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Investigation the Effect of Silver Nanoparticles and Bioresonance Wave Radiation on *Leishmania major*: An In Vitro Study

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Abstract

Introduction: Cutaneous leishmaniasis (CL) is one of the infectious diseases and health problems in tropical regions. Glucantime is commonly used to treat CL and it, not only has some side effects but also observation shows the drug resistance of some of the various *Leishmania* species. Therefore, the aim of this study was to investigate the effect of silver nanoparticles (AgNPs) and bioresonance waves on *Leishmania*, in vitro.

Materials and Methods: In the present experimental study, *Leishmania major* promastigotes were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin and streptomycin at 23°C. After 6 days, the parasites achieved stationary phases of promastigotes, Then the effects of different concentrations of AgNPs (1, 3, 5, 10 and 25 µg/mL) and different radiation times of bioresonance wave (5 and 20 minutes) were investigated. Herein, the effects of various treatment on parasites proliferation were evaluated with live promastigotes counting after 24, 48 and 72 hours treatment.

Results: The parasite count showed that the various concentrations of AgNPs, radiation of bioresonance wave and combination significantly decreased the numbers of live promastigotes over time compared with the control group after 72 hours. The highest antileishmanial activity was seen for AgNPs at concentration of 1 µg/mL when combined with 20 minutes radiation of bioresonance wave (proliferation inhibition: 79.92%)

Conclusions: Based on our result, AgNPs and bioresonance waves are potent antileishmanial agents. The authors declare that the more studies should be done.

Keywords: Bioresonance, Leishmaniasis, Promastigote Stage, Silver Nanoparticles

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Introduction

Leishmaniasis is a disease caused by protozoans of the Leishmania. It is endemic in tropical regions and accounts for a wide variety of diseases with clinical manifestations and different health consequences. Leishmaniasis appears in 3 major forms of cutaneous, mucocutaneous, and visceral leishmaniasis. The cutaneous type of the disease has symptoms such as skin ulcers and is called Salak in Persian. Cutaneous leishmaniasis (CL) is more common than other types of leishmaniasis, with approximately 70% of all new cases reported annually (approximately 1 to 1.5 million).[1] This disease has many socio-economic implications on human societies annually. On the other hand, due to the epidemiological complexities found in the transmission process of this disease (the existence of different reservoirs and vectors), self-care against this disease is of high importance.

Antimony compounds are used for treating CL, which have multiple side effects,[1] and their effects are diminished due to drug resistance. In addition, changes in drug production protocols have been satisfactory in some cases but are not a definitive cure for the disease. Given the endemic nature of leishmaniasis in Iran and its high prevalence as the most important parasitic disease in the country, research should be directed to new methods with low side effects as well as greater impact.

There has been extensive research to improve the treatment of CL in vitro and in vivo, for example, studies of new drug compounds and solving the problem of proto-drug resistance, examining the antileishmanial effects of medicinal plants,[2] the use of nanoparticles, especially silver nanoparticles

(AgNPs),[3,4] and using complementary therapeutic approaches (e.g., the use of direct electric currents),[5] and electromagnetic waves (e.g., ultraviolet [UV], visible and infrared light).[6-9]

In fact, nanotechnology is the recognition and control of materials in the dimensions of 1 to 100 nm, giving rise to unusual physical, chemical, and biological properties compared to the macro state, which enables new and unique applications.[10] The use of AgNPs is influenced by the type of application, particle characteristics, and organisms. In addition, the nanoscale dimensions of these particles make it easier to pass through the membrane and affect the cell physiology. Moreover, as the diameter decreases, the surface area of contact increases, and the effect and penetration of these particles enhance. On the other hand, antimicrobial properties of silver have been known for over 100 years. Silver has very little chemical activity, but when exposed to water, small amounts of silver ions are released into the aqueous medium, which has antimicrobial activity. Therefore, AgNPs can be used as an efficient factor in the treatment of infectious diseases. Argyria is the only disease related to the accumulation of silver in the human body. The presence of more than 2 mg of silver per liter of water or the constant ingestion of silver by drinking water causes the disease. However, a healthy person should consume about 48 times the normal daily dose for one year to develop symptoms of argyria. This toxicity may begin with skin bruising and discoloration of the eyelids and mucous membranes of the mouth. It may eventually lead to the death of the person being poisoned by side effects.[11]

It is widely accepted that bioresonance is an experimental method that has been proposed in the field of medicine as a test-driven approach. Bioresonance therapy works on the basis of the body bioelectricity of living creatures.[12] In fact, in addition to chemical reactions, electrical aspects also play a very important role in the cell, so that the cells can detect themselves by electric currents and electromagnetic waves. For example, a study showed that 3 different processes can produce electromagnetic fields in the cell that include mechanical vibrations of polar structures (proteins), free ions, and electronic oscillations. On the other hand, the emerging evidence indicate the effects of external electromagnetic waves on the body tissues. The electromagnetic waves with very low frequency produce effects such as induction of proliferation and differentiation, apoptosis, DNA synthesis, RNA transcription, protein expression, micro-vesicles translocation, ATP synthesis, hormone production, and so forth at the cellular scale.[13] In other words, all living things have their own frequency patterns, which is called body vibrations. Therefore, cells can communicate directly with each other by means of the waves. Hence, it is possible for these signals to be recorded by devices and then corrected and returned to the body if disturbed, so that they can create the desired effects.[12]

According to a study conducted on the bioresonance device, the waves are received by the electrodes from the body and are matched to the body's natural frequency patterns. Then, abnormal waves are detected by applying different filters, and the cause of the disease is determined by the information stored in the device. Afterwards, depending on the type of pathogen, an appropriate selected treatment and the modified waves will be returned to the patient's body. Since electromagnetic waves can regulate the biochemical activities of the organs, adjusting and correcting fluctuations of the electromagnetic fields can have proper therapeutic effects[13]; therefore, treatment with the help of information from natural body fluctuations or positive external fluctuations would have beneficial effects on the function of tissues and organs. Thus, this study aimed to investigate the effect of bioresonance wave along with AgNPs on the protozoan parasite *Leishmania major* in vitro condition for the first time.

Materials and Methods

Culture of *Leishmania* Parasite

Leishmania major promastigotes (MRHO/IR/75/ER) were prepared from the Department of Parasitology and Mycology, Shahid Beheshti University of Medical Sciences. The parasite was first cultured in RPMI-1640 containing glutamine (Biosera) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin and streptomycin (Sigma-Aldrich) to inhibit bacterial growth. The culture containers were then transferred to the shaker incubator with 40 rpm at 23°C, and the daily growth of promastigotes was examined by microscope (Zeiss, model: Axiovert 40 CFL). After 6 days, promastigotes entered the stationary phase. Then, fresh media were added to reproduce the parasite as needed.

Preparation and Characterization of AgNPs

This study used US silver nanomaterials with 99.99% purity. Transmission electron microscopy (TEM) (Philips, model: CM 30 T/STEM) with light background was used to examine the microstructure of AgNPs. The size of AgNPs in the TEM images was measured using ImageJ software. In addition, the X-ray diffraction (XRD) technique was used to determine the crystal structure of the nanoparticles (Siemens, model: D500).

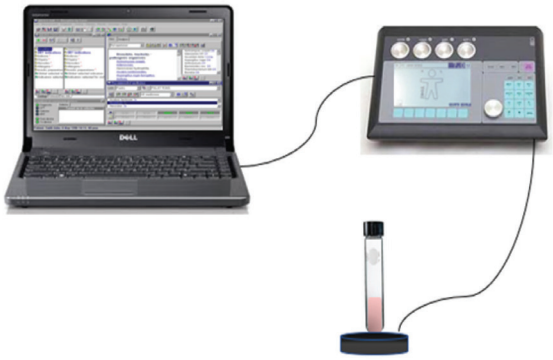
Preparation of Different Concentrations of AgNPs Solution

According to the research design, 2 mg of AgNPs powder was weighed to prepare the initial solution. Then, 20 mL of complete culture medium (RPMI-1640 containing glutamine with 10% FBS and 1% penicillin and streptomycin) was added. In this way, concentration of the initial solution was equal to 100 µg/mL. Then, a sonicator was used to dissolve the nanoparticles and homogenize the solution for 15 minutes. Finally, the AgNPs solution was filtered and purified. The other tested concentrations (2, 6, 10, 20, and 50 µg/mL) were prepared by diluting the base solution and stored in a refrigerator at 5°C.

Bioresonance Radiation

Using the bioresonance device (IMEDIS, model: MINI-EXPERT-DT) at Amir Kabir University of Technology Bioresonance Research and Medical Laboratory, the waves were irradiated directly to the culture containing promastigotes through special probes that connect the MRT outputs. Therefore, glass culture tubes were mounted on the probes through the non-conductive stands on a standing position. Then, given the device software-defined programs

that include certain frequencies for various diseases, the F 195 treatment program that was defined for CL was selected. By setting the device intensity to 100 µT and adjusting the duration of radiation, the treatment process was followed up in 2 independent groups of radiation of 5 and 20 minutes for 3 consecutive days (Scheme 1).



Scheme 1. Schematic image of how the *Leishmania major* promastigotes were placed under the irradiation of the bioresonance device made by IMEDIS.

Examination of Protozoan Growth Rate

To investigate the proliferation of the single-celled promastigotes of *Leishmania*, the number of promastigotes in the stationary phase of growth was counted using the Neubauer chamber and optical microscope (Nikon, model: YS100) by the following formula. 1 mL of the single celled suspension was added to each of the screw cap glass tube.

$$N = M \times Cn \times Cd$$

Where N is the number of protozoa per milliliter of culture medium, M represents the average number of protozoa in 4 WBC cells of Neubauer, Cn refers to the Neubauer constant, and Cd stands for dilution constant (which equals 1 in our calculation). To evaluate the proliferation of promastigotes under the influence of nanoparticles, 1 mL of different concentrations of AgNPs was added to the 1 mL culture tubes containing 1×10^6 promastigotes of *L. major*. The ultimate concentration of AgNPs near promastigotes reached 1, 3, 5, 10, and 25 µg/mL. The culture dishes were then placed in the shaker incubator at 40 rpm and 23°C for 24 hours. After 24 hours, the number of live promastigotes was counted with Neubauer chambers. The counting process was also performed at 48 and 72 hours after the nanoparticle contact. Finally, the percentage of inhibition against proliferation compared to the control group was calculated by the following formula. The control group consisted of protozoans that did not receive treatment; however, their storage and culture conditions were similar to the treated groups. All experiments were repeated 3 times.

$$MIC\ (\%) = (Nc - Nt) / Nc \times 100$$

The above equation is used to compare the proliferation of live promastigotes in the control and treated groups. In this equation, MIC indicates the rate of inhibition of proliferation, Nc refers to the number of live promastigotes in the control group, and Nt represents the number of promastigotes in the treated group. One milliliter of protozoan culture medium containing 1×10^6 promastigote of *L. major* was added to the tubes to determine the effect of radiation by the bioresonance device on proliferation. After 24 hours incubation at 23°C, tubes containing promastigotes were placed in independent groups for 5 and 20 minutes on direct transduction probes. At the time of irradiation, the ambient temperature was kept constant at 23°C, and the number of viable promastigotes was counted 24 hours after irradiation. The wave irradiation and counting process was similarly followed in the second and third days. Finally, the percentage of inhibition of proliferation was calculated in the control group. All experiments were performed with 3 replications. The control groups did not receive radiation, but had conditions similar to those in the irradiated groups.

After completing the 3-day treatment period, the results of the 2 treatment groups (bioresonance radiation and addition of AgNPs) were analyzed and finally 2 optimal concentrations of AgNPs that have the most effect were selected to evaluate simultaneous interactions of the 2 treatment groups. On the other hand, First, 1 mL of protozoan culture medium containing 1×10^6 *L. major* promastigote was added to the tubes. Then, 1 mL of 2 optimum concentrations of AgNPs (1, 3 µg/mL) was added.

After 24 hours, the promastigotes were exposed to electromagnetic waves as in the previous experimental conditions for 5 and 20 minutes. After 4 hours of radiation, the number of viable protozoans was counted, and the percentage of proliferation inhibition was controlled compared to the non-treated control group. This group of experiments was followed up until day 3. All experiments were performed with 3 replications. In this study the groups included untreated groups (Control), treated groups with 2 different concentrations of AgNPs, and treated groups with 2 different irradiation times.

Statistical Analysis

The results are expressed as mean ± SD. Statistical analysis was run between the treatment and control groups using the F-test (to compare the variance between 2 groups) and t test with Microsoft Excel 2016 (to examine the significance, $P<0.05$ as the level of significance).

Results

Characterization of Silver Nanoparticles

TEM was used to evaluate the size and microstructure of AgNPs. TEM images analysis with ImageJ software showed that AgNPs are about 20 nm in size, have a spherical shape with smooth surfaces (Figure 1A), and tend to agglomerate. The results obtained from XRD indicated that the peak location corresponds to the peak in the JCPDS 04-0783 reference card (Figure 1B). In addition to the reference card peaks, there are some extra peaks in the graph, possibly due to the presence of stabilizers added to the nanoparticles process.

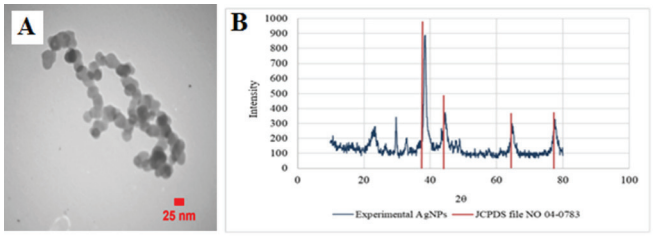


Figure 1. TEM image of spherical silver nanoparticles with smooth surfaces (A). Matching the XRD peaks of the tested nanoparticles with the pattern peaks (B).

The Effect of AgNPs on the Promastigotes Proliferation

The effect of AgNPs on the proliferation of *L. major* promastigotes (Figure 2) demonstrated that after 72 hours, there was a significant decrease in the number of promastigotes in all groups ($P=0.005$). As the concentration increases, the percentage of inhibition of proliferation decreases. In addition, the greatest effect of nanoparticles occurred at concentration of 1 µg/mL on day 3 (72.8% inhibition of proliferation). On the other hand, the percentage of inhibition was significantly different on different days ($P=0.02$).

The Effect of Bioresonance Wave on the Promastigotes Proliferation

The effect of bioresonance radiation at both intervals (Figure 3) showed that the ratio of the control group is significant only on the third day ($P=0.01$). There was also no predictable trend in the percentage of the proliferation inhibition. However, as the promastigotes entered the growth phase, the percentage of inhibition in the second day decreased compared to the first day. Nonetheless, the percentage of inhibition of reproduction increased again on the third day. On the other hand, the highest effect occurred in the 20-minute irradiation on the third day (48% inhibition of proliferation).

Simultaneous Effect of the AgNPs and Bioresonance Wave on the Promastigotes Proliferation

According to the research design, the 2 most effective concentrations of AgNPs (1 and 3 µg/mL) were used in 2 intervals of 5 and 20 minutes. Finally, the number of the

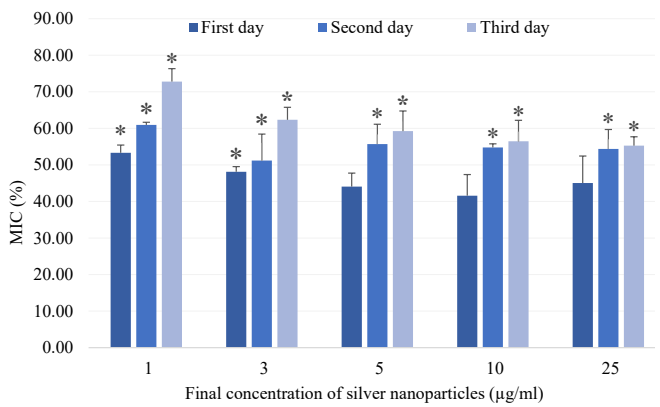


Figure 2. The Percentage of the Proliferation Inhibition of *Leishmania major* Promastigotes Adjacent to Different Concentrations of Silver Nanoparticles. (*) indicates $P<0.05$.

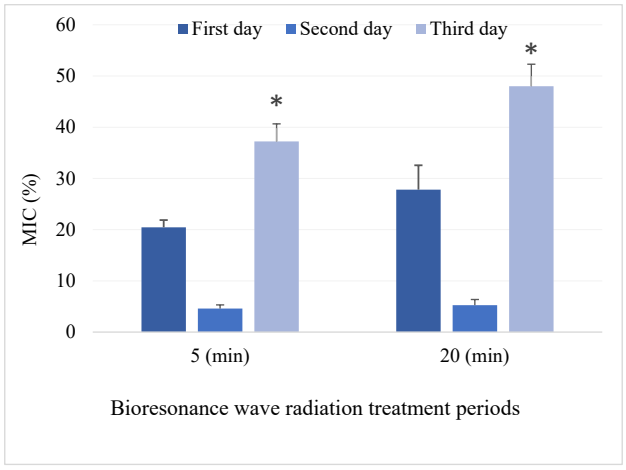


Figure 3. The Percentage of the Proliferation Inhibition of *Leishmania major* Promastigotes Under the Irradiation of Bioresonance Waves of Varying Duration. (*) indicates $P<0.05$.

treated promastigotes was counted, and the results were calculated as the percentage of proliferation inhibition. According to the results (Figure 4), prolongation of treatment for 3 days significantly increased the rate of inhibition of proliferation ($P=0.04$). The highest effect occurred at 1 µg/mL concentration and 20 minutes irradiation (79.92% inhibition of proliferation). In this case, the percentage of inhibition of proliferation was increased compared to the individual use of AgNPs as well as radiation, meaning that the simultaneous use of both methods could produce more favorable effects (Figure 5).

Discussion

Concomitant use of both therapies has advantages, and the World Health Organization (WHO) has also recommended the use of supplementary and multi-therapeutic approaches, which means that the dose of treatment will be reduced by increasing the effectiveness, which will result in the prevention of drug resistance in microorganisms.[2]

To the best of the researcher's knowledge, this is the first study which was evaluated the in vitro effect of bioresonance wave

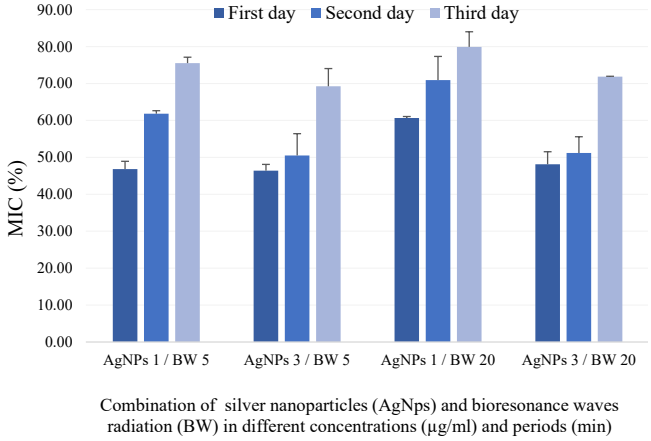


Figure 4. Simultaneous Effect of Silver Nanoparticles and Bioresonance Waves on the Proliferation of *Leishmania major* Promastigotes.

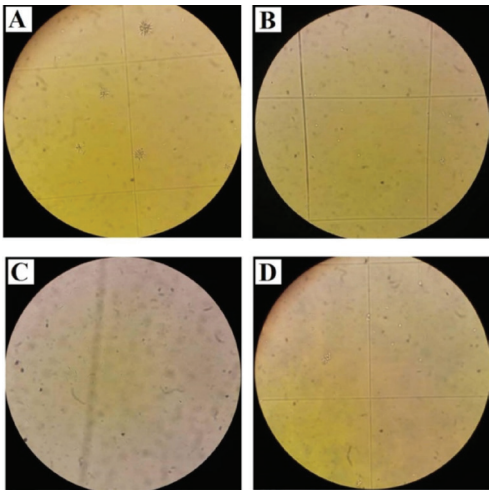


Figure 5. *Leishmania major* Proliferation Count on the Third Day. Control group (A), Silver nanoparticles (1 µg/mL) (B), The irradiation of bioresonance waves after 20 min (C), Simultaneous effect of silver nanoparticles and bioresonance waves (1 µg/mL and 20 min respectively) (D).

singly and in combination with AgNPs on *Leishmania* spp. In this research, we used different concentration of AgNPs (1, 3, 5, 10, 25 µg/mL) with size of 20 nm and bioresonance wave to provide an alternative therapeutic approach for *L. major*. Previous studies showed that AgNPs disrupt cell wall permeability by binding to the microorganisms and have toxic effects on them. The smaller diameter of the AgNPs increases the penetration of the microorganism and makes complex with sulfur in thiol groups of cysteine amino acids and in this way they deactivate vital enzymes. These nanoparticles also produce free radicals such as superoxide, hydrogen peroxide, and hydroxyl ions in the target cells and by affecting the cellular respiration kill them. For this purpose, AgNPs with a mean diameter of ≈20 nm were used.[14]

AgNPs were shown to have antileishmanial effects by inhibiting the proliferation of promastigotes. It was also demonstrated that antileishmanial effects of AgNPs increased due to radiation of bioresonance waves. AgNPs in several studies have been shown as appropriate antimicrobial agents since one of the increasing problems all over the world is resistance of the various *Leishmania* species against common drugs.[15] It was found that the antimicrobial property of AgNPs is due to their small size, high surface-to-volume ratio, and the production of reactive oxygen species (ROS).[6] In addition, AgNPs can also kill *Leishmania* protozoans by various methods. Nanoparticles are highly prone to sulfide and phosphorus bonding, through which they bind to lipophosphoglycan and glycoprotein 63, which are on the protozoa membrane, cause infection, and impair membrane function.[6] The effects of AgNPs and UV light on biological parameters of *Leishmania tropica* studied by Allahverdiyev et al showed significant antileishmanial effects in different concentrations of AgNPs (25, 50, 100, 150, 200 µg/mL) with size of 46 nm by inhibiting the proliferation and metabolic activity of promastigotes, which is in line with the results of our study.[6] A study conducted on the effects of AgNPs on the morphology and infectivity of promastigotes also enhanced under UV light, they used non-toxic concentrations of TiO2@Ag NPs on J774 macrophage cells (5, 10, 15 µg/mL) as antileishmanial agent. Their result represented that TiO2@Ag NPs decreased viability of promastigotes in the presence of visible light. In this study, it was also indicated that amastigote forms of *Leishmania* is more susceptible to NPs than promastigotes which confirms the results of our research.[7] In addition to use of different waves of electromagnetic spectrum, some studies used electrical current in combination with AgNPs. Dalimi et al evaluated the effects of half-wave rectified sine (HWRS) current and AgNPs on *L. major* promastigotes. They demonstrated survival rates of promastigotes by flow cytometery. Their result showed that simultaneous use of AgNPs with concentration of 160 µg/mL and 3 mA HWRS electrical current destroyed all of the promastigote. The results of the present study were similar to those of the above study.[5] The results of Dolat et al indicated that pores formation on the cell membranes as a result of electrical pulses caused higher entrance of AgNPs. Simultaneous use of AgNPs with concentration of 2 µg/mL and 700 V/cm with 100 ms duration of electroporation have higher toxicity on promastigotes, which was similar to the results of the present study.[15]

However, a decrease in the number of promastigotes compared to the control group under the radiation of bioresonance waves may have different causes, such as conversion of promastigotes to amastigotes, disruption of proliferative processes, as well as apoptosis. In this study, we observed the conversion of promastigotes into amastigotes-like in vitro for specimens under irradiation. Investigations showed that amastigote forms of parasites are more susceptible than promastigotes to AgNPs. So, if treatments are effective for promastigotes, may also have effects on amastigote so conversion by irradiation and creation susceptible form showing a synergic effect on promastigotes.[6] Studies in the field of electromagnetic waves showed that these waves affect different targets in cells (here the *Leishmania* protozoa). The ornithine decarboxylase enzyme is one of these targets that contributes to the production of polyamines as the primary enzyme by converting ornithine into putrescine. Disruption of this enzyme results in the inhibition of cell proliferation. Some research in the field suggested that very low frequency electromagnetic waves can affect this enzyme.[16,17] Studies have also shown that cells are exposed to increased intracellular calcium ions immediately after exposure to electromagnetic waves.[18] The results indicated that higher calcium levels can impair mitochondrial function and ultimately activate caspases to induce cell death.[19] Numerous studies on the effect of chitosan-based nano-scaffolds containing various extracts and substances on *Leishmania* parasites, microbes, cancer cells, bone defects, and limited mobility have also been performed.[20-24] Moreover, the use of the above nanomaterials containing AgNPs on these protozoa is recommended. The novelty of this study is to investigate the effect of bioresonance waves on *Leishmania* parasite compared to AgNps for the first time. One of the limitations of the present study is that this test was not administered on laboratory animals.

Conclusions

In brief, the effect of different concentrations of AgNPs and different irradiation times of bioresonance waves on the proliferation of *L. major* under in vitro conditions showed that simultaneous application of AgNPs and bioresonance waves was more effective than their single use. As observed, AgNPs at low doses can have significant effects on the promastigotes proliferation of *L. major*. On the other hand, bioresonance waves with a specific effect on the protozoa can also resolve the problem of toxicity to other cells.

Authors' Contributions

All authors had equal role in design, work, statistical analysis, and manuscript writing.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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