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Original Article

Pathogen Reduction In Human Plasma Using Biosynthesized AgNPs With Juglans Regia Green Husk Aqueous Extract

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Abstract

Background and Aims: To prepare human plasma for therapeutic purposes and serum with a low risk of contamination for cell culture use, a technique is needed to reduce blood borne pathogens. Silver nanoparticles (AgNPs) are a suitable option due to their low-risk of microbial resistance and strong antimicrobial activity.

Materials and Methods: As representative microorganisms, we tested Herpes simplex type 1 (HSV-1), Escherichia coli (ATCC 25922), and Staphylococcus aureus (ATCC 1413). The cytotoxicity of biosynthesized AgNPs was assessed on the Vero cell line. The Maximum Non-Toxic Dose (MNTD) of AgNPs did not affect the virus. In order to address the high toxicity of AgNPs and utilize the highest colloidal concentration of AgNPs (300 µg/ml) that did not precipitate within the 24-hour examination period, we developed a removal process. AgNPs were removed after being exposed to viruses and bacteria. This removal step was done by addition of magnetic iron oxide nanoparticles (iron oxide NPs). Then, treated virus and bacteria were exposed to neodymium magnet, and were used for cell inoculation.

Results: Both AgNPs and iron oxide NPs reduced the titer of virus by at least 3.5 log when used together. Additionally, they inactivated 106 CFU/ml of bacteria in human plasma. While each nanoparticle exhibited antiviral activity individually, but also their effect was enhanced when used simultaneously.

Conclusion: Human plasma pathogens were reduced using AgNPs and/or iron oxide NPs. The plasma could be treated with these nanoparticles, and subsequently the magnetic nanoparticles that adsorbed silver nanoparticles were separated from the plasma using a neodymium magnet, as one of the techniques for pathogen reduction.

Keywords: Pathogen reduction, Human plasma, Herpes simplex type 1, Silver nanoparticles, Iron oxide nanoparticles

Introduction

Human blood is a valuable source of several components such as red blood cells, platelets, and fresh-frozen plasma (FFPB) (1). The plasma products were used to treat many diseases, such as defective multi coagulation factors or Disseminated Intravascular Coagulation (DIC) (2).

Blood and its components can transmit bacteria, viruses, and parasites, which can cause several serious infections (3).

Additionally, serum can serve as a medium for transmitting viral infections in cell culture, leading a significant issue in this field. Viral infections that can be transmitted through blood transfusion include Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Viruses (HIV-1/2), Human T-cell Lymphotropic Viruses (HTLV-I/II), Cytomegalovirus (CMV), Parvovirus B19, West Nile Virus (WNV), and Dengue Virus (4).

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Blood donors have been tested for viral infections using antibody or nucleic acid amplification methods (5) and bacterial contamination is assessed using a questionnaire (6). As global population mobility increases, bacterial and viral infections have become a new challenge in blood and plasma transfusion (5). Pathogens can be inactivated in multiple ways (7), but most chemicals can destroy proteins and reduce the activity of coagulation factors (1). Also, the effectiveness of safe therapies for viral diseases is diminishing due to the development of virus resistance and the emergence of new viral diseases. As such, researchers and pharmaceutical companies focus on new antiviral drugs. AgNPs belonging to nanoscale materials, are particularly attractive in this field (8).

Model virus share envelope and nucleic acid properties with the viruses mentioned. HSV-1 is affiliated with the Herpesviridae family, which includes 200 species with standard viral structures. Viral particles consist of double-stranded DNA, an icosahedral capsid, and an envelope. Human herpes viruses include nine types, and HSV-1 is one of them (9). Several gram-negative bacteria can be found in blood and its products, including Pseudomonas aeruginosa, Pseudomonas fluorescens, Yersinia enterocolitica, and gram-positive species like Staphylococcus and Streptococcus spp (6).

It has been shown that AgNPs can kill bacteria, viruses, and certain microorganisms. Because of their small size and unique properties, these particles interact with bacteria, fungi, and viruses (10,11). In addition, resistance to AgNPs is unlikely. They can prevent viral particles from penetrating into cytoplasm and inhibit virus replication (8). The antibacterial properties of AgNPs can be explained by their interaction with the bacterial cell wall. AgNPs also increase membrane permeability, leading to cell death. AgNPs can interact with intracellular components such as proteins and DNA, as well as extracellular proteins that are rich in sulfur and phosphorus (12, 13).

The function of AgNPs as antiviral and antibacterial agents is sorted according to their therapeutic applications. Additionally, other therapeutic applications include antifungal, anticancer, catalytic, anti-diabetic, and other biomedical applications (14).

Biosynthesized AgNPs, obtained by aqueous extract of dried Juglans regia green husk, were assessed against modeled pathogens in human plasma. Additionally, free AgNPs were adsorbed by iron oxide NPs. Subsequently, the use of neodymium magnet facilitated the removal of these nanoparticles.This research represents a novel approach by evaluating antimicrobial activity within the environment and innovatively addressing nanoparticle removal after their interaction with pathogens.

This method holds potential for generating pathogen-reduced plasma products suitable for therapeutic and cell culture applications.

Materials and Methods

Viral Propagation and Quantification

The HSV-1 was isolated from the infected person's herpes and cultured in cell culture, and its type was determined with fluorescent antibody in the Iranian Blood Transfusion Organization (IBTO) laboratory. Virus titer was determined on Vero cells using the Reed and Muench method (15). Viral stock then was stored at -70°C until the day of use. All the steps in this part were done in the Iranian Blood Transfusion Organization. The selected virus serves as a model for Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Viruses (HIV-1/2), Human T-cell Lymphotropic Viruses (HTLV-I/II), Cytomegalovirus (CMV), West Nile Virus (WNV), and Dengue Virus (4).

Bacterial Preparation

Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 1413) are the most frequent bacterial contaminants in plasma and are gram-negative and gram-positive bacteria, respectively. They were grown on nutrient agar and incubated for 24 hours at 37° C. The bacterial suspensions were prepared in sterile Phosphate Buffer Saline (PBS), and adjusted to the 0.5 McFarland scale. It was then diluted to reach a concentration of 106 CFU/ml (16).

FFP Preparation

The O-positive blood obtained from the Iranian Blood Transfusion Organization. It tested negative for HIV, HCV and HBV using ELISA and molecular screening tests. Fresh plasma from the O-positive blood separated by centrifugation for 15 minutes at 7000 rpm at room temperature (20°C). Plasma then was stored at -20°C until required.

AgNPs Preparation

AgNPs were purchased from Nanoziran, a start-up based in Alzahra University, Tehran, Iran. AgNPs were prepared at a concentration of 500µg/ml and were sonicated for 2 hours before use.

Iron oxide NPs Preparation

Iron oxide NPs were purchased from Nanoziran, a start-up based in Alzahra University, Tehran, Iran. The iron oxide NPs were dried for 2 hours at 60°C and stored at room temperature until use.

MTT Assay

Vero cells were seeded in a 96-well microplate, and the highest stable concentration of AgNPs for 24 hours, was prepared. Final dilutions ranging from 5 µg/ml to 200 µg/ml were prepared in each well by adding 50 µl of AgNPs at the calculated concentrations and 150 µl of RPMI medium containing 20% FBS. Afterward, the plate followed by 24 hours incubation at 37°C and 5% CO2.

After the incubation, following the kit protocol (MTT cell proliferation assay kit, CIB Biotech Company, Tehran, Iran), 10µl of MTT color solution was added to each well (100 µl of wells contents), and incubated for 4 hours. Then, the contents of the wells were removed, and 100 µl of DMSO was added to each well. ELISA reader assays cell viability at 750nm after incubation in the dark for approximately 30 minutes. Subsequently, the data was exported to Microsoft Excel, and a chart depicting cell viability and AgNPs concentrations was obtained (17).

Viral Inactivation Tests

The most stable concentration of AgNPs in 24 hours, 300 µg/ml, was used to examine the antiviral activity of AgNPs. For this test, the method described by Mwilu et al. (18) was followed. One ml solution consists of iron oxide NPs (300 mg/ml), sonicated AgNPs (500 µg/ml), and 400 µl combination of plasma and modeled virus (1:1 ratio) was prepared. In addition, controls including a mixture of virus, plasma, iron oxide NPs and distilled water, as well as a mixture of virus, plasma and distilled water, were performed to estimate the effect of iron oxide NPs and the sonication process on virus, and viral cytopathic effects (CPE), respectively. These controls were conducted alongside the initial test.

Before adding the combinations of virus, plasma, and nanoparticles, as well as virus, plasma, and distilled water to the weighed dried iron oxide NPs to prepare the test and control mixtures, they were incubated for 1 hour at 37°C with 5% CO2. Then the test, iron oxide NPs control, and sonication control samples were vortexed for 30 seconds. Each samples were sonicated twice, with each sonication lasting 15 minutes using a bath sonicator. The water temperature of the sonicator was initially 24°C and reached a maximum temperature of 48°C by the end of the sonication process. Plasma was sonicated based on Pförringer and colleagues' protocol (1).

The supernatant from the test and iron oxide NPs control vials was transferred into glass containers and placed in a shaker-incubator along with two other controls. The containers were shaken at 119 rpm and temperature of 25°C for 1 hour. Afterward, they were incubated at room temperature for 15 minutes. Before being exposed to cells, the samples were subjected to a neodymium magnet for 10 minutes to separate the iron oxide NPs with adsorbed AgNPs. The supernatants were inoculated on Vero cells in a 96-well microplate seeded the previous day. Before adding the test and controls supernatants, the cells were rinsed with PBS.

Modeled virus titer after being exposed to both AgNPs and iron oxide NPs, as well as just iron oxide NPs, was determined using the Reed and Muench method (15) and a 10-fold dilution series. A schematic illustration of the steps

involved in this test is shown in the graphical abstract.

Bacterial Inactivation Tests

Prepared 10^6 CFU/ml bacteria suspensions, according to part 3.2, were mixed with calculated volumes of FFP and AgNPs. The mixture was then incubated for 1 hour at 37°C before being poured onto weighed dried iron oxide NPs in vials. Then, the vials containing these mixtures were vortexed for 30 seconds and sonicated for 2 rounds, with each round taking 15 minutes in a bath sonicator. The water temperature was 24°C at the beginning, and it reached a maximum temperature of 48° C at the end of sonication. Both vials' supernatant was transferred into a glass container and incubated for 1 hour at 119 rpm and 25°C in a shaker-incubator.

After that, they were incubated at room temperature for 15 minutes, then each glass container got exposed to a neodymium magnet for another 15 minutes (18). After separating nanoparticles, 500 µl of supernatant was added to the nutrient agar, and plates were rotating in the shape of 8 until the supernatant was adsorbed to the medium.

Also, 500 µl of each prepared bacteria suspension was cultured in the same way on nutrient agar as the positive control for bacteria growth. The test calculation was performed similarly to the viral inactivation test. 300 mg of dried iron oxide NPs were mixed with FFP, AgNPs that were prepared at a concentration of 500 µg/ml, and a bacteria suspension to reach a volume of 1 ml.

Results

Modeled Virus Titer

HSV-1 titer was 10^6 $TCID_{50}$/ml.

Characterization of Biosynthesized AgNPs from Aqueous Extract of Dried Juglans regia green husk and Iron oxide NPs

AgNPs were spherical in shape with the average size of 7 nm and their hydrodynamic diameter was around 69 nm (19). The average pore width and total pore volume of the iron oxide NPs were 123.59 A° and 0.27 cm^3 /g (20). The iron oxide NPs' size was 10-25nm which was small, so the saturation magnetization was low, about 55.26 emu/g (21).

MTT Assay

The absorption of each well was shown that AgNPs were so toxic and the MNTD of AgNPs was 10 µg/ml. Cells were viable in 10 µg/ml and concentrations lower than MNTD of AgNPs. Cell viability after being exposed to AgNPs concentrations is shown in Figure 1.

Viral Inactivation Tests

Antiviral activity of AgNPs and iron oxide NPs together, and just iron oxide NPs was evaluated by inhibition of CPE formation. Wells of test plate were observed with inverted optical microscope and results compared to controls. Examples for both CPE+, CPE- and treated virus are shown in Figure 2.

Wells with CPE or without it, were counted, and modelled virus titer reduction was calculated based on Ramakrishnan calculations after being treated with both nanoparticles (22). After AgNPs and iron oxide NPs treatment, *HSV-1* titer reached 2.5 log with 3.5 log reduction. Also, after treatment with just iron oxide NPs, *HSV-1* titer reached 5 log with 1 log reduction.

Bacterial Inactivation Tests

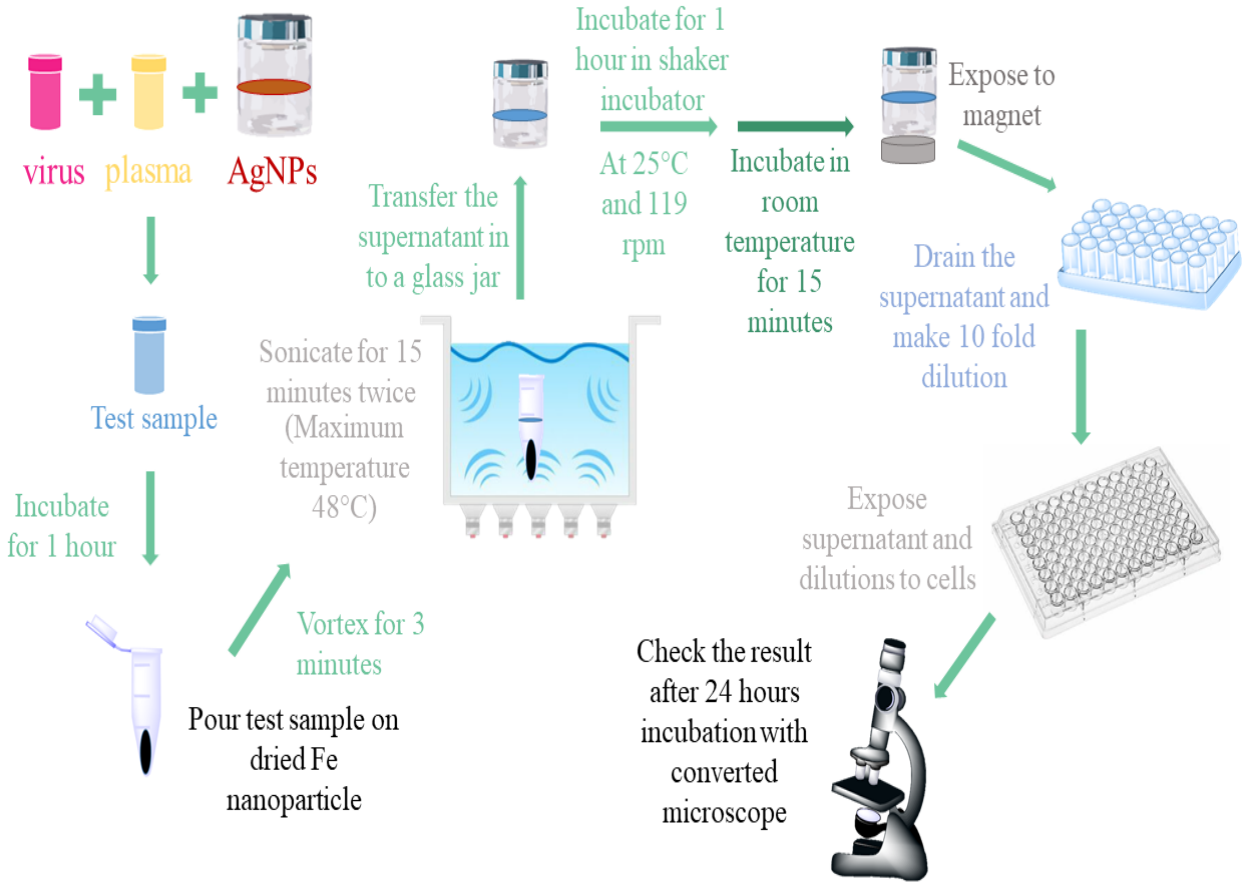
Bacteria titer reduced from 0.15×10^6 CFU/ml to almost zero, and nutrient agar was completely clear without any colony compared to the control medium. Bacteria were entirely inactivated after treatment, as shown in Fig. 2.

Discussion

Selection of plasma/blood donors based on antibodies and nucleic acid amplification assays is so important for therapeutic usage of human plasma (5). New pathogenic viruses' emergence and resistance to common antiviral drugs have become issues that are compelling researchers to search for a special antiviral agent on the nanoscale. The preparation of nanoparticles is more economical than the preparation of antibiotics and other drugs, and they are stable for a longer time.

Table 1. Antiviral and antibacterial activity of other studied AgNPs (in the cell medium) is compared to *Juglans regia* green husk capped AgNPs and iron oxide NPs (in the human plasma).

Type of nanoparticles	Antiviral activity ($TCID_{50}$/ml)	Assayed viruses	Antibacterial activity (CFU/ml)	Assayed bacteria	References
Chitosan capped AgNPs	3 log	*African swine fever virus*	10^8	*Salmonella*	(27)
Polyvinylpyrrolidone capped AgNPs	2.7 log	*Herpes simplex virus type 2*	--	--	(28)
***Andrographis paniculata* capped AgNPs**	≤3 log	*chikungunya virus*	--	--	(29)
***Juglans regia* green husk capped AgNPs and iron oxide NPs**	≥3.5 log	*HSV-1*	10^6	*Escherichia coli* and *Staphylococcus aureus*	--



Graphical abstract. An overview of all steps which were taken in the virus inactivation test is shown in the picture

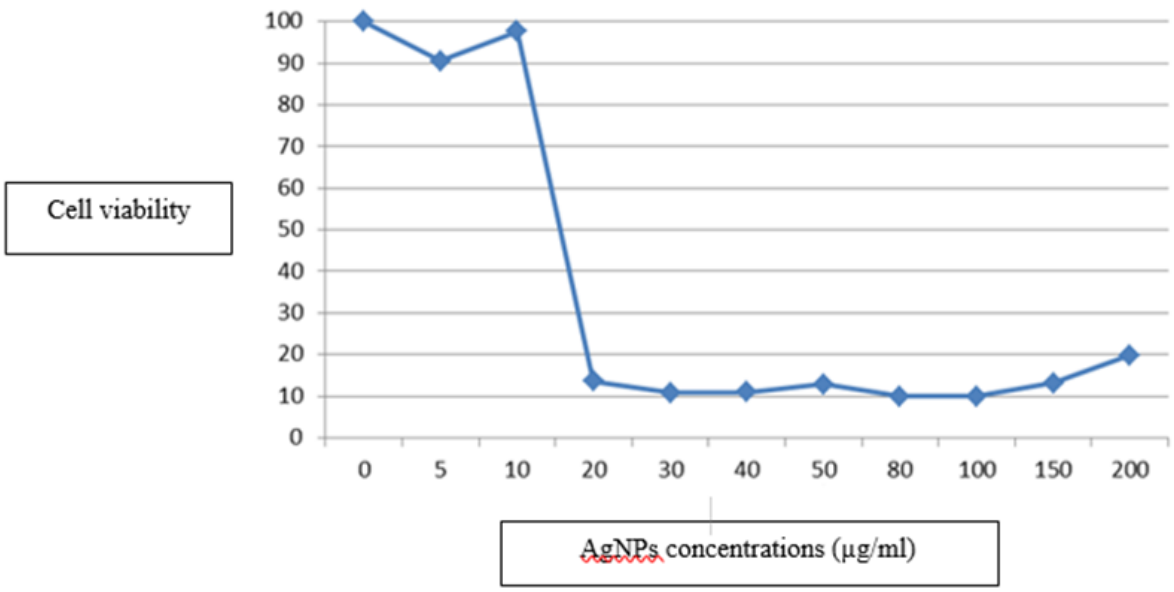


Fig 1. cell viability in MTT test after being exposed to AgNPs concentrations.

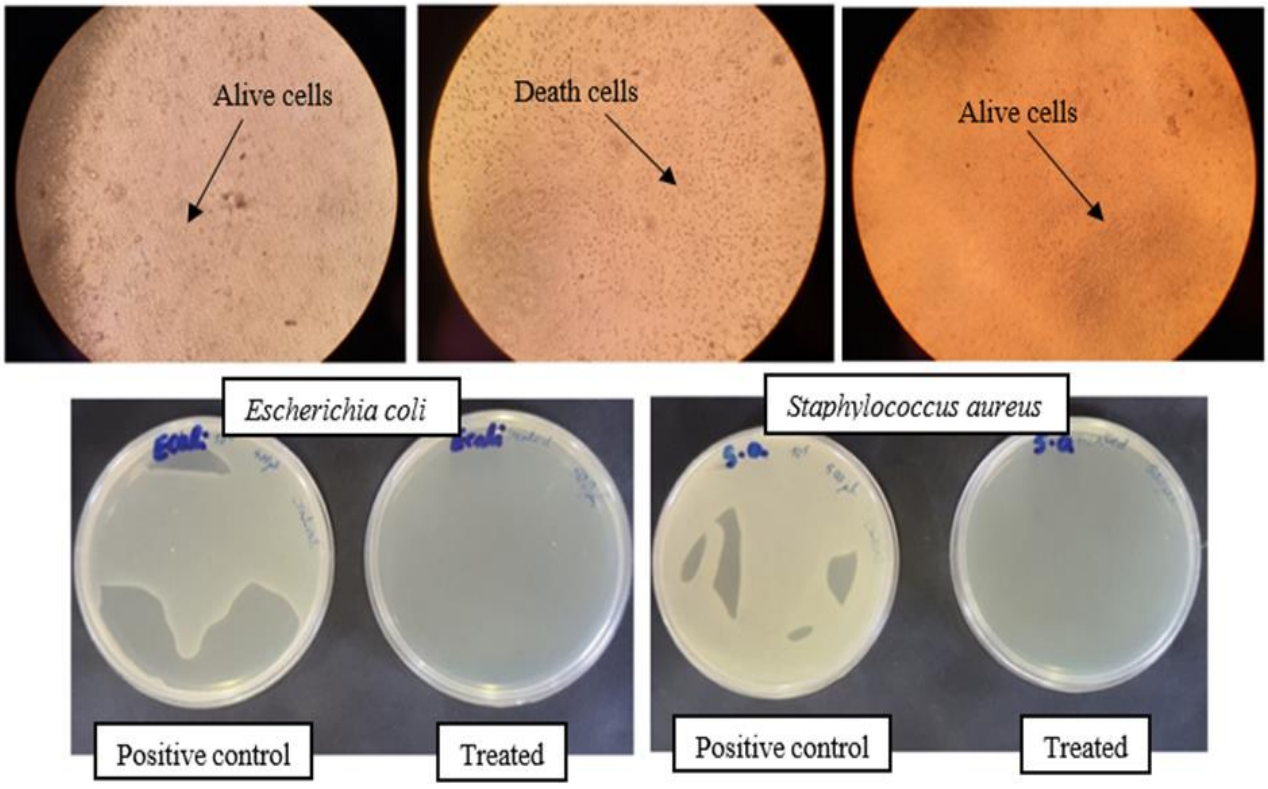


Figure 2. a) Optical inverted microscopy images of living cells without CPE (magnification, 100x). b) Optical inverted microscopy images of CPE formation of Vero cells after 24 hours incubation (magnification, 100x). c) Inactivated *HSV-1* with AgNPs and iron oxide NPs.d) *Escherichia coli* after treatment with AgNPs and iron oxide NPs compare to positive controls. e) *Staphylococcus aureus* after treatment with AgNPs and iron oxide NPs compare to positive controls.

Additionally, nanoparticles are eco-friendly and viruses are less likely to resist them (8).

In this study, biosynthesized AgNPs were evaluated as a material for reducing pathogens in plasma and its products. The aim was to develop a therapeutic application and prepare serum with a low risk of microbial infection, which is essential in the field of cell culture. The use of human serum can overcome major obstacles of interspecies differences when working with human cell lines and reduces the need to translate animal studies to humans. Both bovine and human serum stimulated cell proliferation and migration similarly, whereas HS significantly increased cell invasion (23).

The cytotoxicity of AgNPs was estimated on Vero cells using the MTT assay. The MNTD obtained from the MTT assay and lower concentrations were exposed to modeled virus and cells, but CPE could still be observed. The low MNTD (10 µg/ml) of the AgNPs was toxic to the cells, but it did not affect modeled virus. Therefore, treatment of the plasma was done with the highest colloidal concentration of AgNP (300 µg/ml), which remained stable during in the 24-hour examination period. Then, the AgNPs were adsorbed by magnetic iron oxide NPs. In this study, it was found that 300 µg/ml of AgNPs can be absorbed with a concentration of iron oxide NPs, which is 103 times higher (300 mg/ml). Both types of nanoparticles were then separated from the treated virus using a neodymium magnet (20). So, the virus was inactivated and the nanoparticles were removed.

This method was implemented on modeled virus and bacteria. In this method, AgNPs and iron oxide NPs were used to inactivated modeled virus and bacteria. Iron oxide NPs control also showed antiviral activity themselves, but it was much lower than AgNPs and iron oxide NPs altogether, which can be a sign of synergism between these two nanoparticles (NPs).

Previous research has proposed some mechanisms for AgNPs and iron oxide NPs action. The hypothetical mechanisms for HSV-1 inactivation via AgNPs are that NPs and the virus compete for binding to cell receptors. NPs interfere in the early steps of virus penetration and block their attachment to cell. As a result, they prevent virus entry and cell-to-cell spread (8). AgNPs can also agglutinate viral glycoproteins, preventing virus binding to cell receptors (24). The size of NPs (AgNPs = 7nm and iron oxide NPs = 10-25nm) (18,19) are smaller than virus particles; hence, NPs can move easily between viral particles and interact with them leading to their cell entrance inhibition (25). Another molecular mechanism for iron oxide NPs involves a potential reaction between iron oxide NPs and proteins containing –SH groups. As a result, these NPs may inactivate viruses by interacting with viral proteins. This interaction between iron oxide and –SH groups of proteins can be harmful to proteins in the cell, interfering with important cellular mechanisms (26). However, by using this method where the NPs are extracted using a neodymium magnet, the possibility of this interaction is reduced.

A further three features are proposed for NPs' antibacterial mechanisms: 1) slow release of silver ions, inhibiting ATP synthesis and DNA replication; 2) direct destruction of the cell wall; and 3) ROS generation.

Silver ion connects to the negatively charged components of nucleic acids and proteins and causes changes in bacterial structure, cell wall, membrane and nucleic acid (8).These proposed mechanisms can explain the reactions of NPs against modeled bacteria and virus, and the results can be generalized for a wide range of plasma bacteria and viruses .

The antiviral and antibacterial activities of synthesized AgNPs by different methods have been evaluated in some cases as they were illustrated in Table 1.

A Chitosan capped AgNPs and Polyvinylpyrrolidone capped AgNPs that were synthesized through chemical reduction method caused 103 and 102.7 reduction in African swine fever virus and Herpes simplex virus type 2 titration, respectively (27,28).

Biosynthesized AgNPs using Andrographis paniculata extract and two blue-green algal strains were reduced chikungunya virus and Herpes Simplex virus for ≤103 in titer (24, 29). However, biosynthesized AgNPs with an aqueous extract of dried Juglans regia green husk at a concentration of 300 µg/ml, along with iron oxide NPs, showed higher activity

against modeled virus (≥3.5 log) and inactivated 106 CFU/ml of the most common bacteria. Novelty is that, unlike others, the activity of biosynthesized AgNPs using aqueous extract of dried Juglans regia green husk was estimated in plasma, which can cause differences in the reduction of viruses' titer.

The biosynthesized AgNPs using the aqueous extract of Nigella Arvensis were made from an aqueous plant extract, such as biosynthesized AgNPs with an aqueous extract of dried Juglans regia green husk. Antibacterial activity of these nanoparticles were reported against four bacteria, Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa. The first three bacteria exhibited zone inhibition but Pseudomonas aeruginosa showed resistance to the AgNPs solution. HSV-1, HAV, and adenovirus were tested as high mutation viruses and their replication inhibition was observed. Antiviral and antibacterial activity of these AgNPs were assessed in culture medium without removal process (30). However, the removal process with adsorption by iron oxide NPs that used in this study, made it possible to use concentrations higher than the maximum non-toxic concentration (MNTC), which improved the antiviral and antibacterial activity and reduced the cytotoxicity of nanoparticles.

In order to use this method as one of pathogen reduction methods, it was compared with other conventional methods as shown in Table 2. The limitation of these methods is that they affect plasma proteins, such as coagulation factors and inhibitors.

In recent studies, researchers have even used a combination of two identified pathogen reduction methods against a specific virus. Pathogen reduction with riboflavin and UV radiation is one such combination method that has been shown to effectively reduce the infectivity of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) to the limit of detection in inoculated plasma.

However, its efficacy against a wide range of pathogens has not been measured and evaluated for infectivity (31). Also, this combined method was applied to monkey pox virus and no infectious virus was detected after treatment. However, results from an individual pathogen cannot be generalized. Risk-benefit and cost-benefit are the two main issues that should be considered in choosing the appropriate pathogen reduction method (32). The main challenge associated with biochemical reactions used in pathogen reduction methods, such as riboflavin and methylene blue, is their effect on the immunological quality of the convalescent plasma. This challenge is due to the amount of reactive oxygen species released after photo-activation (33). It is suggested to study the impact of the proposed method on plasma proteins and immunological quality. By determining the effect of this method on plasma proteins and aggregation factors, and addressing its limitation, this method can be utilized as an effective pathogen reduction technique.

Conclusion

Biosynthesized AgNPs and magnetic iron oxide NPs can reduce the titer of modeled virus by at least 3.5 log using this method. Additionally, they can completely inactivate 106 CFU/ml of modelled bacteria in human plasma. By conducting further research, this method can be utilized as an effective technique for reducing pathogens.

Highlights

1. Blood derivatives contaminated with viruses and bacteria cannot be used for therapeutic purposes, as they may cause acute diseases in individuals who receive them.
2. The low MNTD (10 µg/ml) of biosynthesized AgNPs with an aqueous extract of dried *Juglans regia* green husk demonstrated their toxicity when exposed to cells. However, at these low concentrations, they were unable to interact with model virus. Therefore, we developed another assay that allowed us to use higher concentrations of AgNPs without causing any toxic effects on cells.
3. This method can be used as one of the effective techniques for reducing pathogens by studying its effect on plasma proteins and aggregation factors, and overcoming the limitations.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Approvals and Consent to Participate

Not Applicable.

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Authors' contribution

M.S. performed the experiment and wrote the manuscript, A.E. and P.G. designed the experiment and edited the manuscript.

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