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Inhibition of Leishmania major growth by Ultraviolet radiation B with Silver nanoparticles in an animal model

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ABSTRACT

Introduction: Cutaneous Leishmaniasis (CL) is a parasitic disease produced by the different species of flagellated protozoan leishmania. Regardless of numerous studies, there are still serious challenges in CL treatment. This study has evaluated influence of a low dose ultraviolet B (UV_B) radiation with silver nanoparticles (AgNPs) on Leishmania major.

Materials & Methods: Leishmania major promastigots (MRHO/IR/75/ER) extracted from infected mice spleens and 2×10^6 promatigots in 50 µl of phosphate buffer solution were subcutaneously injected into the footpad of Balb/c mice. About two months later, when the ulcers were infected, the animals were divided into 4 groups consisting control with no treatment, receiving AgNPs, receiving UV_B, and receiving UV_B with AgNPs. 2 mg/kg of AgNPs was injected into the lesions and an UV_B phototherapy system was utilized in order to lesions irradiation at 5 minutes after NPs injection. The treatment protocol was repeated for 4 times with the 4 day intervals. The lesions were monitored by measuring the lesions diameters in three dimensions. Also, spleen parasite burden was assessed on the day of 40th after the first treatment. The data were analyzed by static instat, ELIDA and SPSS 16 softwares.

Results: The results showed the most inhibitory effect in the group of receiving AgNPs with UV_B. The simultaneous administration of AgNPs and UVB provided the significant differences in the relative area of the lesions in comparison with the control group. Also there were significant differences between the groups of receiving AgNPs alone and combinational treatment. The data of parasite burden showed the significant differences between the control and the other treatment groups.

Conclusion: UV_B in the presence of AgNPs provides an useful anti-leishmania effect, inhibits extension of the CL lesions and reduces the rate of disease's visceral progress.

Keywords: Phototherapy- nanosilver- Leishmania major- Ultraviolet B- Cuntaneous leishmaniasis- Parasite Burden

1. Introduction

Cutaneous leishmaniasis (CL) is a parasitic disease caused by the flagellate protozoans of Leishmania species transmitted via the bite of female sandflies from the genera Phlehotomus and Lutzomyia. According to the World Health Organization, more than 350 million people worldwide live in areas with the possibility of active transmission. It is estimated that approximately 14 million people in Africa, Asia, Europe and America are directly affected by the disease Igbineweka (2012). In most areas of the world, Meglumine antimoniate (Glucantime) and Sodium stibogluconate (Pentostam) are considered as the first choice drugs that are associated with severe complications such as cardiac, liver, kidney and blood disturbances when be administered intramuscularly Igbineweka (2012), Sazgarnia (2012). But according to reports in recent years, the effectiveness of these drugs has been significantly reduced Sazgarnia (2013).

Silver salts due to strong anti-bacterial and antiseptic properties are widely used as antimicrobial therapy Darroudi (2009), Kvítek (2011). In this regard, some researchers have suggested very small silver particles in the nanometer range to be used in the treatment of infections because of the increased level of germicidal power Kheybari (2010). Some studies have reported their anti-Leishmania effects Khosravi (2011). It is worth noting that due to the toxicity of silver nanoparticles for liver, brain and spleen as well as decreased ovarian follicles Ghorbanzadeh (2011), administeration in low doses is recommended.

Beneficial and harmful health effects of ultraviolet radiation are well known. Vitamin D synthesis is one of the beneficial effects. Harmful effects include: sunburn, premature aging of the skin and the increased risk of skin cancer, the latter is the most important UV rays health outcome. Proteomics studies have shown these results arising from long-term exposure to UV Pastila (2013).

UV-B radiation is currently used to treat skin diseases such as psoriasis and vitiligo Evers (2010), El-Zawahry (2012). The effects of UV-B radiation on the immune system of the animals have been studied via irradiation of animals before infection by different Leishmania species. But UV-B has not been used for the treatment of leishmaniasis so far Giannini (1986), Khaskhely (2002).

We studied the efficacy of combination therapy of UV-B and silver nanoparticles on an animal model of CL caused by Leishmania major according to the lesions improvement and spleen parasitic load.

2. Materials and Methods

Silver nanoparticles synthesis

We used AgNPs with a size 9.67 nm produced by the Department of Pharmaceutical Chemistry, Mashhad University of Medical Sciences. Silver nitrate solution at concentrations 0.01 to 0.001 M is used for the synthesis of silver nanoparticles. This solution is reduced in the container with two electrodes made of platinum. The particles size is dependent on electrical voltage and current of the system. After reduction of the transparent solution of silver nitrate and convert it into a brown colloidal systems, Particle Size Analyzer is used to determine the particles size.

Promastigotes preparation

First, the mice infected with leishmaniasis that developed visceral disease, were killed and splenectomied. Then completely crushed spleen were cultured in blood agar (NNN) and culture medium RPMI 1640 (GIBCO) containing 100 *µ*g/ml penicillin, 100 *µ*g/ml streptomycin, plus 20% fetal calf serum (FCS) purchased from GIBCO company in incubator at 28°C for two weeks. So that, amastigotes released from the spleen cells were converted into promastigotes. Then promastigotes were cultured in medium containing 100 *µ*g/ml penicillin, 100 *µ*g/ml streptomycin, plus 10% (FCS) in incubatot at 28°C. In order to parasite passage, suspension was precipitated in a refrigerated centrifuge for 10 minutes at 4500 rpm and the supernatant culture medium was removed. Thus, parasites achieved the fixed growth phase (stationary-phase) after 6 days of incubation, and they will be suitable for injection and induction of cutaneous leishmaniasis Mohebali (2009), Sazgarnia (2012).

Animal models

Mice 6-8 weeks were purchased from the Pasteur Institute, Karaj Branch. Mice were maintained at 22-25°C and in lighting conditions of 12 h darkness and 12 hours light. The third or fourth passage of promastigotes in the stationary phase of growth was used to infect animals. After proliferation and harvesting, parasites were washed with PBS, and the concentration was adjusted to a PBS so that, each 1 ml contains 40 × 106 Leishmania major. Fifty µL of solution containing live promastigotes (containing 2 × 106 parasite) was injected subcutaneously in the right hind paw of each mouse . After about 20 days the wound was established. After a random sampling of the some animals and confirmation of cutaneous leishmaniasis by a pathologist, animals were randomly divided into four groups and treated by determinate modalities.

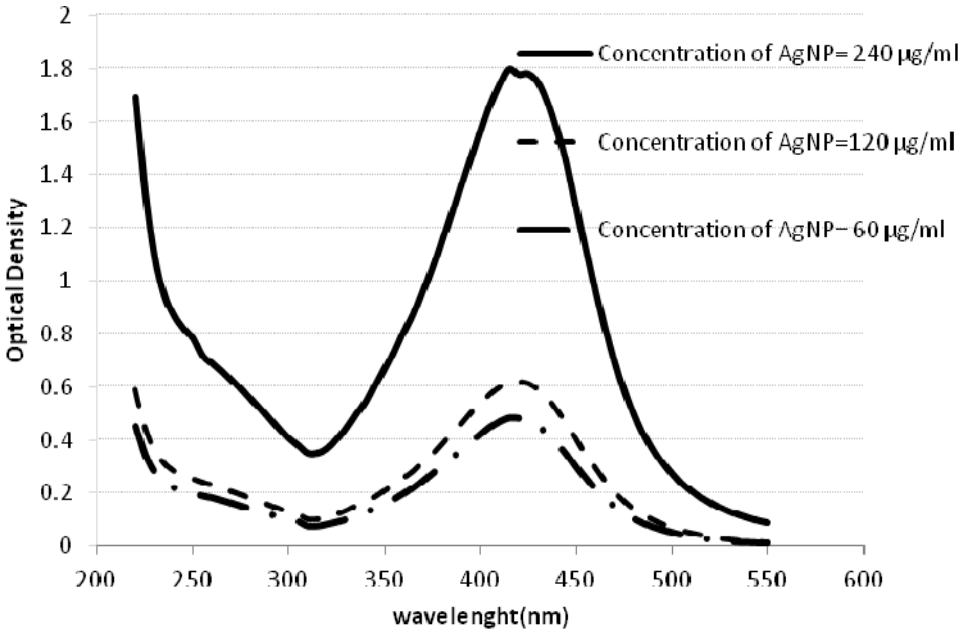

Fig. 1 Absorption spectra of silver nanoparticles at different concentrations

Characterization of silver nanoparticles

Absorption spectra of different concentrations of AgNPs in water at wavelengths ranging from 200 to 550 nm were obtained by UV-visible spectrophotometer Shimadzu model UV1700 (Fig. 1). The absorption peak of nanoparticles is recorded at 420 nm.

Nanoparticles size distribution was determined using a particle size analyzer (Malvern Instruments, Southborough, MA). The most abundance of particles was determined in 9.67 nm (Fig. 2).

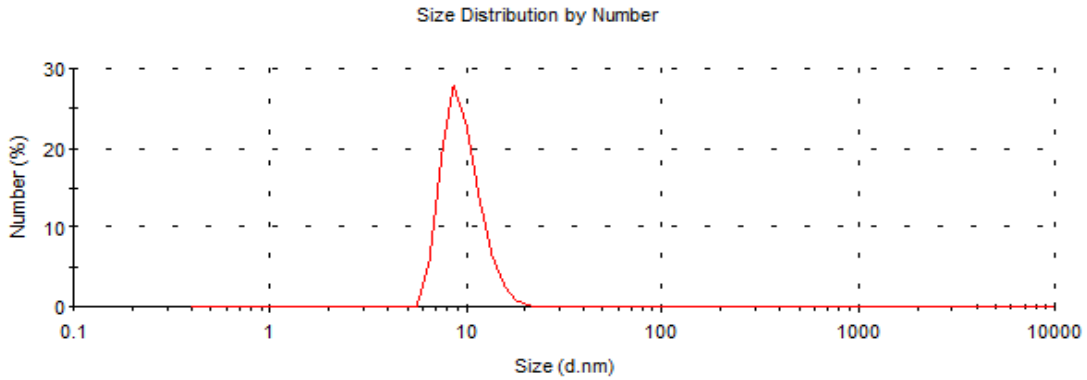

Fig. 2 Size distribution of silver nanoparticles

Phototherapy device

Wounds are irradiated by the UV lamps (PHILIPSULTRAVIOLET-B TL 20W/12RS) with the maximum output of wavelengths 310 and 437 nm manufactured by Philips of the Netherlands (Fig. 3). Lamps with length of 70 cm and intensity of 20 W were placed concentrically at interior walls of a cylindrical tube. To assess the wavelength range of the UV lamps, the spectrometer equipped with a Cooled CCD was utilized. Spectrometer counts the number of photons in terms of the wavelength. To transmit light to the spectrometer, optical fiber with a diameter of 25 mm was used.

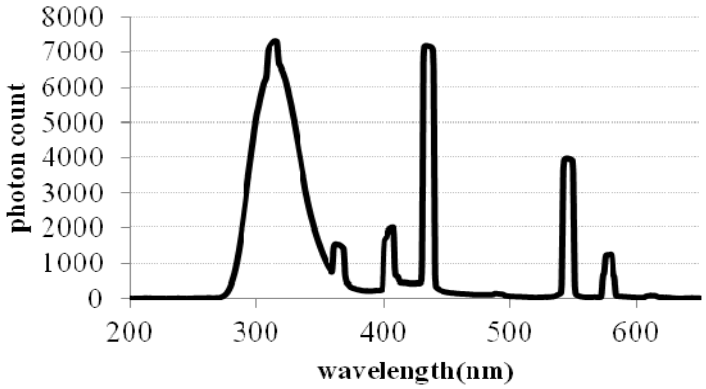

Fig. 3 Output spectrum of a lamp (PHILIPS ULTRAVIOLET-B TL 20W/12RS)

Experiments Conditions

The mice with cutaneous leishmaniasis were injected by nanoparticles at a dose 2mg / kg under anesthesia Xue (2012). Treatment of animals randomly divided into four groups, each consisting of 10 animals was carried out as follows: Group 1: Control; group 2: evaluating the toxicity of silver nanoparticles; Group 3: evaluating the effect of phototherapy alone, Group 4: evaluating the effect of silver nanoparticles combined with UV.

In order to assess the effect of treatment, surface of the lesion was measured using a digital caliper twice a week during and 20 days after the treatment. For treatment of dermatologic conditions, the suberythemogenic dose i.e., 70 percent of minimal erythema dose (MED) is administered to the patient in first session. However, for practical reasons, phototesting is often not done Honigsmann (2012). According to dermatologists' experiences, irradiation was started by 0.03 J/cm^2 UV-B Zanolli (2000). Because of severe erythema, second irradiation was decreased to 0.02 J/cm2. Irradiation was done by 0.05 J/cm^2 in third and forth sessions.

The lamps were placed on inner wall of a cylinder. With radiometric probe placement on the central axis of the cylinder, Intensity of radiation was measured in terms of W/m^2 in the area where the wound was supposed to be the irradiation. Intensity of UV radiation emitted by the lamp m was 0.135 W/cm^2. Treatment time was calculated according to the following formula: time (sec) = (mJ/cm^2)/(mW/cm^2). Time to deliver the desired dose was 188 seconds for 30 mJ/cm^2; 166 seconds for 20 mJ/cm^2; and 377 seconds for 50 mJ/cm^2.

Parameters of comparing treatment

The effects of different treatment were determined on the basis of both changes in the lesion and spleen parasite burden. The size of lesions was recorded by a digital caliper 2 times a week and normalized to change the day before treatment. In order to determine the number of viable parasites in the spleen, animals were killed on day 40 after the first treatment and spleen tissue was removed. Spleen parasite burden was determined in the following manner Zanolli (2000). The two-phase system consisting of a liquid phase containing RPMI-1640, 10% FCS, 100 μg/ml penicillin and 100 μg/ml streptomycin on a solid substrate was prepared from blood agar. Solid agar medium containing 10% sterile defibrinated rabbit blood was prepared and under sterile conditions, complete RPMI-1640 medium (180 ml) was dispensed into each well of 96-well plate. To determine the number of parasites, the spleens of mice were removed after their spinal cord cutting under sterile conditions and placed into 6 well plates containing complete RPMI-1640 medium. After the homogenization of spleen tissue by syringe piston, volume in wells was raised to 200 micro liters with complete RPMI-1640 medium. Seven dilution of a serial dilution of 1/10 from each spleen and four replicates from each mouse were prepared. Prepared plates were kept for 10 days at c ° 28. Positive (with parasite) and negative (without parasite) results were recorded at different dilutions.

3. Data Analysis

Data were analyzed by statistical software SPSS version 11.5. To compare the data with regard to their abnormal distribution, the Mann whitney and Kruskal wallis tests were used. The results of spleen parasite load were reported as number of parasites in the spleen using ELIDA software and comparison between groups was performed by Tukey-Kramer test using Instat software.

4. Results

Effects of UV-B radiation alone and in the presence of AgNPs on the right foot lesion in mice BALB/C were studied. At the end of the 16-day treatment period, the size of lesions increased twice and half as much compared to the day before the treatment, respectively, in the control group and receiving the silver nanoparticles. Ten days after treatment, the lesions size was not significantly increased in nanoparticles group, but in the control group, the lesions size increased nearly twice as much the day before the treatment. No increase in lesions size was observed in the group receiving UV-B radiation in the presence of the AgNPs at the end of 16-day treatment, continued until 10 days after treatment. In the group receiving the UV-B alone, no increase in lesions size was observed until the end of treatment, whereas 10 days after treatment, it increased as 1 times compared the first day before treatment. It is worth noting that in all treatment and control groups, the lesions showed no increase during 5 days after treatment.

Fig. 4 shows that simultaneous presence of AgNPs and UV-B rays provides significant difference in the lesion size compared to the control group ($p < 0.001$). Significant difference in the lesions size was observed in both group receiving AgNPs

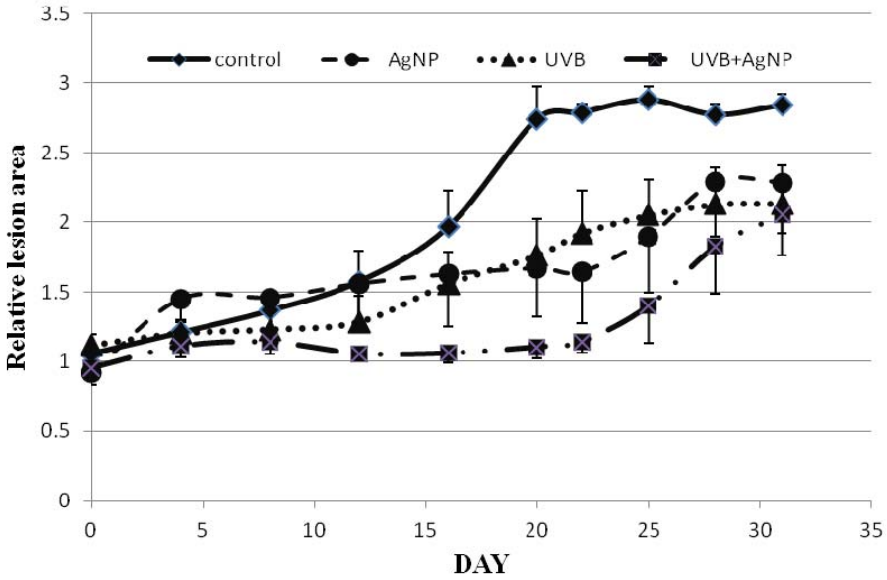

Fig. 4 Variations of the relative lesion area of mice treated in different groups during consecutive days (the data are presented as MEAN ± SEM, number of mice in each group = 10)

and group receiving UV-B radiation alone, compared to the control group ($p < 0.001$). No significant difference in the lesions size was observed in two treatment groups receiving AgNPs and UV-B rays. The group receiving combination of UV-B in the presence of AgNPs showed significant difference in lesion size compared to AgNPs group ($p < 0.029$). Moreover, the treatment groups receiving UV-B radiation alone and UV-B radiation combined with AgNPs revealed significant difference ($p < 0.003$).

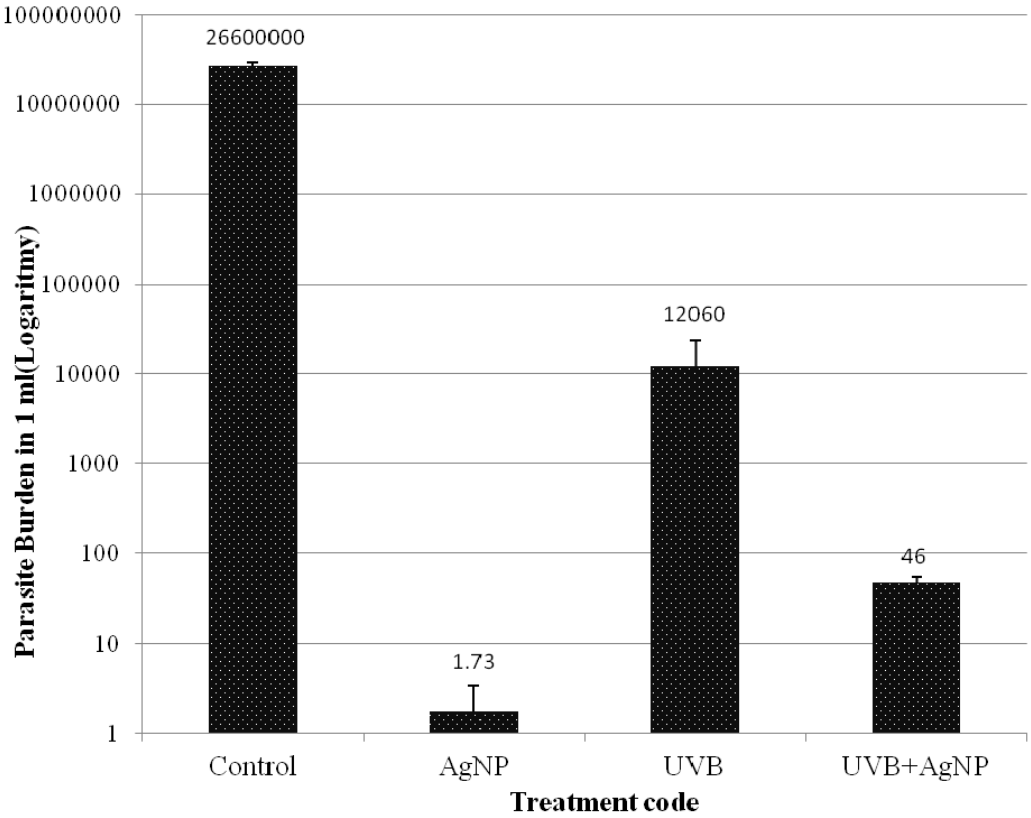

Fig. 5 Count of Leishmania major parasites in Balb/C mice's spleen at different groups 9 weeks after infection with Leishmania major; data represented as MEAN ± SD, number of mice in each group = 10)

There are some qualitative observations. As shown in Fig. 6, the lesions began scaling at the third and fourth sessions of treatment in the groups treated with UV-B radiation alone and UV-B radiation combined with silver nanoparticles, which could be a sign of healing. These observations were associated with pale pink discoloration caused by UV-B radiation, returned to the initial state after 48 hours of irradiation. While in control group, mice lost the leg at day 16 after treatment.

Discussion

The strong point of this study is application of UVB radiation in a dose lower than erythema dose limit Xue (2012), which is safer in human applications, and also lower

radiation period Damian (2001). The use of silver nanoparticles, besides providing for targeting and increased cellular uptake, will not only reduce the required dose of AgNPs but the side effects of silver toxicity. In addition, the likelihood of implementing the topical treatment should not be ignored.

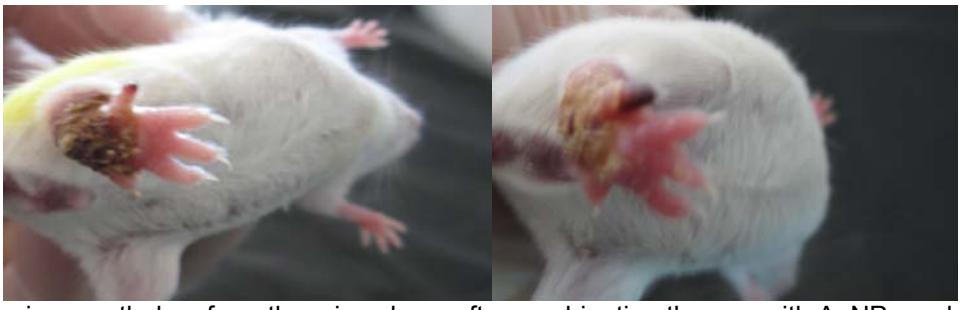

Fig. 6 Two images thaken from the mice ulcers after combination therapy with AgNPs and UV_B rays

An advantage of simultaneous application of AgNPs and UVB radiation to treat cutaneous lesions is that AgNPs can enforce antiparasitic effects (through ROS), and the effect of ultraviolet radiation on AgNPs gradually releases Ag+ ions, which give rise to silver-cysteine complexes by affecting cysteine groups in the structure of enzymes and proteins. Application of ultraviolet photons on these complexes generates monosulfide radicals, resulting in eventual death of the organism Khaskhely (2002).

The advantage of silver nanoparticle over colloidal solution of silver is that the latter has plenty of Ag^+ ions, being diluted in wounded site by blood and uselessly distributed in all the organs. In contrast, AgNPs can remain in wound site for longer periods of time, gradually releasing Ag^+ ions and causing toxicity for the parasites. In the study of Darudi et al, it has been shown that the size of nanoparticles is decreased by applying ultraviolet radiation on AgNPs Darroudi (2011), enhancing the toxic effects. The production mechanism of silver ions during ultraviolet radiation is presented in the following Eqs. (1), (2), (3)

$$(Ag)_n + 2h\vartheta \rightarrow (Ag)_n^+ + e_{aq}^- \quad (1)$$

$$(Ag)_n^+ + e_{aq}^- \rightarrow (Ag)_n \quad (2)$$

$$(Ag)_n^+ \rightarrow (Ag)_{n-1} + Ag^+ \quad (3)$$

Application of ultraviolet photons on $(Ag)_n$ nanoparticle releases an aqueous electron (e_{aq}^-), and an ion is generated from $(Ag)_n^+$ silver nanoparticles. This ion may be combined with an aqueous electron (e_{aq}^-) and revert to its original status or my convert to an n-1 $(Ag)_{n-1}$ nanoparticle and produce an Ag^+ ion Darroudi (2009).

Several studies emphasize that ultraviolet radiation can suppress cellular immune responses, and the shift from T1 to T2 helper cells is attributed to response to radiation Damian (2001). In a study by N Khaskhely in 2002, low-dose UVB was applied to mice before infection by L.amazonensis. In this study, the theory that UVB radiation

invariably causes suppression of the immune system was rejected, and low dose UVB was reported to be useful for L.amazonenis infection Khaskhely (2002).

In the study of Allahverdiyev *et al*. (2011), the anti Leishmania effect of nanosilver in the presence of UVB was evaluated on promastigotes and amastigotes of L.tropica. The results indicated enhanced anti-leishmania effect by nanosilver, so that after UVB irradiation on L.tropica promastigotes and amastigotes, the proliferation and metabolic activity of the parasite was inhibited 1.5 to 3 times and 2 to 6.5 times, respectively Allahverdiyev (2011).

The findings of this research indicate that synergism of UVB radiation with a cumulative dose of 150 mJ/cm^2 and AgNPs with a concentration of 2 mg/kg (in each treatment round) has been capable of inhibiting the extension of the lesion and controlling disease visceralization in groups receiving AgNPs or UVB radiation and combined treatment. The following points should be mentioned with respect to possible mechanisms of function of AgNPs and UVB radiation:

AgNPs with small size and vast surface area capable of attachment to sulfur and phosphorus groups have increased anti-leishmania effects, providing a high capacity of ROS production, to which leishmania parasite seems to be vulnerable.

Once again the hypothesis of immune suppression by UV radiation is questioned, and UVB radiation at low doses is reported to be useful for Leishmania major infection.

Irradiation of UV photons in the presence of AgNPs has increased its toxic effects by releasing silver ions.

On the other hand, a part of the output light of the lamp used in our study has been in visible region of the spectrum in addition to UVB. Therefore, we suggest comparison of separated treatment effects of narrow band UVB and blue light on cutaneous Leishmania lesions in animal models in the presence of AgNPs to distinguish the effect of any of the regions.

Conclusions

The presence of AgNPs in low concentration (which is not toxic for liver and spleen) simultaneous with UV_B irradiation in low cumulative dose is effective to treat the L.major lesions, and can prevent the visceral course of this disease and reduce the parasite load in spleen.

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