Nanosilver in the treatment of localized cutaneous leishmaniasis caused by *Leishmania major* (MRHO/IR/75/ER): an *in vitro* and *in vivo* study

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ABSTRACT

Background and the purpose of the study: This study was designed to evaluate the effectiveness of different concentrations of Nanosilver solution against Iranian strain of *Leishmania major* (MRHO/IR/75/ER) both *in vitro* and *in vivo* on BALB/c mice model for the first time.

Methods: this is an interventional study which was conducted on the infected macrophages by *L. major* amastigotes *in vitro*. In order to confirm the *in vitro* results, various concentration of Nanosilver solution were administered topically on skin lesions caused by *L.major* in 78 inbred BALB/c mice as test or interventional and 52 mice as control groups once or twice daily for 14 days.

Results and major conclusion: Results of this study showed that different concentration of Nanosilver reduced proliferation of *L. major* amastigotes compared with the control wells but the differences were not statistically significant. Also, different concentrations of Nanosilver did not decrease the lesion sizes and amastigote counts in the BALB/c mice significantly. Secondary infection was significantly decreased in Nanosilver- treated groups compared with control groups ($p<0.01$). In conclusion, Nanosilver seems to be effective for control of secondary infection of localized cutaneous leishmaniasis.

Keywords*:* Nanosilver, *Leishmania major,* BALB/c mice

INTRODUCTION

In the absence of a desirable vaccine, the use of pentavalent antimonials is still the first line treatment for all forms of leishmaniasis in Iran and other countries (1,2). Nevertheless, these drugs usually have 80-85% cure rate and clinical signs and relapses are frequent particularly, in anthroponotic cutaneous leishmaniasis (ACL) (3)**.**Costly and painful injections are the major drawbacks of treatment by antimonial compounds (4).Other alternatives such as amphotericin B, aminosidine, allopurinol and miltefosine have also shown variable results (5-7). During the last two decades, many attempts have been made to develop effective new compounds for treatment of cutaneous leishmaniasis (CL) that would be economical, applicable topically to lesions and could avoid development of resistance (8). For decreasing side effects of drugs in treatment of lesmonarsis , local application has been considered in recent years, but the results have not been satisfactory because their penetration have been variable (5-9). Before penicillin, colloidal silver was a treatment of choice for many illnesses and infections. There are many reports that show colloidal silver is effective on about 650 different micro-organisms (10, 11). Therefore, it seemed Nonosilver particles with highly small sizes, have a good penetration into the cutaneous lesions and a good efficacy on *Leishmania* spp. In order to investigate the effectiveness of Nanosilver solution topically, its in vivo efficacy on experimentally induced lesions in BALB/c mice and *In vitro* amastigote proliferation of Iranian strain of *L.major,* was investigated.

MATERIALS AND METHODS

Preparation of Nanosilver solutions

Nanosilver powder was purchased from Quantum Sphere (USA) (www.qsinano.com) with mean particle size of 50 nanometers (nm). Dilutions of 100, 500 and 1000 part per million (ppm) in double

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deionized water were prepared immediately before use. Iranian Nanosilver was prepared in faculty of Pharmacy, Tehran University of Medical Sciences by heating silver nitrate solution containing fructose as a reducing agent. The mean particle size of this sample was 40 nm as determined by dynamic light scattering on a Nano ZS (Nanoseries, Malvern Instruments). Glucantime® (Rorer Rhone-Poulenc Specia, Paris, France) was received from the Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences.

Parasite

Iranian reference strain *L.major* promastigotes (MRHO/IR/75/ER) were grown in RPMI-1640 supplemented with 10% inactivated fetal calf serum (FCS), 100 mg/ml streptomycin and 100 IU/ml penicillin G at 23-25°C (1). The promastigotes from stationary-growth phase of culture were used to infect both mouse peritoneal macrophages and also inbred BALB/c mice.

In vitro study

Mouse peritoneal macrophages

The macrophages of peritoneal fluid of male BALB/c mice were collected and re-suspended at concentration of 5×10^4/ml in RPMI 1640 supplemented with 15% FCS. Cells were plated in eight-chamber LabTek tissue-culture slides, and adherent macrophages were infected with late logarithmic promastigotes at a parasite to macrophage ratio of 5:1. After 2 hrs of incubation at 34 °C, extracellular parasites were removed by washing and treated with Nanosilver solutions at concentration of 1.6, 3.2, 6, 25, 12/5, 25, 50 and 100 ppm. No drug was added to the control wells. Each experiment was carried out in triplicates. The tissue-culture slides were incubated for 3 days, and then drugs were added to each well, and slides were incubated for 24, 48 and 72 hrs. The slides were fixed and stained with Giemsa. The proliferation of parasites was evaluated by counting of amastigotes inside macrophages on tissue-culture slides stained by Giemsa. The percentage of infected macrophages and the number of parasites per infected cell was evaluated by microscopic examination of at least 100 macrophages at the end of the experimental period.

In vivo study

Male BALB/c mice, 6-8 weeks old with a body weight of approximately 20 g, were used in this study. The animals were obtained from the Animal Breeding Stock Facility of Razi Institute of Iran, Hesarak, Karaj, Iran. The animals were inoculated subcutaneously with approximately 2×10^6 *L. major* promastigotes (MRHO/IR/75/ER) at the base of tail. Cutaneous leishmaniasis progression was monitored by parasitological examination of lesions before and 2 and 6 weeks after inoculation respectively. Experiments with animals were carried out according to the ethical protocol in the Declaration of Helsinki, and measures were taken to protect animals from pain or discomfort. The protocol was approved by Tehran University of Medical Sciences ethical review board.

Experiments and groups

Treatment was initiated after inoculation, when the infection was well preformed and local lesions were obvious. Before treatment the diameter of lesions was measured and the mice were randomly divided into 10 groups of at least 8 animals in each group. The animal used in this experiment including control 1 (silver nitrate, 1000 ppm twice daily for 14 days), control 2, (Glucantime®, 20 mg Sb^{5+}/kg twice daily for 14 days), control 3 (Iranian Nanosilver, 40 ppm) and control 4 (no treatment). Nanosilver solutions were used in 6 groups at doses of 100 ppm, 500 ppm and 1000 ppm once and twice daily for 14 days. Nanosilver and silver nitrate groups were administered topically on the local lesions for 14 days.

Effects of treatment with Nanosilver solutions were compared with Glucantime®, silver nitrate, Iranian Nanosilver and non-treated control groups by measurement of the size of the skin lesions in millimeters (before treatment, two and 6 weeks after beginning of the treatment) and observation of amastigote forms before and at the end of experimental periods by light microscopy with high magnification(X1000) on smear prepared from lesion.

Statistical analyses

Statistical significance between groups was analyzed by Student's t test and two-way analysis of variance (ANOVA) using SPSS version 13.5. Values of $P < 0.05$ were considered statistically significant.

RESULTS

In vitro studies

In vitro efficacy of Nanosilver solutions on *L. major* amastigotes were determined after 24, 48 , and 72 hrs exposure. The data represent the means ± standard deviations (SD) of three independent experiments. The mean of the proliferation of *L.major* amastigotes decreased in both Nanosilver and Glucantime® groups (Figure1) but the difference in Nanosilver groups in contrast to Glucantime® group, was not statistically significant ($P = 0.135$). More than 85% of *L.major* amastigotes-infected macrophages were damaged after 24 hrs when treated with ≥ 100 ppm of Nanosilver which was cytotoxic for both parasite and macrophage.

In vivo studies

Effect on size of lesions

In all six Nanosilver treated groups compared

Table 1. Therapeutic effects of different concentrations of Nanosilver solution on the lesion sizes (mm) of localized cutaneous leishmaniasis induced by *L.major* in BALB/c mice compared to control groups.

Group	Concentration (ppm)	No of daily administration	Lesion size (mm) Before intervention	Lesion size (mm) after intervention		*P* value
				2- week	6 -week	
			X* ±2SD**	X ±2SD	X ±2SD	
Nanosilver	**100**	**once**	**7.18 ± 2.20**	**11.99± 4.38**	**14.90± 5.15**	***p*> 0.05**
Nanosilver	100	**twice**	**6.8 ±2.28**	**11.3± 4.99**	**11.9± 7.38**	p> 0.05
Nanosilver	500	**once**	**5.7± 2.07**	**14.0± 5.5**	**16.77± 6.77**	p> 0.05
Nanosilver	500	**twice**	**5.9 ±1.43**	**11.30± 6.33**	**10.6 ±6.14**	p> 0.05
Nanosilver	1000	**once**	**6.7± 5.92**	**11.20± 4.72**	**11.7± 5.04**	p> 0.05
Nanosilver	1000	**twice**	**6.1 ±2.56**	**13.0± 5.58**	**15.92± 5.62**	p> 0.05
Control(1) (Silver -nitrate)	**1000**	**twice**	**6.6± 3.40**	**11.8± 4.71**	**12.0± 5.18**	p> 0.05
Control(2) (Glucantim^{R})	**60 mg/kg**	**twice**	**6.7± 3.03**	**13.1± 3.47**	**15.3± 6.26**	p> 0.05
Control(3) (Iranian Nanosilver)	**40 ppm**	**twice**	**7.6± 1.76**	**6.7 ±2.12**	**6.8± 2.1**	p> 0.05
Control(4) (Not treated)	0	0	**7.4 ±3.07**	**10.7± 5.50**	**12.0± 6.39**	p> 0.05

with control groups, the mean lesion sizes did not decrease significantly after treatment (Table 1). Amastigote counts (mean ± SD) of the skin lesions decreased in all Nanosilver and control groups except for Nanosilver 1 (100 ppm, once daily), control 1 (silver-nitrate) and control 4 (no treatment) groups, respectively (Table 2). No statistically significant difference were found between interventional and control groups before ($P = 0.241$) and 6 weeks after treatment ($P = 0.131$). Secondary infections in Nanosilver-treated groups compared to all control groups decreased significantly at the end of treatment course ($P = 0.001$) (Table 3).

DISCUSSION

Nanosilver may be synthesized in any particle size ranging from 10-100 nm (10). In this study; different concentrations of Nanosilver solution of the 10-100 nm sizes on *L.major* by both in *vitro* and *in vivo* methods were studied.

Results of this investigation showed that despite of relative effectiveness of different concentrations of Nanosilver on proliferation of *L. major* in macrophage cultivation, the mean of mouse skin lesion sizes and amastigote counts in the lesions, did not decrease significantly at the end of the treatment courses. Interestingly, secondary infections in Nanosilver-treated groups compared to all control groups decreased significantly at the end of treatment courses.Based on the previous studies, silver compounds have many effects on the vast amount of microorganisms including bacteria, viruses, fungi, protozoa and even they have protective effects against colds and flu (10, 11).

In this study, a reduction of intracellular amastigotes of *L. major* was observed in Nanosilver and Glucantime® groups *in vitro*. In contrast in vivo experiments showed that amastigotes of *L. major* is susceptible to Nanosilver. From these results it may be assumed that the inbred BALB/c mouse is a highly susceptible animal model against *L.major* infection because, host immunity may modify clinical signs and could affect the size of lesions, the survival rates and the percentage of amastigotes in lesion smears.

A recent study that was carried out in Pasteur Institute of Iran, pathogenicity differences were compared in both susceptible BALB/c and resistant C57BL/6 mice infected with *L. major* (MRHO/IR/75/ER), (12). The results showed that proliferation of amastigotes inside macrophages, pathogenicity, even clinical signs in both susceptible and resistant hosts were completely different (12). Moreover, *in vitro* results showed that optimal dose of Glucantime® as the first line drug for the treatment of all clinical forms of leishmaniasis (1), did not have any effect on the lesion sizes in infected BALB/c mice. Therefore, it seems that variation in results depend completely to the mice strain.

Also, it was found the Iranian Nanosilver solutions reduced the size of lesions produced by experimentally induced infection with *L. major* in susceptible BALB/c mice compared to other groups but this difference was not statistically significant. Since, the study was performed by using only one concentration (40 ppm) of Iranian Nanosilver;

Table 2. Effects of different concentrations of Nanosilver solutions on the No.of amastigotes in localized cutaneous leishmaniasis induced by L.major on BALB/c mice compared to control groups.

Group	No of amastigotes		P value
	Before intervention	6 weeks after intervention	
Group 1	3.0 ± 3.03	9.8 ± 9.49	p > 0.05
Group 2	27.5 ± 58.25	10.40 ± 10.33	p > 0.05
Group 3	23.5 ± 49.39	20.13 ± 24.17	p > 0.05
Group 4	8.6 ± 20.93	5.8 ± 6.94	p > 0.05
Group 5	19.7 ± 23.97	8.1 ± 5.43	p > 0.05
Group 6	16.85 ±13.05	15.6 ± 20.77	p > 0.05
Contol 1	6.4 ± 7.6	8.4 ± 11.86	p > 0.05
Contol 2	29.4 ± 48.18	21.7± 35.45	p > 0.05
Contol 3	18.9 ± 41.25	17.8 ± 25.15	p > 0.05
Contol 4	7.4 ± 3.07	7.6 ± 4.50	p > 0.05

P > 0.05, comparing lesion sizes before and after treatment

Table 3. Secondary infections after treatment by Nanosilver groups Compared to the control groups.

Group	**No.of mice**	**Secondary infection**			
		Positive		**Negative**	
		No	%	**No**	%
Treated with Nanosilver	78	3	**3.85**	75	**96.15**
Control	52	15	**28.85**	37	**71.15**
Total	130	18	**13.84**	112	**86.16**

further investigation on larger groups of animals and different concentrations of the solutions are required before any conclusion about the efficacy of Iranian Nanosilver could be made.

CONCLUSION

These results encourage the use of Nanosilver in the treatment of secondary infection of cutaneous leishmaniasis in clinic. Further studies are required to clarify the role of Nanosilver concentrations in treatment of human cutaneous leishmaniasis.

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