Tropical Biomedicine 35(1): 50–58 (2018)

The killing *in vitro* effect of Half-Wave Rectified Sine electricity plus silver nanoparticle on *Leishmania major* promastigotes and BALB/C mice skin leishmanial lesion healing

Dalimi, A.[1*], Karimi, M.[1], Jameie, F.[1], Ghafarifar, F.[1] and Dalimi, Ab.[1]
[1]Department of Parasitology and Entomology, Medical Sciences Faculty, Tarbiat Modares University, P. Box: 14115-331, Tehran, I.R. Iran
[*]Corresponding author e-mail: dalimi_a@modares.ac.ir
Received 28 June 2017; received in revised form 9 October 2017; accepted 14 October 2017

Abstract. In this experimental study, first the killing effect of silver nanoparticles alone or in combination with 3mA of Half-Wave Rectified Sine current was assessed in promastigote culture for 10 minutes. The survival rate of infected promastigotes was evaluated by Flow cytometery. In the second step, BALB/c mice were infected experimentally with *L. major*, followed by silver nanoparticles injected inter-lesion and simultaneously 3 mA a Half-Wave Rectified Sine current induction was applied directly into the wound. Finally, the lesion size and the mice body weight changes were measured during 5 weeks. Results indicated that simultaneous use of nanoparticle and electricity increased the mortality of promastigotes significantly. However, when 3 mA of HWRS and 160 µm/ml nanosilver were used alone in medium culture only 73.4% and 32% of promastigotes were killed respectively but the combined use destroys the promastigotes completely. The diameter of the lesions after six weeks in the control group; group treated with meglumine antimoniate and the group treated with HWRS increased to 6.01, 0.02 and 0.52 mm but in group treated with HWRS plus Nanosilver was reduced to -0.14 mm. The results showed that, when silver nanoparticles with HWRS current electricity were used in mice, the skin lesions were reduced in size but like Glucantime, complete healing was not achieved.

INTRODUCTION

Leishmaniasis is a predominantly rural disease that is prevalent in 98 countries from 4 continents. The disease is reported to cause 1.6 million new cases annually (WHO 2008, 2010), of which 1.1 million cases are due to cutaneous leishmaniasis (90.0% of them occurring in Afghanistan, Algeria, Brazil, the Islamic Republic of Iran, Peru, Saudi Arabia, Sudan and the Syrian Arab Republic). Of the 1.6 million estimated cases, only about 600 000 are actually reported (WHO 2008). Since 1993, the distribution of leishmaniasis has expanded, and there has been a sharp increase in the number of cases recorded (Desjeux 2001). This disease is the second most common insect-borne disease in almost all parts of the country.

Effective treatment is one of the most crucial ways that can greatly reduce the pain, psychological, and social side effects of the disease (Reithinger *et al.*, 2007). Various drugs, including chemical and herbal compounds have been used to treat leishmanial skin lesions. Several cases of the disease have been reported, particularly in patients with immunodeficiency and also due to problems such as disease recurrence, drug resistance, drug side effects, and secondary bacterial infection. Sodium antimony gluconate and meglumine antimoniate are the most common used compounds in the treatment of leishmaniasis. Due to the

appearance of *Leishmania* resistance to antimony compounds (Brogden *et al.*, 1998), efforts are being made to find new effective medications or therapies that are still ongoing.

In addition to the chemical compounds, some other methods such as thermotherapy, cryotherapy (WHO 2010) or electrical current were applied for the management of *Leishmania* infections. There is considerable evidence that electrical current can inhibit the growth of microorganisms (Rowley *et al.*, 1974; Barranco *et al.*, 1974). On the other hand, electrical current has been used for wound healing for many years (Reger *et al.*, 1999; Alvarez *et al.*, 1983; Wolcott *et al.*, 1969; Gaulth and Gatens 1976; Carley and Wainapel 1985; Gentzkow *et al.*, 1991).

Rectification is defined as the process of conversion of alternating current (AC) to direct current (DC). In half wave rectification of a single-phase supply, either the positive or negative half of the AC wave is passed, while the other half is blocked. Because only one half of the input waveform reaches the output, mean voltage is low. Half-wave rectification requires a single diode in a single-phase supply, or three in a three-phase supply (http://en.wikipedia.org/wiki/Rectifier Lander and Cyril 1993).

Currently there are limited number of studies on the use of electrical current to treat leishmanial ulcers. Shaquie *et al.* (1998) and Hijazi *et al.* (2004) have used electricity to treat leishmanial skin lesions.

Furthermore, various nanoparticles have been evaluated against *Leishmania in vitro* and *in vivo* (Soflaei *et al.*, 2014; Beheshti *et al.*, 2013; Soflaei *et al.*, 2012; Elmi *et al.*, 2013; Jebali *et al.*, 2013; Delavari *et al.*, 2014; Torabi *et al.*, 2012; Andreadou *et al.*, 2014). And several researchers have used silver nanoparticles to treat leishmanial ulcers (Baiocco *et al.*, 2011; Allahverdiyev *et al.*, 2011; Mohebali *et al.*, 2009).

The main objective of the present study was to evaluate the efficacy of a new protocol for the treatment of cutaneous leishmaniasis. It consisted of using HWRS plus silver nanoparticle in the killing of *Leishmania major* promastigotes *in vitro* and in healing the leishmanial ulcer.

MATERIALS AND METHODS

Parasite

The standard strain of *Leishmania major* (e.i., MRHO/IR/75/ER) prepared from Razi Vaccine and Sera Institute (Iran). The parasite cultured in NNN medium, then into RPMI1640 culture medium (Gibco Company). To prevent the growth of bacteria 100unit / ml penicillin and 100µg / ml streptomycin and fetal calf serum as a supplement (FBS) 15-12% was added to the medium. Then incubated at 24°C, transferred to a flask and was checked by invert microscope every day. This was done until the required number of parasites obtained. The Neubauer counting slide was used for counting the number of parasites.

Silver nanoparticle

The 3000ppm Nanosilver solution manufactured by the US Research Nanomaterials Company was used. The particle size was 20 nm and its purity was 99.99%. The concentrations of 20, 40, 80, 160 µg/ml were prepared from nanoparticles.

For *in vivo* assay, the nanoparticle was injected at a concentration of 250mg / kg into three points of the wound.

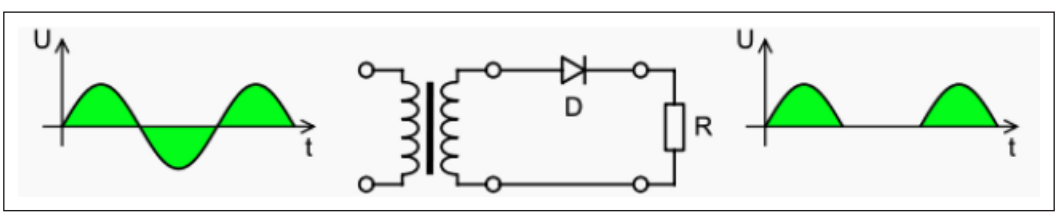

Figure 1. Half-wave rectifier (http://en.wikipedia.org/wiki/Rectifier Lander and Cyril 1993).

Assessment of apoptosis by flow cytometry

Promastigotes exposed to nanoparticle for 10 minutes into 1.5 ml microtubes were centrifuged at 4000 RPM for 5 minutes. Supernatant was discarded and the precipitate obtained, then according to the kit instructions 500µL of Binding buffer was added to the precipitation. Then 5µL Propidium Iodide and 5µL annexin were added to the samples and was incubated for 5 min at room temperature and darkness. Annexin-V and Propidium Iodide staining intensity absorbed by the cells were measured by BD FACSCanto II Flow cytometer. Finally, the results were analyzed using FlowJo software.

Device for use in medium culture

A device was designed with optimized voltage and current output to be used in medium culture. Since, RPMI 1640 medium containing water and salt so, two gold wires as electrode was used with the highest conductivity, the lowest level of decomposition and the least amount of heat. A multimeter device was used to measure electrical current and voltage into medium culture.

Mice

The 6-4 week old female BALB/c mice purchased from Razi Vaccine & Serum Research Institute (Iran). Animals were kept in the animal house of the University. The mice were divided into 4 groups of six mice per group; infected control group without treatment; infected control group treated with meglumine antimoniate; infected group treated with HWRS and infected groups treated with HWRS plus silver nanoparticle.

Infected mice

To infect mice, 0.1 ml of a solution containing 2×10^6 promastigotes in the stationary phase was injected subcutaneously at the base of the tail of the mice by insulin syringe. After 2 weeks of infusion parasites, small nodules at the injection site induration appeared. To ensure the presence of *Leishmania* in the wound, microscopy examination was used on the direct smear taken from the wound.

Inducing HWRS in the mice

The Variac Transformer device was used for induction different voltages of HWRS in the ulcer of the mice.

Course of treatment and measuring the diameter of the ulcer

HWRS induction of 1 mA was applied for 10 min on alternate days for 6 weeks. It should be noted that the mice were anesthetized with ketamine 10 minutes before applying electric current. To induce electrical current in the ulcer, two gold wire electrodes were used. To more accurately measuring the voltage a multi-meter device and to measure the diameter of the wound a digital caliper was applied.

Statistical Analysis

The mean variables in the different groups were analyzed using ANOVA. To detect significant differences between the two different test groups, T-test was used. It is noteworthy that the one sample Kolomogrov-Smirnov test was used to check the assumption of normality of variables. For data analysis, SPSS version 16 was used and the significance level was set at 0.05.

RESULTS

In vitro:

HWRS plus nanosilver

The viability percent of promastigotes under induction of different mA of HWRS as well as under induction of 10 min of 3 mAmp HWRS plus different concentrations (20, 40, 80, 160 µg/ml) of nanosilver is shown in Figures 2 and 3 respectively. The viability of promastigotes under induction of 1 and 3 mA was calculated 40.53% and 26.6% respectively.

According to Figure 3, the use of nanoparticle and electricity simultaneously has increased the lethality of promastigotes significantly. However, when 3 mAmp of current half-wave and 160 µm/ml nanosilver promastigotes were used alone in medium culture only 73.4% and 32% were found

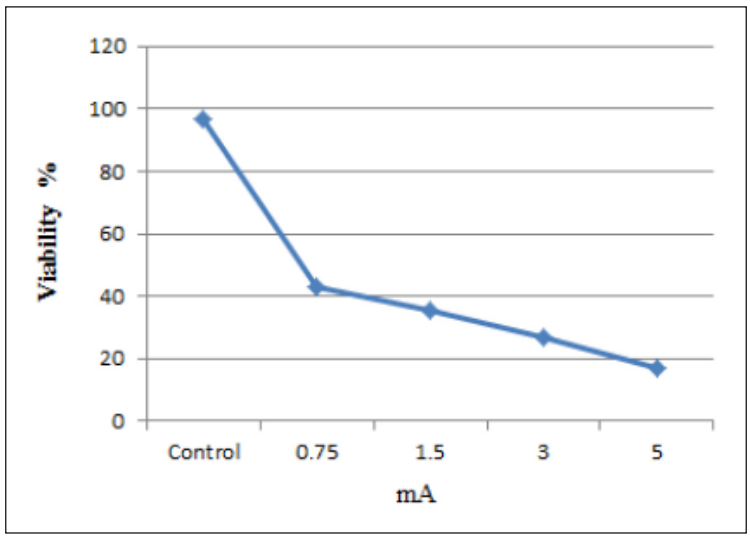

Figure 2. The viability percent of promastigotes of *L.major* under induction of different mA of HWRS.

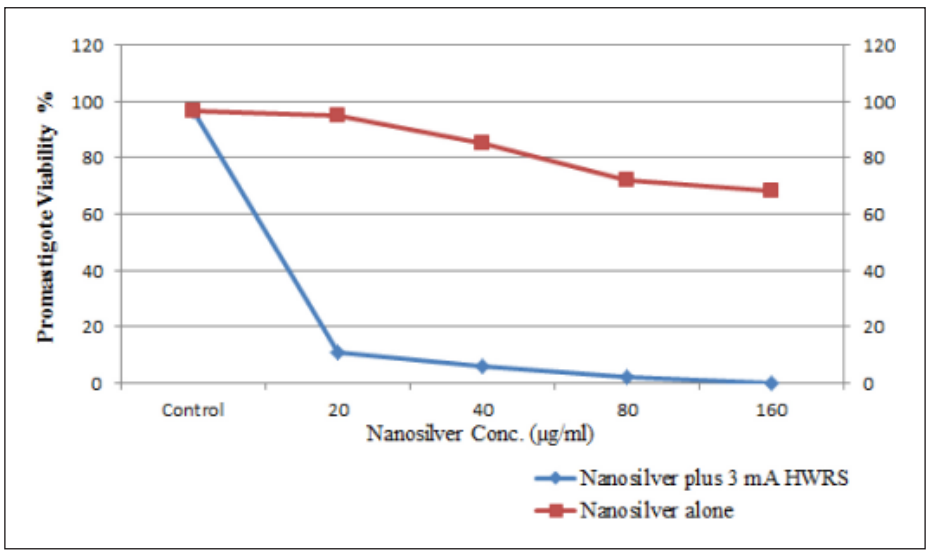

Figure 3. Viability percent of promastigotes of *L.major* under induction of 3 mAmp HWRS plus using different concentrations of nanosilver and nanosilver alone (µg/ml).

killed respectively but combine use of them destroyed the promastigotes completely.

Flow cytometry result

Apoptosis occurs in 0.96% of promastigotes of the control group 10 minutes after culture (Figure 4). In the group treated with 3mAmp HWRS 10 minutes after culture, apoptosis was seen in 87.40% of promastigotes (Figure 4).

In vivo:

Leishmanial ulcer diameters of the treated and non-treated mice

The mean diameter of the lesions in treated and non-treated mice during six weeks are shown in Table 1. The ulcer did not fully recover in the groups after six weeks. According to the result, the diameter of the lesions in the control group; group treated

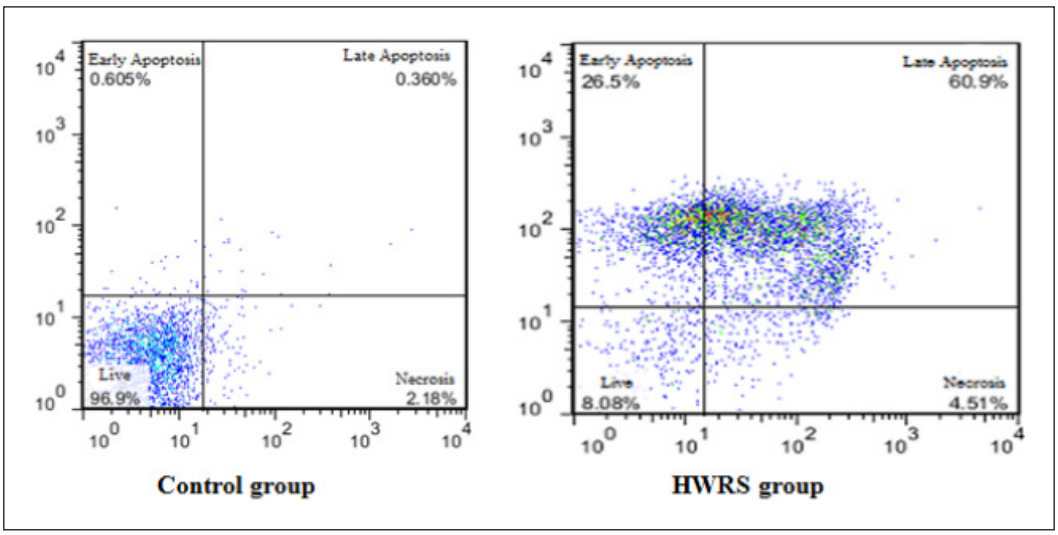

Figure 4. Flow cytometry result: Apoptosis in promastigotes of *L.major i*n the control group (left) and the group treated with 3mAmp HWRS (right).

Table 1. Mean leishmanial lesion diameters of treated and non-treated mice

Weeks	Lesion Diameters (mm) in			
	Control (Group 1) Mean±SE	Glucantime (Group 2) Mean±SE	HWRS (Group 3) Mean±SE	Nanosilver Plus HWRS (Group 4) Mean±SE
1	6.56±1.59	5.19±1.24	6.72±2.18	7.30±2.68
2	8.60±1.12	5.59±1.08	7.37±2.21	8.44±2.65
3	9.44±1.35	5.44±1.45	8.94±1.48	8.76±2.42
4	10.01±2.42	5.38±1.94	9.44±1.66	8.12±1.64
5	11.69±2.74	5.32±2.04	8.48±1.58	7.91±1.87
6	14.57±3.75	5.21±1.96	8.24±1.74	7.08±1.72
Sig. Diff. with Group*	2, 3, 4, 5	1, 3	1, 2	1

*Significant Difference P<0.05.

with meglumine antimoniate and in the group treated with HWRS were increased 8.01, 0.02 and 1.52 mm after six weeks but in group treated with HWRS plus Nanosilver was decreased about 0.22 mm. Between the group treated with HWRS plus Nanosilver and the group treated with meglumine antimoniate no significant difference was observed. (P<0.05). The image of skin lesions after 6 week treatment with Nanosilver plus HWRS is shown in Fig. 5.

Body weights of treated and non-treated mice

The body weights of treated and non-treated mice during six weeks are shown in Table 2. According to the result, the body weights after six weeks in the control group; group treated with meglumine antimoniate; in the group treated with HWRS1 and in the group treated with HWRS plus Nanosilver were increased 2.12, 3.78, 3.15 and 3.77 g. Between the group treated with HWRS plus Nanosilver

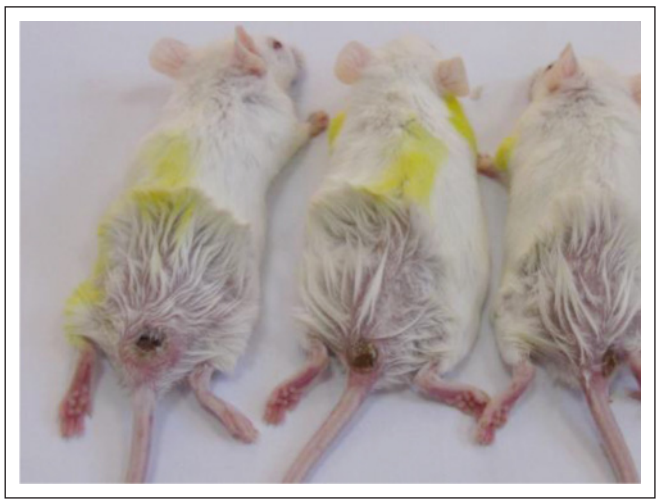

Figure 5. Image of skin lesions after 6 week treatment with Nanosilver Plus HWRS.

Table 2. Mean body weights of treated and non-treated mice infected with *L.major*

Weeks	Mice Body Weights (gr) in			
	Control (Group 1) Mean±SE	Glucantime (Group 2) Mean±SE	HWRS (Group 3) Mean±SE	Nanosilver Plus HWRS (Group 4) Mean±SE
1	23.74±1.06	24.85±2.69	25.26±0.85	22.39±4.13
2	24.42±1.24	24.89±2.45	26.11±1.21	23.85±3.74
3	25.05±1.40	25.81±2.51	26.47±0.13	23.91±3.81
4	25.54±1.80	26.19±3.09	26.92±0.66	24.01±3.25
5	25.79±1.60	28.12±3.23	27.36±1.20	25.74±3.54
6	25.86±2.20	28.63±3.67	28.41±2.19	26.16±5.01
Sig. Diff. with Group*	2, 3, 4	1	1	1

*Significant Difference P<0.05.

and the group treated with meglumine antimoniate no significant difference was observed. (P<0.05).

DISCUSSION

In general, the impact of electricity on the ulcer healing process can be attributed to the following reasons. Electrical stimulation of epidermal cell causes migration and recruitment of neutrophils and macrophage cells that can stimulate proliferation of fibroblasts and DNA synthesis. (Bourguignon & Bourguignon1987; Orida & Feldman 1982). The cell migration to the site of damage is done by the negative electrode. Usually the charge around the cells are positive. Electrical stimulation of the lateral cutaneous nerve activation by negative electrode can cause increased blood circulation in the region. What is striking is that if the intensity of stimulation is caused by excessive electrical activity it may cause intense muscle contraction (Baker *et al.*, 1997). So the low-intensity electrical stimulation should be applied to prevent pain caused by muscle contraction (Baker *et al.*, 1997).

The electricity effect on ulcer healing can be justified due to the following points: If a tissue is damaged in the body, lesion improvement will happen spontaneously under biological phenomena. In fact, a series of modifications and electrical mechanisms occurs during wound healing. The lesion initially becomes a positive charge after the injury, but four days after healing, it changes to the negative charge, and it remains negative till complete healing (Burr *et al.*, 1940)

So, the healing is associated with negative electric potential. Most cells are electrically active in that they generate a membrane potential. This electrical potential, which is negative inside the cell compared with the outside, is generated by active pumping of ions across the membrane (Brighton 1977; Burr *et al.*, 1940).

This study was conducted in two stages; in the first, promastigotes were cultured in RPMI1640 medium to reach their proper developmental stage, then they are exposed to the influence of electric currents plus different concentrations of silver nanoparticles concurrently. The combined use of a 3 mAmp of HWRS and 160 µm/ml of nanosilver destroys the promastigotes completely in medium culture. However, when the 3 mA of the current half-wave and 160 µm/ml nanosilver were used alone only 73.4% and 32.0% of promastigotes were found killed respectively in media culture.

Electricity currents were applied for a period of 6 weeks on alternate day for all groups. The results obtained in this phase of the study groups were significant. The electricity was applied for 150 minutes on average to each mouse for a period of 6 weeks. The wound almost completely recovered and the leishmanial ulcer was not observed in any of the mice after treatment.

In Hejazi *et al.* (2004) study's, direct electric current was found to be effective for the treatment of ulcers in BALB/c mice infected with *Leishmania major*. The voltage of direct current electricity was 3, with an average current of 0.065 mA and the average time of 30 minutes (range of 10-50 minutes) (Hejazi *et al.*, 2004). Full improvement of the mice was achieved with the improvement of 42.69% in the week and mean of 1.5 weeks. In addition to differences in the type of electricity and the use of silver nanoparticles used in this study was different from Hijazi *et al.* (2004). In Hijazi *et al.* (2004) study, stainless steel electrode was used and the initial ulcer size was very small, only about 2 mm (Hejazi *et al.*, 2004). However, in our study, gold electrode was applied and an average diameter of the ulcer was about 6 mm. These can be effective factors in achieving different results in the two studies.

CONCLUSION

The survival rate of promastigotes reaches zero after using 160 µm/ml of silver nanoparticles plus HWRS simultaneously. The results of study in animal model showing that silver nanoparticles plus HWRS can limit growth or reduce the diameter of leishmanial ulcer in treated mice.

Acknowledgement. The present work is part of MSc thesis, funded by Tarbiat Modares University. All authors contributed equally in this research and they declare that they have no conflict of interest. The authors would like to thank the personnel and staff of the Parasitology Department of Medical Sciences, Faculty of Tarbiat Modares University for their kind help during this research.

REFERENCES

Allahverdiyev, A.M., Sefik, Abamor, E., Bagirova, M., Ustundag, C.B., Kaya, C., Kaya, F. & Rafailovich, M. (2011). Antileishmanial effect of silver nanoparticles and their enhanced antiparasitic activity under ultraviolet light. *International Journal of Nanomedicine* **6**: 2705-2714.

Alvarez, O.M., Mertz, P.M., Smerbeck, R.V. & Eaglstein, W.H. (1983). The healing of superficial skin wounds is stimulated by external electrical current. *Journal of Investigative Dermatology* **81**: 144-148.

Andreadou, M., Liandris, E., Gazouli, M., Taka, S., Antoniou, M., Theodoropoulos, G., Tachtsidis, I., Goutas, N., Vlachodimitropoulos, D., Kasampalidis, I. & Ikonomopoulos, J. (2014). A novel non-amplification assay for the detection of Leishmania spp. In clinical samples using gold nanoparticles. *Journal of Microbiological Methods* **96**: 56-61.

Baiocco, P., Ilari, A., Ceci, P., Orsini, S., Gramiccia, M., Di Muccio, T. & Colotti, G. (2011). Inhibitory effect of silver nanoparticles on trypanothione reductase activity and *Leishmania infantum* proliferation. *ACS Medicinal Chemistry Letters* **2**: 230-233.

Baker, L.L., Chambers, R., De Muth, S.K. & Villar, F. (1997). Effects of electrical stimulation on wound healing in patients diabetic ulcers. *Diabetes Care* **20**(3): 405-425.

Barranco, S.D., Spadro, J.A., Berfer, T.J. & Becker, R.O. (1974). *In vitro* effect of weak direct current on staphylococcus aureus. *Clinical Orthopaedics and Related Research* **2**: 13-16.

Beheshti, N., Soflaei, S., Shakibaie, M., Yazdi, M.H., Ghaffarifar, F., Dalimi, A. & Shahverdi, A.R. (2013). Efficacy of biogenic selenium nanoparticles against *Leishmania major in vitro* and *in vivo* studies. *Journal of Trace Elements in Medicine and Biology* **27**: 203-207.

Bourguignon, G.J. & Bourguignon, L.Y. (1987). Electric stimulation of protein and DNA synthesis in human fibroblasts. *The FASEB Journal* **1**(5): 398-402.

Brighton, C.T. (1977). Bioelectric effects on bone and cartilage. *Clinical Orthopaedics and Related Research* **124**: 204.

Brogden, R.N., Goa, K.L. & Coukell, A.J. (1998). Amphoterion B Colloidal dispersion. *Drugs* **56**(3): 365-83.

Burr, H.S., Taffel, M. & Harvey, S.C. (1940). An electrometric study of the healing wound in man. *The Yale Journal of Biology and Medicine* **12**(5): 483.

Carley, P.J. & Wainapel, S.F. (1985). Electrotherapy for acceleration of wound healing: Low intensity direct current. *Archives of Physical Medicine and Rehabilitation* **66**: 443-446.

Delavari, M., Dalimi, A., Ghafarifar, F. & Sadraei, J. (2014). *In vitro* study on cytotoxic effects of ZnO nanoparticles on promastigote and amastigote forms of *Leishmania major* (MRHO/IR/75/ER). *Iranian Journal of Parasitology* **9**(1): 6-13.

Desjeux, P. (2001). Worldwide increasing risk factors for leishmaniasis. *Medical Microbiology and Immunology* **190** (1-2): 77-79.

Elmi, T., Gholami, Sh., Fakhar, M. & Azizi, F. (2013). A review on the use of nanoparticles in the treatment of parasitic infections. *Journal of Mazandaran University Medical Sciences* **23**(102): 127-134.

Gaulth, W.R. & Gatens, P.F. (1976). Use of low intensity direct current in management of ischemic skin ulcers. *Physical Therapy* **70**: 37-40.

Gentzkow, G.D., Pollack, S.V., Kloth, L.C. & Stubbs, H.A. (1991). Improved healing of pressure ulcers using dermapulse, a new electrical stimulation device. *Wounds* **3**(5): 158-170.

Hejazi, H., Eslami, G. & Dalimi, A. (2004). The parasiticidal effect of electricity on *Leishmania major*, both *in vitro* and *in vivo*. *Annals of Tropical Medicine and Parasitology* **98**(1): 37-42. http://en.wikipedia.org/wiki/Rectifier

Jebali, A. & Kazemi, B. (2013). Nano-based antileishmanial agents: A toxicological study on nanoparticles for future treatment of cutaneous leishmaniasis. *Toxicology in vitro* **27**: 1896-1904.

Lander, Cyril W. (1993). "2. Rectifying Circuits". Power electronics (3rd ed.). London: McGraw-Hill. ISBN 97800770 77143.

Mohebali, M., Rezayat, M.M., Gilani, K., Sarkar, S., Akhoundi, B., Esmaeili, J., Satvat, T., Elikaee, S., Charehdar, S. & Hooshyar, H. (2009). Nanosilver in the treatment of localized cutaneous leishmaniasis caused by *Leishmania major* (MRHO/IR/75/ER): an *in vitro* and *in vivo* study. *Daru* **17**(4): 285-289.

Orida, N. & Feldman, J.D. (1982). Directional protrusive pseudopodial activity and motility in macrophages induced by extracellular electric fields. *Cell Motility* **2**(3): 243-255.

Reger, S.I., Hyodo, A., Negami, S., Kambic, H.E. & Sahgal, V. (1999). Experimental wound healing with electrical stimulation. *Artificial Organs* **23**(5): 460-462.

Reithinger, R., Dujardin, J.C., Louzir, H., Pirmez, C., Alexander, B. & Brooker, S. (2007). Cutaneous leishmaniasis. *The Lancet Infectious Diseases* **7**(9): 581-596.

Rowley, B.A., McKenna, J.M., Chase, G.R. & Wolcott, L.E. (1974). The influence of electrical current and infecting microorganism in wounds. *Annals of the New York Academy of Sciences* **238**: 543-552.

Sharquie, K.E., Al-Hamamy, H. & El-Yassin, D. (1998). Treatment of cutaneous leishmaniasis by direct current electrotherapy: The Baghdadin device. *Journal of Dermatology* **25**: 234-237.

Soflaei, S., Dalimi, A., Abdoli, A., Kamali, M., Nasiri, V., Shakibaie, M. & Tat, M. (2014). Anti-leishmanial activities of selenium nanoparticles and selenium dioxide on *Leishmania infantum*. *Comparative Clinical Pathology* **23**(1): 15-20.

Soflaei, S., Dalimi, A., Ghaffarifar, F., Shakibaie, M., Shahverdi, A.R. & Shafiepour, M. (2012). *In vitro* anti-parasitic and apoptotic effects of antimony sulfide nanoparticles on *Leishmania infantum*. *Journal of Parasitology Research*, doi:10.1155/2012/756568.

Torabi, N., Mohebali, M., Shahverdi, A.R., Rezayat, S.M., Edrissian, Gh., Esmaeili, J. & Charehdar, S. (2012). Nanogold for the treatment of zoonotic cutaneous leishmaniasis caused by *Leishmania major* (MRHO/IR/75/ER): An animal trial with methanol extract of Eucalyptus camaldulensis. *Journal of Pharmaceutical & Health Sciences* **1**(1): 13-16.

Wolcott, L.E., Wheeler, P.C., Hardwicke, H.M. & Rowley, B.A. (1969). Accelerated healing of skin ulcers by electrotherapy. *The Southern Medical Journal* **62**: 795-801.

World Health Organization (2008). The global burden of disease: 2004 update. Geneva.

World Health Organization (2010). First WHO report on neglected tropical diseases: working to overcome the global impact of neglected tropical diseases, Geneva, P: 91-97.

World Health Organization (2010). Control of Leishmaniasis. WHO Technical Report Series, 949: 1-187.