by intra-vaginal inoculation of 25 µl of GBS suspension ($1× 10^8$ CFU / ml.). Animals were immobilized and the tip of the sterile pipette was inserted 5-10 mm into the vaginal cavity and the bacterial suspension was transferred. The mouse was lifted from the tail and placed in this position for a minute. If the reverse flow was observed, a fresh pipette tip was used to transfer and return the bacterial contents to the vagina.[24]

### Investigation of infection and treatment in mice

Mice were examined for vaginal infections on days one, three, and five, after inoculation with GBS bacteria and during treatment with antibiotics, VC, SNPs, and the mixture of VC and SNPs. 100 µl of normal saline solution was added to the sterile tube. A sterile swab soaked in serum was inserted a few millimeters into the vaginal lumen and gently rotated several times in the vaginal area. This swab was transferred to 100 µl of physiological saline into a tube. The tube was vortexed for 15 seconds to remove bacteria from the swab. Relatively, each bacterial sample in the tube was diluted with physiological serum, and the concentration of GBS in the vaginal area of mice was calculated using the serial dilution method. 20 µl of different dilutions were cultured on streptococcus-specific THA media using the pure plate method. The plates were incubated for 24 hours at 37 ° C and the number of bacterial colonies was counted in the dilutions that could be counted.[25]

### Determination of estrus cycle

In order to determine the estrus cycle of mice, vaginal smear preparation was started daily from two days before treatment and continued until the end of the experiment. To perform this process, 20 µl of sterile physiological serum was gently inserted into the mice vaginal cavity. The vaginal fluid was spread on the glass slide. After drying the smears, the samples were fixed with 96% ethanol and then stained using the Papanicolaou method. The stained slides were examined under a light microscope and the ratios of nucleated epithelial cells, epithelial cells, and leukocytes were determined to evaluate the sexual phase of mice, proestrus, estrus, metestrus, and diestrus.[26]

### Blood sampling

After the treatment period, blood samples of three mice in each group were collected in the morning. The mice were anesthetized by ether inhalation. Immediately after anesthesia, the mice were kept in a supine position on a fixed plate. To prepare a blood sample, the mouse abdomen and chest were gently cut. Using a 2 ml syringe, 0.5 ml of blood was taken from the left ventricle of the mice and collected in special blood sample tubes containing anticoagulants. Blood samples were then centrifuged for 20 minutes and after separating the blood serum, blood cells were counted using a cell counter.[27]

### Murine vaginal histology

After infection and treatment duration, four animals in each group were sacrificed, and vaginal tissues were collected. The vaginal tissues of mice were fixed in formalin solution, dehydrated in increasing percentages of ethanol, clarified in xylene, and then embedded in paraffin. The prepared paraffin blocks were cut 6 µm in size by a microtome apparatus (YIDI china). The tissue sections of vaginal mice were stained with hematoxylin and eosin (H&E) and analyzed under a light microscope.[28]

### Statistical analysis

Data from this study were analyzed using the software SPSS version 26. One-way ANOVA analysis and post hoc tests were applied for data comparison. Data were represented as mean ± std deviation. P- value ≤ 0.05 was considered significant in all analyses.

## Results

### The Results of disk diffusion test

Figure 1 represents the results of the antibacterial activity of SNPs. The antibacterial activity of SNPs against GBS was evaluated by measuring the diameters of inhibition zones. The disk diffusion result of GBS against penicillin and SNPs in different concentrations (64, 128, 256, 512, and 1024 ppm) are represented. The SNPs showed dose-dependent antimicrobial activity against GBS. GBS showed great susceptibility to SNPs at 1024 ppm (20.8±0.46 mm). No susceptibility was observed towards VC (250 mg/ kg) and DMSO 5 % disks.

### The results of antibacterial activity of SNPs by microdilution method

SNPs in different concentrations (8, 16, 32, 64, 128, 256, 512, and 1024 ppm) showed potential antibacterial activity versus GBS, as a gram-positive bacterium (Figure 2). MIC and MBC values were used to identify the antibacterial properties of nanoparticles. There were significant differences (P< 0.05) between inhibitions values for all concentrations of SNPs compared with control. Our results represented the potential antibacterial activity of SNPs against GBS. SNPs exhibited potent antibacterial activity against GBS, with a MIC of 512 ppm. The MIC of commercial antibiotics was 256 ppm for penicillin. For GBS, SNPs, and penicillin represented two-fold higher MBC values than their MIC values, representing the bactericidal activity (1024 and 512 ppm, respectively).

### SNPs inhibits the biofilm of GBS

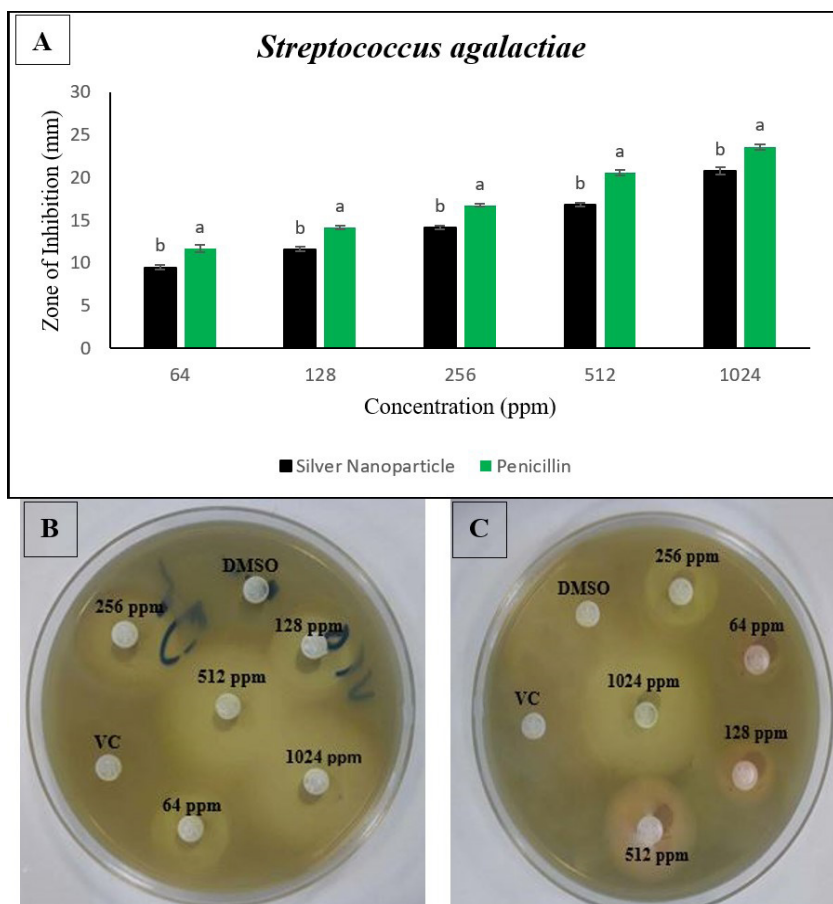GBS has the ability to biofilm formation on different

**Figure 1.** The results of the disk diffusion method. a) The zone of GBS inhibition around the disks i millimeters in different concentrations of SNPs and penicillin (64, 128, 256, 512, and 1024 ppm). a) significant difference with SNPs and b) significant difference with penicillin (p< 0.05). b) zone of GBS inhibition around the penicillin disks, and c) SNPs disks.
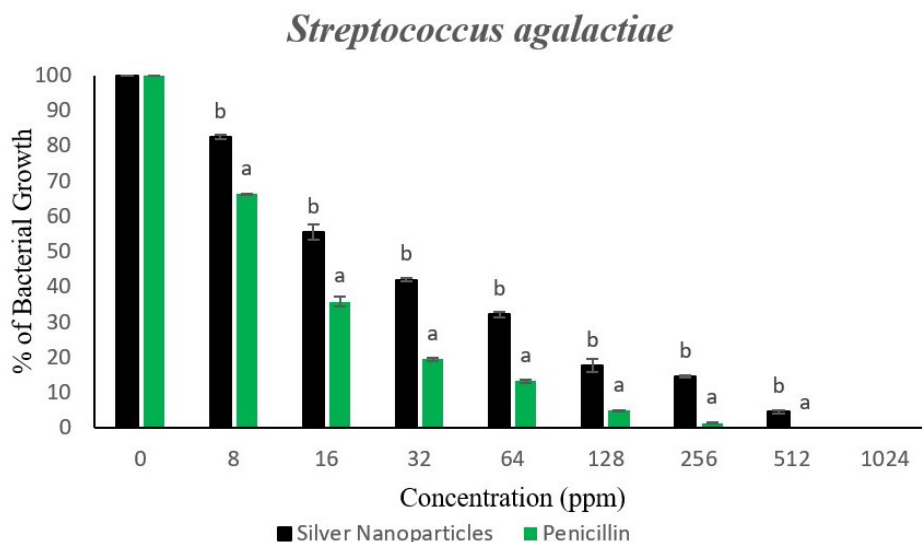


**Figure 2.** Antibacterial effect of SNPs and penicillin (8, 16, 32, 64, 128, 256, 512, and 1024 ppm) on GBS by broth microdilution method. a) significant difference with SNPs and b) significant difference with penicillin (p< 0.05).

surfaces. The ability of biofilm formation by GBS was assessed using CV staining method. The anti-biofilm effects of SNPs and penicillin in different concentrations (8, 16, 32, 64, 128, 256, 512, 1024, and 2048 ppm) are represented in Figure 3. For GBS biofilm inhibition, a dose-dependent effect of SNPs was observed. The results of this study revealed a great anti-biofilm activity of SNPs against the biofilms of GBS with the MBIC-90 of 512 ppm. But, the anti-biofilm activity of penicillin was lower than nanoparticles with the MBIC-90 of 1024 ppm. So, the GBS
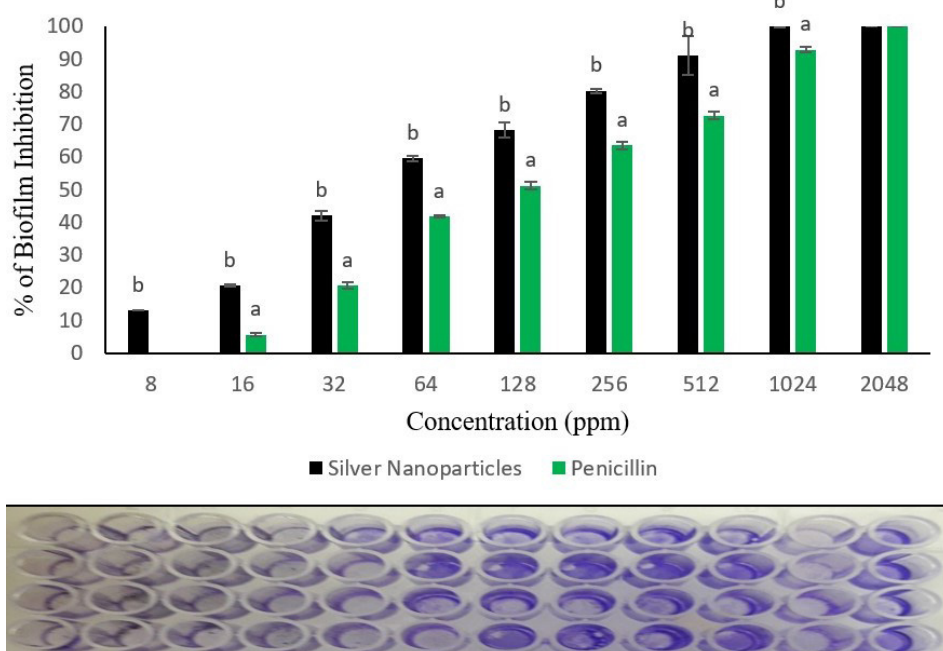
## *Streptococcus agalactiae*



**Figure 3.** The effect of SNPs and penicillin (8, 16, 32, 64, 128, 256, 512, 1024, and 2048 ppm) on biofilm inhibition created by GBS using CV staining method. a) significant difference with SNPs and b) significant difference with penicillin (p< 0.05).

biofilm was sensitive to different concentrations of SNPs with the percentage of inhibition ranging from 13.14±0.34 % to 100 %, with concentrations ranging from 8 to 2048 ppm. Our results of this experiment were compared with positive controls (untreated GBS biofilm) and negative controls (without biofilm).

### *Cytotoxicity effect of SNPs and protective effect of VC*
The cytotoxicity effect of SNPs was evaluated by the MTT test (Figure 4). These results showed a decrease in viability in a dose-dependent manner after 24 hours of exposure to SNPs (16, 32, 64, 128, 256, 512, and 1024 ppm). The

cell viability percentage was calculated in different concentrations of SNPs (single form and its combination with 250 mg/ kg of VC). The lethal dose of 50 % (LD-50) was measured at 128 ppm of SNPs. By the dose-response pattern of SNPs, the protective effect of VC on SNPs cytotoxicity was calculated. The LD-50 of simultaneous exposure of VC (250 mg/ kg) with SNPs (8- 1024 ppm) was measured at 1024 ppm. After treatment with VC increased cell viability at 512 ppm of nanoparticles to 65.32%. The OD values of unexposed cells and the VC exposed cell was taken as 0% cytotoxicity (100% viability).
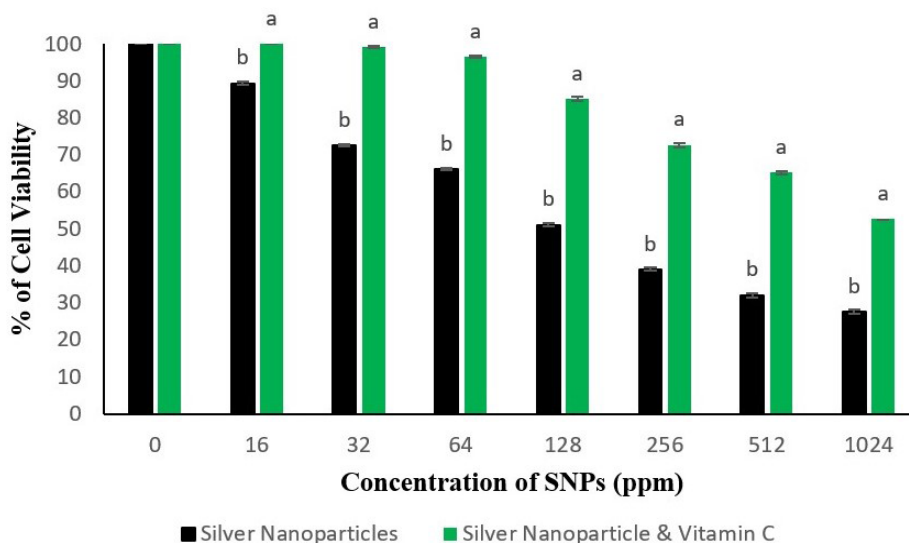


**Figure 4.** The percentage of cell viability in 16, 32, 64, 128, 256, 512, and 1024 ppm concentrations of SNPs alone and in the combined state of SNPs with 250 mg/ kg VC. a) significant difference with SNPs and b) significant difference with SNPs and VC (p< 0.05).

## The results of investigation the infection and treatment duration in mice

Vaginal infection by GBS was established in animals through intra-vaginal delivery of $1\times10^8$ CFU/ ml of bacteria. We monitored the mice during infection and treatment, respectively. Vaginal fluids of mice on days one, three, and five, after inoculation with GBS bacteria and also on days three, five, seven, ninth, and eleventh during treatment with antibacterial agents, in terms of the presence of bacteria in the vagina were collected. Vaginal specimens were cultured in the pure-plate method on a THA medium. After 24 hours of incubation, the number of bacterial colonies grown on the plates was calculated. Figure 5 shows the growth of GBS in the vaginal fluids of mice during the infection, which shows a significant increase in the number of bacteria isolated from the secretions. As shown in Figure 6, during the 14-day treatment period, a mixture of SNPs and VC removed the bacteria and resulted in a treat of vaginal infection.
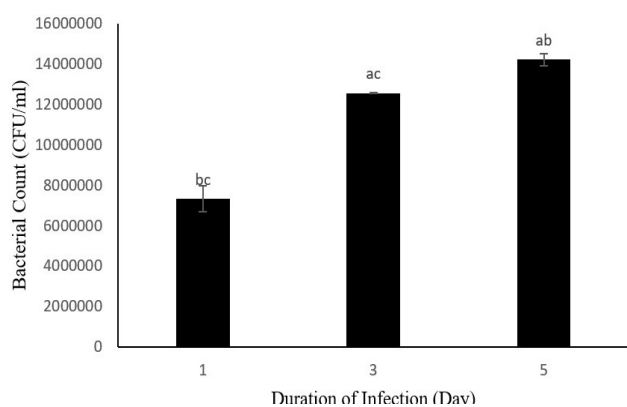


**Figure 5.** Evaluation of the number of GBS bacteria in mice vaginal fluids during infection. Animals were colonized with 1× 108 CFU/ml of GBS. a) significant difference with the first day of infection, b) significant difference with the second day of infection, and c) significant difference with the third day of infection (p< 0.05).
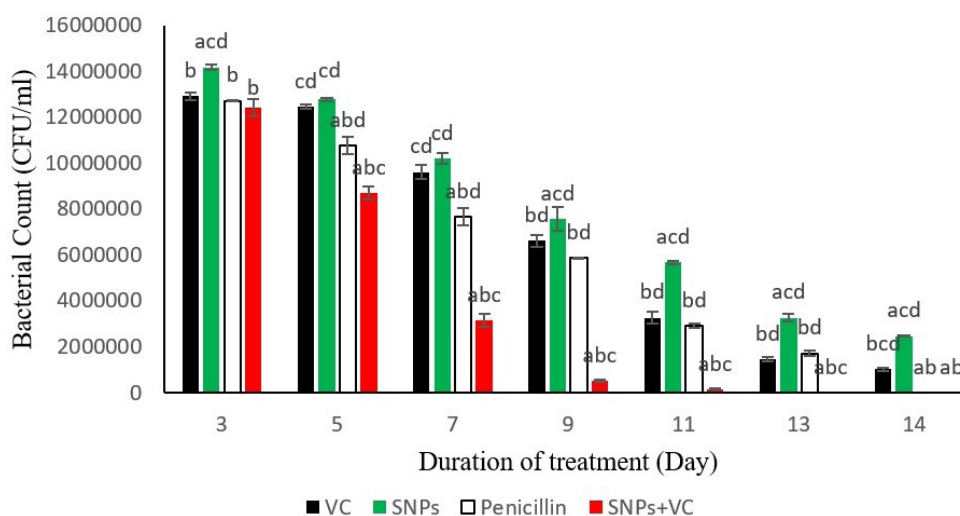
### Estrus cycle measurement

In this study, the estrus cycle stages of mice were evaluated by Papanicolaou staining of vaginal smear (vaginal cytology). The mice were given estradiol in oil prior to infection with GBS to synchronize their estrus cycles. The results of this test are summarized in Table 1. Estrus cycle monitoring of mice using vaginal fluid smears obtained at daily intervals revealed reversion to regular sexual cycling during the day. In terms of the length of the pro-estrus and estrus phases, there was a significant decrease between the I, P, SNPs, I+ P, I+ SNPs, and I+ VC groups compared with the control group. The difference in the length of the met-estrus stage between I and control groups was increased significantly. Increased significance in di-estrus length was observed in the P, SNPs, I, I+ P, I+ SNPs groups compared with control. Further, prolonged length of the estrus cycle with increased met-estrus or di-estrus stages were presented in the I, P, SNPs, I+ P, I+ SNPs, and I+ VC groups compared with the controls. The difference between VC, VC+ SNPs, and I+ SNPs+ VC mice groups in the length of the cycle was not significant. Based on the results, the GBS vaginal infection disturbs the normal sexual cycling of mice. When mice were treated with penicillin, GBS bacteria were eliminated from the vagina, but the blocking of the estrous cycle remained. We observed in this research that SNPs, penicillin, and GBS infection block the estrus cycle at the diestrus stage, but VC and the combined use of SNPs+ VC do not block the cycle.

### Results of the study of blood factors in mice

At the end of the experiment, blood samples of three mice in each group were collected. Cells in blood samples were analyzed, and their results are presented in Table 2. In this study, white blood cell count (WBC), red blood cell (RBC), hemoglobin (HB), hematocrit (HCT), mean red blood cell volume (MCV), mean hemoglobin (MCH), mean red blood cell hemoglobin concentration (MCHC), Platelets



**Figure 6.** Evaluation of the number of GBS bacteria present in mice vaginal secretions during treatment with VC, SNPs, penicillin, and the simultaneous combination of SNPs and VC (SNPs + VC). a) 21 significant difference with VC, b) significant difference with SNPs, c) significant difference with penicillin, and d) significant difference with simultaneous consumption of SNPs and VC (p< 0.05).

**Table 1.** Changes in the estrous cycle in different groups of mice.

| Groups of mice | Pro-estrus | Estrus | Met-estrus | Di-estrus |
|---|---|---|---|---|
| Control | 3±0.51 | 3.25±0.5 | 0.5±0.58 | 7.25±0.5 |
| P | 1.25±0.5* | 1±0.82* | 1.5±0.58 | 10.25±1.5* |
| VC | 3±0.82 | 2.75±0.5 | 1.5±1.26 | 6.75±0.5 |
| SNPs | 0* | 0.25±0.5* | 1.7±1.25 | 12±1.63* |
| VC+ SNPs | 3±0.81 | 3.5±0.58 | 0.5±0.58 | 7±0.82 |
| I | 0.25±0.5* | 0.5±0.28* | 2±1.41* | 11.25±1.89* |
| I+ P | 0.5±0.57* | 0.5±0.58* | 1.5±0.57 | 11.5±1.29* |
| I+ VC | 2* | 2.25±0.5* | 1.75±0.57 | 8±0.82 |
| I+ SNPs | 0* | 0* | 0.5±0.25 | 13.5±0.57* |
| I+ VC+ SNPs | 3±0.82 | 3 | 0.5±0.25 | 7.25±0.5 |

*Significant difference with control group.
Control: healthy mice that received only water and pellets diet and were not treated
P: healthy mice that received intraperitoneal injection of 1024 ppm of penicillin
VC: healthy mice that received intraperitoneal injection of 250 mg/ kg VC
SNPs: healthy mice that received intra-vaginal inoculation of 512 ppm of SNPs
VC+SNPs: healthy mice that received both of VC and SNPs
I: the animals that were infected by intra-vaginal inoculation of $1×10^8$ CFU/ ml of GBS
I+P: infected mice that received intraperitoneal injection of penicillin
I+VC group: infected mice that received intraperitoneal injection of VC
I+SNPs: infected mice that received intra-vaginal inoculation SNPs
I+ SNPs+ VC: infected mice that received co-administration VC and SNPs

**Table 2.** The changes in blood factors of mice in different groups.

| Groups | WBC | RBC | HB | HCT | MCV | MCH | MCHC | PLT | LYM | NEU |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | 5.66 ±0.06 | 8.67 ±0.1 | 14.73 ±0.15 | 44.03 ±0.08 | 51.23 ±1.62 | 15.15 ±0.3 | 32.08 ±0.29 | 373.19 ±5.83 | 83.82 ±0.92 | 51.51 ±0.52 |
| I | 6.85 ±0.26* | 10.88 ±0.27* | 13.56 ±0.43* | 44.19 ±0.74 | 51.23 ±0.55 | 15.27 ±0.35 | 29.14 ±1.25* | 257.24 ±11.76* | 77.21 ±2.71* | 56.44 ±0.51* |
| SNPs | 7.86 ±0.72* | 6.31 ±0.28* | 12.35 ±0.33* | 32.74 ±0.84* | 42.56 ±0.78* | 12.85 ±0.32* | 28.37 ±0.52* | 377.39 ±15.64* | 80.17 ±0.94* | 46.5 ±1.05* |
| VC | 5.66 ±0.12 | 8.55 ±0.16 | 14.46 ±0.42 | 43.8 ±0.46 | 51.23 ±0.99 | 14.99 ±0.02 | 31.23 ±0.65 | 369.71 ±3.35 | 83.19 ±0.27 | 51.12 ±1.74 |
| P | 3.83 ±0.31* | 8.11 ±0.12* | 13.99 ±0.02* | 41.83 ±0.51* | 49.61 ±0.55 | 15.07 ±0.09 | 32.08 ±0.19 | 347.26 ±6.42* | 80.75 ±0.55* | 51.41 ±1.12 |
| SNPs+ VC | 5.7 ±0.1 | 8.77 ±0.13 | 14.87 ±0.43 | 43.03 ±1.54 | 50.99 ±1.12 | 15.19 ±0.31 | 31.12 ±0.13 | 369.56 ±7.08 | 83.61 ±0.73 | 51.32 ±0.79 |
| I+SNPs | 11.12 ±0.98* | 4.96 ±0.39* | 11.11 ±0.94* | 29.79 ±1.28* | 42.66 ±1.41* | 12.66 ±0.56* | 25.02 ±1.07* | 252.98 ±18.68* | 72.83 ±1.72* | 42.85 ±0.75* |
| I+VC | 6.65 ±0.41* | 8.73 ±0.54 | 13.89 ±0.18* | 41.87 ±2.42* | 51.84 ±1.46 | 14.16 ±0.15* | 29.84 ±1.02* | 344.53 ±11.65* | 80.21 ±0.99* | 53.47 ±0.81* |
| I+P | 4.11 ±0.14* | 7.58 ±0.53* | 13.86 ±0.32* | 41.31 ±0.96* | 48.88 ±0.57* | 15.31 ±0.11 | 31.52 ±0.53 | 332.21 ±2.91* | 80.47 ±1.01* | 51.74 ±0.45 |
| I+SNPs+ VC | 5.7 ±0.17 | 8.56 ±0.28 | 14.99 ±0.02 | 43.84 ±0.53 | 51.82 ±1.24 | 15.08 ±0.15 | 31.79 ±0.48 | 371.99 ±6.08 | 83.42 ±0.63 | 51.17 ±0.88 |

*Significant difference with control group.
(WBC (white blood cell), RBC (red blood cell), HB (hemoglobin), HCT (hematocrit), MCV (mean red blood cell volume), MCH (mean hemoglobin), MCHC (mean red blood cell hemoglobin concentration), PLT (Platelets), LYM (lymphocytes), and NEU (neutrophils).
Control: healthy mice that received only water and pellets diet and were not treated
I: the animals that were infected by intra-vaginal inoculation of $1×10^8$ CFU/ ml of GBS
SNPs: healthy mice that received intra-vaginal inoculation of 512 ppm of SNPs
VC: healthy mice that received intraperitoneal injection of 250 mg/ kg VC
P: healthy mice that received intraperitoneal injection of 1024 ppm of penicillin
VC+SNPs: healthy mice that received both of VC and SNPs
I+SNPs: infected mice that received intra-vaginal inoculation SNPs
I+VC group: infected mice that received intraperitoneal injection of VC
I+P: infected mice that received intraperitoneal injection of penicillin
I+ SNPs+ VC: infected mice that received co-administration VC and SNPs

(PLT), lymphocytes (LYM) and neutrophils (NEU) were evaluated. The changes in WBC, in four groups including I, SNPs, I+SNPs, and I+VC, a significant increase was seen. In two groups of P and I+P groups, a significant decrease in WBC was seen, while in other groups it was similar to control. Decreased significance in RBC values was observed in SNPs, P, I+SNPs, and I+P groups compared with control. Increased significance was seen in the I group. In terms of

changes in HB, in six groups including I, SNPs, P, I+SNPs, I+P, and I+VC, a significant decrease was seen with the control group. Decreased significance in HCT was seen in P, I+SNPs, I+P, and I+VC groups compared with the control. Increased significance was observed in the SNPs group. Decreased significance in MCV was seen in SNPs, I+SNPs, and I+P groups compared in control. Decreased significance in MCH was seen in SNPs, I+SNPs, and I+VC groups compared with control. Decreased significance in MCHC was observed in SNPs, I, I+SNPs, and I+VC groups compared with the control. Increased significance was observed in the SNPs group. Decreased significance in PLT and LYM was seen in six groups including P, I, I+SNPs, I+P, and I+VC compared with control. Increased significance in NEU was observed in I and I+VC groups compared with the control. Decreased significance was seen in two including SNPs and I+SNPs compared with the control. The results of this experiment showed that blood factors in three groups of mice after the treatment period were similar to the control group, which included VC, VC+SNPs, and I+VC+SNPs groups (p <0.05).

## The results of vaginal histology analyses of mice

The efficacy of the SNPs and VC was measured on the vaginal mucosa of adult NMRI mice. After two weeks of agent application, vaginal tissues of mice were excised and prepared for histological evaluation (Figure 7). In the group of infected mice (Figure 7B), examination of the prepared the vaginal tissue samples showed remarkable changes compared to the vagina of the control group. The stained sections of mice vaginal tissues revealed an increased presence of inflammatory cells in the epithelial surface, a high thickness of the vaginal epithelium, irregular structure of lamina propria, a high rate of epithelial keratinization, an increase in blood vessels, and hyperplasia in infected mice. In the groups of mice treated with SNPs, significant changes were observed in tissue samples compared to the healthy mice. In the SNPs (Figure 7D) and I+ SNPs (Figure 7I) groups, there were increases in the density of the vaginal tissue layers, epithelial keratinization, and blood vessels. Bleeding and hyperplasia were seen in these tissues. In the I+ VC group (Figure 7H), the results showed that 250 mg/ kg of VC can be somewhat effective in improving the GBS vaginal infection. The architecture of vaginal tissue was almost similar to the control group but there was some increase in blood vessels and bleeding in the tissues. In treating with penicillin, large changes were made in the vagina compared with the control group. In the P (Figure 7B) and I+ P (Figure 7G) groups, high rates of blood vessels, bleeding, and Hyperplasia were seen. The
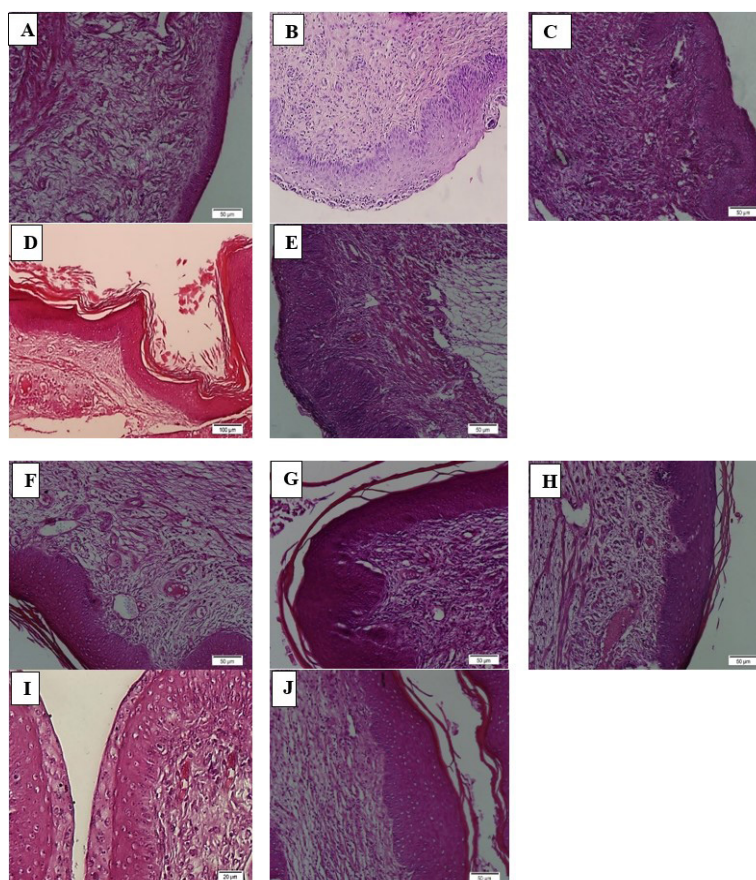


**Figure 7.** Histological analyses of vaginal tissues of NMRI mice. A) Control group, B) mice were treated with 1024 ppm of penicillin, C) mice were treated with 250 mg/ kg of VC, D) mice were treated with 512 ppm of SNPs, E) mice were treated with co-administration of SNPs (512 ppm) and VC (250 mg/kg), F) infected mice with GBS, G) infected mice were treated with penicillin, H) infected mice were treated with VC, I) infected mice were treated with SNPs, J) infected mice were treated with co652 administration of SNPs and VC.

vaginal epithelium of healthy mice (control group) showed normal thickness and architecture, representing well-defined layers of epithelial with normal keratinization, and the lamina propria layer intact (Figure 7A). Similarly, no damage for the epithelium of mice vaginal tissue and no inflammation after two weeks in three groups, VC (Figure 7C), VC+ SNPs (Figure 7E), and I + VC+ SNPs (Figure 7J) were observed.

## Discussion

Vaginal infections are common gynecological disorders, characterized by unpleasant signs.[29] GBS is an asymptomatic bacterium isolated from the reproductive tract system of women.[2,3] GBS bacteria cause acute infection in the female genital tract. As Gram-positive bacteria, GBS is able to form a biofilm that could boost its potential to infect and persist in the vagina.[3] Our study showed similar results that in the model of GBS vaginal infection, GBS disrupted the estrus cycle of mice, and caused damage to the vaginal mucosa.

Antibiotics are used as a medicine for human health worldwide. But, the emergence of multi-drug resistant microorganisms to antibiotics, resulted in the request for costly and stronger therapy.[30] Ampicillin or penicillin is the first-choice antibacterial medicine, cefazolin, clindamycin, and vancomycin being alternative antibiotics in specific conditions. Many studies analyze the susceptibility of GBS against commercial antibiotics.[2,31] A potential side effect related to the use of antibiotics in the vagina is the interference in normal microbiota such as Lactobacillus that have an important function in the female genital tract.[22] Recent studies evaluated the side effects of the broad-spectrum antibiotic agent on hematopoiesis in a mouse model. On the other hand, antibiotic treatment reduced bone marrow cellularity and peripheral blood counts. For instance, b-lactams are most generally implicated in the suppression of bone marrow.[32] The results of our studies also showed that the use of penicillin to treat the infection removed the bacteria from the vaginal fluid, but the estrus cycle was disrupted, reducing the number of blood factors, and tissue damage was seen in mice treated with the antibiotic.

Recently, nonmetals with diameters less than 100 nm have made a remarkable impact in different biomedical uses. In particular, SNPs have been considered a great antimicrobial factor that can combat multidrug-resistant Gram-positive and Gram-negative bacteria.[11] With the necessity of finding new suitable antimicrobial agents, our study aims to evaluate the antibacterial and cytotoxic properties of SNPs. With the increasing application of SNPs, concern about their toxicity is escalating. Recent studies aimed to calculate the toxicity of different sizes of SNPs. Bio-distribution and toxicity studies, following various ways of exposure, such as instillation, inhalation, dermal, oral, and intravenous have established silver translocation, toxicity, and accumulation in different organs.[33] Various studies revealed that SNPs are toxic nanoparticles because of high biological activity and Ag+ ions released[9]. SNPs are known to cause oxidative stress and induce cytotoxicity in different cells.[34] Elkhawass et. al. investigated LD50 of SNPs with two sizes (20 nm and 50 nm). The results of their research revealed that the size 20 nm SNPs were more toxic than nanoparticles with a size of 50 nm.[35] In this study, we evaluated the cytotoxicity of SNPs (20 nm) by MTT assay. Our results, like previous studies, showed a dose-dependent decrease in cell viability. The results of our studies showed that an LD-50% was measured at 128 ppm SNP. Studies about the toxicity and accumulation of SNPs in the vaginal are lacking. One study showed that, following the vaginal inoculation of SNPs, nanoparticles accumulate in the vagina. They demonstrated that this mechanism caused structural changes not only in vaginal tissues but also in the rectum and urethra. These data revealed a new migration pathway of SNPs when inoculated intravaginally. They suggested that SNPs can enter blood circulation.[36] AL-Baker et. al, evaluated the effect of SNPs on the hemopoietic system. They concluded that SNPs administration led to a decrease in the RBC, Hb, PCV, MCH, MCHC, and MCV, but an increase in WBCs and PLTs. It means that SNPs have a potentially toxic effect on blood parameters.[37] Therefore, the small size of SNPs easily enters the different parts of the body and is transported through the barrier. It is likely that the smallest SNPs are more toxic properties to reproductive organs. Inflammation and oxidative stress have been indicated to happen after the administration of SNPs. These results suggest that the causal reason for the developmental and reproductive toxicity of SNPs is the high generation of reactive oxygen species.[38] Our results showed, in the groups of mice treated with SNPs, significant changes such as increases in the density of the vaginal tissue layers, epithelial keratinization, high blood vessels, bleeding, and hyperplasia were observed in tissue samples compared to the healthy mice. The results of the study of hematological factors showed similar results in that the number of factors RBC, Hb, PCV, MCH, MCHC, and MCV decreased and WBCs and PLTs increased. Many studies have shown that some compounds with antioxidant effects can reduce the toxicity of nanoparticles. For example, Faedmaleki et. al, examined the cytotoxic effects of SNPs on mouse cells. They calculated the cell viability with the MTT method after cell exposure to SNPs at different concentrations for 24h. They showed that SNPs caused a decrease in cell viability in a concentration-dependent manner ($IC_{50}$ value = 121.7 ppm). Then they evaluated the protective effect of vitamin E and silymarin on SNPs cytotoxicity. Their results indicated that silymarin and vitamin E have protective effects on SNPs cytotoxicity and the viability percentage was increased.[39] In this study, we used 250 mg/kg of VC to eliminate the toxic effects of exposure to SNPs. Our results indicate that vitamin C has a protective effect against toxicity caused by nanoparticles. After treatment with VC increased cell viability at 512 ppm of nanoparticles to 65.32%. Our results showed that VC supplementation

may reduce hemotoxicity in mice exposed to the SNPs. VC is one of the most essential and common vitamins with protective activity. The supplementation of VC could protect the body against toxification, infections, cancer, and other diseases.[40] On the other hand, Guo et. al, showed that oxidative stress and reactive oxygen species caused by are the major mechanisms for vaginal infection. Their data offered that antioxidant agents may improve vaginitis, and antioxidants decrease can be related to oxidative stress, ROS overproduction, and vaginitis. Therefore, the antioxidant treatment seems to be useful for vaginal infection treatment.[41] In clinical research, Petersen and Magnani[42] evaluated the effect of VC (250 mg/ kg) in the treatment of vaginitis. They found that VC administration improved the main signs of infection in the study group. In the study by Chen *et al.*,[4] women with vaginal infections were cured with 250 mg VC every day. Their results showed that VC can control oxidative stress and cell apoptosis in vaginal epithelial cells. VC has long been related to fertility. Over the past few decades, data indicate that steroid secretion, and protects gametes against free radicals during fertilization and production. It is revealed that VC is a key agent in gonadal physiology[21]. VC has also been reported to be useful in the improvement of RBC reduction. The antioxidant activity of the VC has been shown to prevent apoptosis[19]. The group of infected mice that received 250 mg/ kg of VC, somewhat improved the GBS vaginal infection. We observed in this research that SNPs and GBS infection block the estrus cycle at the diestrus stage, but VC and the combined use of SNPs and VC regular the cycle. SNPs were given alone, and no treatment was seen in mice vaginitis, but a mixture of SNPs and VC removed the bacteria and resulted in a treatment of vaginal infection. No damage for the epithelium of mice vaginal tissue and no inflammation after two weeks in I + VC+ SNPs were observed. The result indicates SNPs in combination with VC possess the therapeutic capability in mice vaginal infection.

## Conclusion

This study established the mice model of GBS vaginal infection by intra-vaginal inoculation of bacteria. Nanoparticles are one of the most promising and interesting approaches for the treatment of vaginal infection and the removal of bacteria from the vagina. According to studies, during the vaginal infection and also during treatment with SNPs, oxidative stress occurs, which makes the condition worse. So, the development and discovery of antioxidant agents is one of the most interesting fields for the treatment of vaginitis and the toxicity of nanoparticles. Antioxidants, like VC, have protective effects in proteins, lipids, and nucleic acids against oxidative damage. Based on our result, when VC and antibiotics were given alone to infected mice as a single-dose component, a little curative was observed. But SNPs were given alone, and no treatment was observed. We demonstrate that SNPs in combination with VC possess therapeutic capability in mice vaginal infection. Therefore,

the co-administration of SNPs and VC has curative effects against vaginal infection.

## Author Contributions

Zhale Mansoori: Investigation, Formal Analysis, Writing - Original Draft. Khosrow Chehri: Conceptualizatio Methodology, Supervision, Writing - Review & Editing. Ahmad Gharzi: Conceptualization, Methodology, Writing - Review & Editing.

## Conflict of Interest

The authors declare that there is no conflict of interest.

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