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Original Article

Evaluation of Nanonanoliposomal Curcumin on Cutaneous Leishmaniasis Skin Lesions Caused by *Leishmania major* in BALB/c Mice

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Abstract

Background: Curcumin is an extract of rhizome turmeric (diferuloylmethane), with antioxidant, anti-inflammatory, antimicrobial, and anti-parasitic properties, which making it a potential candidate for the treatment of leishmaniasis. The aim of the presented study was to evaluate curcumin as possible candidate for treatment of cutaneous leishmaniasis.

Methods: We investigated the physicochemical properties and anti-leishmanial effects of nanoliposomal curcumin (40, 80, and 120 µM) in *Leishmania major* (MRHO/IR/75/ER) infected BALB/c mice at the faculty of Veterinary Medicinem University of Tehran, Iran. For this aim, *L. major* promastigotes (MHROM/IR/75/ER) at stationary phase (2×10^6) were inoculated sub-cutaneously into the upper area of the tail in BALB/c mice (six groups, n= 10 per group). For evaluation of nanoliposomal curcumin, the zeta potential, particle size and stability of nanoliposomal curcumin was determined. Furthermore, the anti-leishmanial effects of nanoliposomal curcumin formulation on the lesion sizes was determined and the parasite burden in the leishmania induced lesion was performed using semi quantitative PCR.

Results: Treatment of *L. major* infected BALB/c mice with nanoliposomal curcumin led to a reduction in the kinetic of the skin lesion size development. The semi quantitative PCR analysis of DNA extracted from the lesions showed reduction of parasite burden. The most effective treatment could be found in 80 µM nanoliposomal curcumin. Treatment with Glucantime, as a positive control, also showed a nearly similar effect compared to the effect of 80 µM nanoliposomal curcumin.

Conclusion: Nanoliposomal curcumin could be considered as a potential drug against cutaneous leishmaniasis caused by *L. major* in susceptible animal models.

Introduction

Leishmaniasis is a parasitic disease that is caused by parasites of the genus *Leishmania* sp., classified as one of the Neglected Tropical Diseases (NTDs) (1, 2). The disease has wide clinical spectrums from self-limiting cutaneous leishmaniasis (CL) as the most common form of leishmaniasis worldwide (3), to fatal visceral leishmaniasis (VL) making the disease a serious public health problem worldwide (3). Despite universal scientific community efforts to eradicate leishmaniasis, there was no available perfect vaccine or suitable drugs (4).

The pentavalent antimonial, meglumine antimonite, and Sodium stibogluconate (SSG) are the commonly used drugs of choice to treat CL in most endemic countries including Iran (5). The disadvantage of these drugs is their toxicity, high cost, severe side effects, and drug resistance (6). In the absence of an effective vaccine and the appearance of drug resistance, there is a necessary need for new and more effective drugs (7).

Curcumin is an extract of rhizome turmeric (diferuloylmethane), which had antioxidant, anti-inflammatory, anti-cancer, apoptotic, and anti-proliferative activity. It has a suppressive effect on reciprocal talk in the tumor microenvironment (8-12). Additionally, it modulates many enzymes like proteases and kinases, transcription and apoptotic factors, and cell surface receptors (13). Interestingly, curcumin had antiproliferative effect on promastigote and amastigote forms of *Leishmania major* in *in vitro* model which was analyzed by Giemsa staining, MTT, and semi-quantitative PCR methods (14, 15).

More of the antileishmanial properties of curcumin have been investigated in the promastigote rather than the intracellular amastigote stage (16). Curcumin has significant anti-leishmaniasis activity. Therefore, we aimed to investigate the efficacy of nanoliposomal curcumin on CL lesions caused by Iranian *L. major* amastigotes (MRHO/IR/75/ER) in BALB/c mice.

Materials and Methods

Ethical statement

This study was approved by the Ethical Committee of Faculty of Veterinary Medicine, University of Tehran under ethical code IR.UT.VETMED.REC.1402.004.

Parasite culture

The standard reference strain of *L. major* (MRHO/IR/75/ER) was obtained from the School of Public Health, Tehran University of Medical Sciences. For mass production, promastigotes were transferred to RPMI 1640 medium (Gibco, Life Technologies GmbH, Germany) with 10% heat-inactivated fetal bovine serum (Gibco, Germany), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco, German), and incubated at 23-25 °C.

Animals and housing

Sixty BALB/c mice (4–6 weeks old, weighing about 20–25 gr, procured from Faculty of Veterinary Medicine University of Tehran) were used for experiments. BALB/c mice were housed in six groups in standard laboratory cages [light/dark cycles, controlled temperatures (21 ± 1°C) and relative humidity (50 ± 5%)], and the mice had free access to food and fresh drinking water.

Preparation of nanoliposomal curcumin formulation and determination of its stability

Nanoliposomal curcumin was prepared in a solution containing lecithin, tween, and glycerin. Briefly, lecithin (2%) was dissolved in 4 ml tween, 0.72 ml glycerin in end volume of 40 ml. After mixing, curcumin from stock solution (5000 µM/DMSO) was added to the liposome achieving concentrations of 40, 80, and 120 µM and the solution was sonicated by 0.8 cycles, 1 min, amplitude 75% for 2 times. Subsequently, the curcumin-liposome was analyzed for size and zeta potential particles.

The stability of the nanoliposomal curcumin was examined by High-performance liquid chromatography (HPLC) (Knauer, Germany) equipped with four pumps, degassing chamber, and photodiode array detector, also an autosampler was used to measure the phoxim. Isolation was performed with a C_{18} column with dimensions of 25 cm in length and 4 mm in inner diameter and a particle size of 5 µm made by Waters (USA). EZ Chrom software was used to control the instrument. The mobile phase of 0.1 phosphoric acid in water and acetonitrile with a ratio (60:40) at a flow rate of 1.5 mL min^{-1} and a temperature of 25 °C (ambient temperature) was isocratically used and a detector wavelength of 420 nm was used for isolation and measurement. The curcumin was measured in the nanoliposomal curcumin at days 0, 10, 20, 30 and 40.

Analysis of size and zeta potential of particles

The prepared nanoliposomal curcumin was sent to Day Petronic Company (Zetasizer Nano-ZS) for measuring zeta potential and particle size (DLS). Zeta potential of the nanoparticles was measured three times in the device ZetaSizer Nano-ZS (Malvern Instruments Ltd., Red Lable, Worcestershire, United Kingdom). The size of the particles distributed was measured by Dynamic light scattering (DLS).

In vivo experiments

L. major promastigotes (MHROM/IR/75/ER) at stationary phase (2×10^6) were inoculated sub-cutaneously into the upper area of the tail in BALB/c mice (six groups, n= 10 per group). Following lesion development, mice were treated subcutaneously with a concentration of 40 µM (group 1), 80 µM (group 2), and 120 µM (group 3) of nanoliposomal curcumin once every two days. As positive control (group 4), the mice were treated with 20 mg/kg Glucantime®. As negative control (group 5), the mice were treated with liposome without curcumin. As additional negative control (group 6), the mice remain untreated. After 4 weeks, the size of cutaneous lesions was measured by colis in 2 diameters. The smears from skin lesion were prepared for microscopic examinations, parasitological assessment, and quantitative PCR.

Polymerase chain reaction

The genomic DNA of *Leishmania* and BALB/c mice was extracted using MBST kit (Tehran, Iran) according to the manufacturer's instructions. To evaluate the semi quantitative PCR, the DNA extracted from healthy skin of BALB/C mouse and from the *L. major* infected lesion was used. For this aim, the DNA extracted from *L. major* infected lesion was diluted (1:10, 1:100 and 1:1000) and tested. The amplification of the DNA extracted from the skin lesion was done using specific common primers, forward (5'AGAGGTGAAATTCTTGGACCG-3') and Reverse (5'-TTCCGTCAATTCCTTTAAGTTTCA-3') derived from 18S rRNA genes of *L. major* and mouse, that resulted simultaneously a PCR product with 255 bp in length for DNA originated from BALB/c mice and 360 bp in length for DNA originated from *L. major*. Amplification reactions were set for a total volume of 25 µl, containing 12.5 µl of Taq PCR Master Mix (Master Mix RED, Denmark), 1 µl of each forward and reverse primers (10 pmol), and 3 µl DNA templates. The final volume was made 25 µl by the addition of double-distilled water.

The PCR conditions consisted of initial denaturation of DNA strands at 95 C for 5 min followed by 35 cycles of denaturation at 94° C for 45 s, annealing at 53° C for 45 s, and extension at 72° C for 45 s. The PCR reaction was followed by a final 72° C for 10 min. PCR products were analyzed by 2% agarose gel electrophoresis using SYBR safe gel stain (Thermo Fisher Scientific, USA) or ethidium bromide.

Statistical analysis

The difference between diameter before and after treatment (after minus before) was analyzed using SPSS program version 22 (IBM Corp., Armonk, NY, USA). The chi-*squared* test was used for the determination of any sta-

tistically significant differences. The statistical significance was chosen at *P*-value<0.05.

After that, the intensity of PCR product deriving from the parasite was divided through the intensity of PCR product deriving from mouse genome was used as a marker for parasite burden in the lesion using SPSS program version 22. The one-way analysis of variance (ANOVA) was used for the determination of any statistically significant differences between the mean ratio resulted by abovementioned division. Tukey post hoc tests were also used for determination of the final difference between ratios on the last day of experiments.

Results

Zeta potential, particle size and stability of nanoliposomal curcumin

The Zeta potential of the prepared nanoliposomal curcumin was measured by a nano Zetasizer (Malvern Zetasize, UK), which showed a negative zeta potential (-3.7 mV) (Fig. 1A), and therefore the particles can be considered as approximately neutral. The Dynamic Light Scattering (DLS) analysis of nanoliposomal curcumin showed by mean intensity of 25.6% particle size between 5.6-13.544 nm, by mean intensity of 48.8% particle size between 24.36 – 78.82 nm and by mean intensity of 25.6% particle sizes between 122.42 – 295.4 nm with Z-average of 151.6062 (Fig. 1B). The stability of the curcumin in nanoliposomal condition was performed by HPLC. The results of HPLC showed that the amount of curcumin in the nanoliposomal solution was approximately constant and all three phenolic fractions di-feuloylmethane, desmetoxycurcumin and bi-desmetoxycurcumin were detectable and comparable over the analysis interval times. That means that the curcumin was stable in the nanoliposomal condition throughout the experimental times.

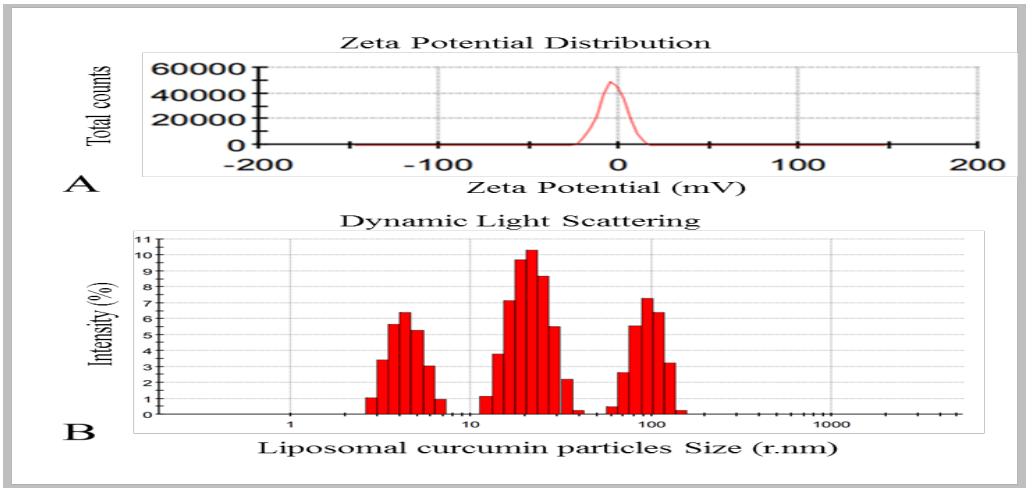

Fig. 1: The Zeta potential of the prepared liposomal curcumin was measured by a nano Zetasizer (Malvern Zetasize, UK) (**A**), the curcumin particle size was measured using the Dynamic Light Scattering (DLS) (**B**)

Anti-leishmanial effects of nanoliposomal curcumin formulation on the lesion sizes

In general, the sizes of lesions determined by subtraction of the diameter of the skin lesion after treatment from diameter of the skin lesion before treatment showed a reduction in kinetics of lesion development by mice treated with nanoliposomal curcumin (40, 80, and 120 µM) and with Glucantime compared to control groups (Table 1). The most effect dealing

with the shrinking in the kinetics of the lesion size development could be observed significantly in the mice of the group treated with the nanoliposomal curcumin concentration of 80 µM with P-value of 0.0226 (Table 1).

Table 1: The size of lesion was measured before and after treatment. a :The sum of differences between final size of lesions and primary size of lesions of mice in mm, b: The average of differences between final size of lesions and primary size of lesions of mice in mm.
c: Standard error of mean, d: *P*-value <0.05 is significant

Animal groups	*Drug used*	*Number of animals*	*Sum* [a]	*Mean* [b]	*Std.Error of Mean* [c]	*Sig.* [d]
1	Curcumin (40µM)	9	3.00	0.2727	0.46887	0.8782
2	Curcumin (80µM)	9	-7.00	-0.7778	0.43390	0.0226
3	Curcumin (120µM)	8	-3.00	-0.3000	0.33500	0.2403
4	Glucantime	8	-4.00	-0.4444	0.33793	0.2753
5	Without treating	8	23.00	3.2857	0.94401	0.0957
6	Liposome	9	11.00	2.2000	1.01980	0.1685

Determination of parasite burden using semi quantitative PCR analysis

The evaluation of the semi quantitative PCR was performed using DNA extracted from healthy skin of BALB/C mouse and from the *L. major* infected lesion in different dilutions. Fig. 2 showed that the intensity of the amplicons decreased for both amplified DNA fragments DNA concentration dependent. Two days after the last treatment, the DNA was extracted from skin lesions infected with *L. major* and amplified as above-mentioned. Fig. 3 showed exactly that in comparison to the skin lesion of untreated infected mouse, and mouse treated with basic nanoliposom, the parasite burden was significantly decreased in the mice treated with Glucantime, curcumin 40, 80 and 120 µM. For the quantitative analysis, the ratio of intensity of PCR products derived from 18s rRNA gene from genomic DNA of *L. major* to 18S rRNA gene from genomic DNA of mouse was calculated using GelQuant.Net program.

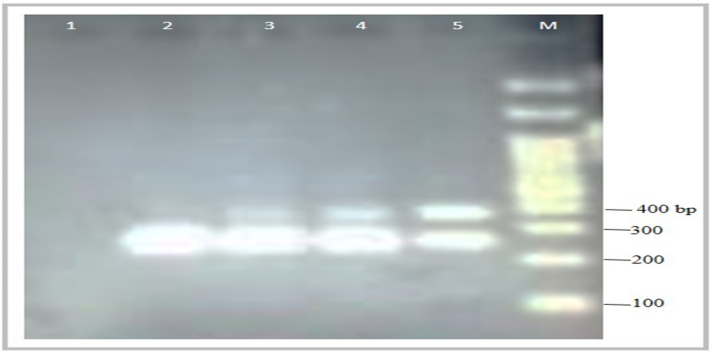

Fig. 2: DNA was extracted from mouse skin (lane 2), DNA from mouse skin plus 1:1000 diluted DNA extracted from strain of L. major (MRHO/IR/75/ER) (lane 3), DNA from mouse skin plus 1:100 diluted DNA extracted from strain of L. major (MRHO/IR/75/ER) (lane 4), DNA from mouse skin plus 1:10 diluted DNA extracted from strain of *L. major* (MRHO/IR/75/ER) (lane 5) were amplified using common primers derived from 18S rRNA gene. 1 is negative control and M is 100 bp DNA marker

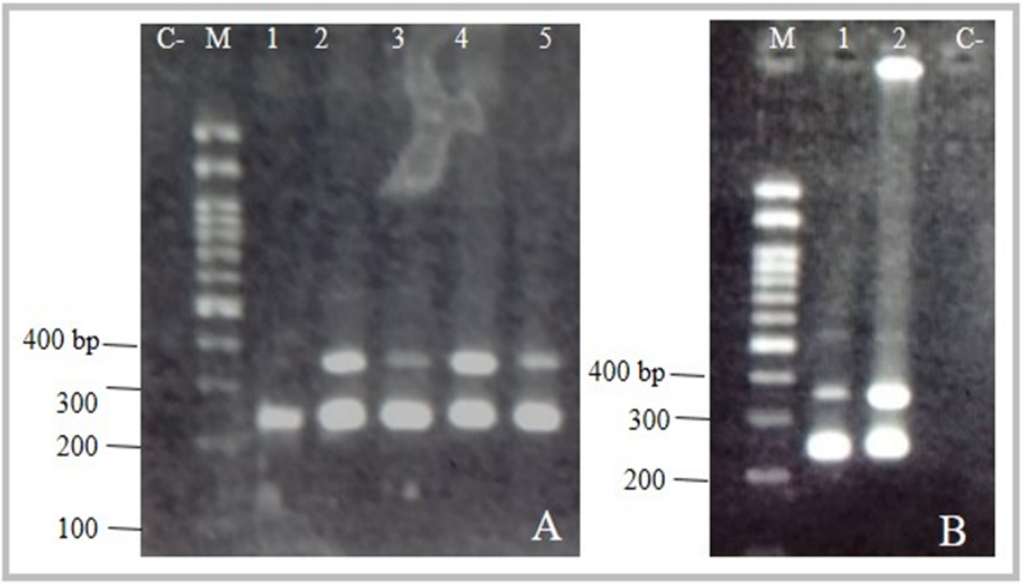

Fig.3: The DNA was extracted from samples prepared from lesion before and after euthanasia of mice and amplified by PCR resulting two PCR products with 255 bp for parasite and 360 for host. A: C-: is negative control, 1: treated with gluauntime, lane 2: treated with basic nanoliposom, lane 3: treated with curcumin nanoliposome (80 µM curcumin), 4: untreated infected mouse, 5: treated with curcumin nanoliposome (120 µM curcumin). B: lane 1: treated with curcumin nanoliposome (40 µM curcumin), Lane 2: untreated infected mouse, M is 100 bp DNA marker

As could be observed in Table 2, the *P*-value for curcumin (40 µM), curcumin (80 µM), curcumin (120 µM) and Glucantime were 0.001, 0.000, 0.004, and 0.001 respectively. The parasite burden was reduced by all treated animals compared to the animals in control groups. The most parasite burden reduction could be demonstrated in the mice of the group treated with 80 µM curcumin ($P<$ 0.001).

Table 2: The intensity of PCR product deriving from parasite was divided through the intensity of PCR product deriving from mouse genome (see fig. 3) a: The average of the intensity of PCR product deriving from parasite was divided through the intensity of PCR product deriving from mouse genome, b: standard error of mean, c: *P*-value <0.05 is significant

Animal groups		*Number of animals*	*Drug used*	*Mean* [a] *Leish/Mac*	*Std.Error of Mean* [b]	*Sig.* [c]
PCR	1	8	Curcumin (40µM)	0.41	0.12	0.001
	2	8	Curcumin (80µM)	0.29	0.05	0.000
	3	8	Curcumin (120µM)	0.49	0.08	0.004
	4	9	Glucantime	0.39	0.08	0.001
	5	8	Without treating	1.07	0.15	0.782
	6	8	Liposome	0.85	0.11	0.782

Discussion

It is known that in CL patients, cutaneous lesions will often heal without treatment (17). However, treatment can speed healing, reduce scarring, and decrease the risk of disease progression (18). The most used drug against leishmaniasis is the pentavalent antimony (SbV), Glucantime in Iran (19). Unfortunately,

drugs based on pentavalent antimony have many side effects. The very serious side effects associated with Glucantime are high toxicity, low efficacy, difficulty in administration, and parasitic drug resistance (20). The other drug against leishmaniasis is the phospholipid analogue miltefosine, which first was used against cancer and in 2002 was approved in India for application against *Leishmania* infection (21, 22). This drug is also accompanied by serious side effects. The most side effects of this drug are vomiting, fever, headaches, abdominal pain, low blood platelets and possible severe skin reaction that can be observed by Stevens-Johnson syndrome (23). Amphotericin B is also a candidate to be used against leishmaniasis and acts by binding to ergosterol precursors of parasite and disrupted it from the membrane (24). Unfortunately, this drug has also serious side effects. The side effects of amphotericin B can be summed up in fever, hypoxia, vomiting, nausea, rigors, hyper- or hypo-tension, and especially in renal toxicity (25).

Due to the side effects of the above-mentioned drugs, it is of great importance to find a drug against leishmaniasis with the minimum of side effects. It is known, that many people have employed herbal remedies to alleviate symptoms of leishmaniasis since the ancient age (26, 27).

One of these herbal remedies is curcumin. Curcumin, a major isolated polyphenol from the rhizome of turmeric (*Curcuma longa*), has a surprisingly wide range of beneficial properties. The antileishmanial activity of curcumin compounds via modulations in signaling pathways of inflammation is confirmed by increasing oxidative stress and inducing apoptosis (16). Since nanotechnology has proven to be an effective technique to accelerate wound healing (28) and the nanoliposomes as drug delivery systems, which increase the solubility of hydrophobic drugs in plasma and skin tissue as well as a decrease in the drug release rate (29), in the present study nanoliposomal curcumin against cutaneous leishmaniasis was used. Our results showed that the nanoliposomal curcumin was effective in reducing the kinetic of lesion development by *Leishmania*-infected BALB/c mice. The nanoliposomal curcumin showed suitable anti-leishmanial activity (30). Nanoliposom formulation was also used with the other drugs with positive results. For example, the antileishmanial effect of nanoliposomal miltefosine was stronger than miltefosine, which was speculated to be due to better penetration into the dermal infected macrophage (31). Interestingly, Akbari et al. also used a nanoliposomal formulation of curcumin in topical form against leishmaniasis in BALB/C mouse model. They showed no significant difference between treated and control BALB/c regarding lesion size; in contrast to our results. They reported additionally that splenic parasite burden was not significantly changed in tested animal groups (32).

We have yet to investigate the distribution of *Leishmania* in different mouse organs not only by nanoliposomal curcumin but also by Glucantime as a drug recommended by official global authorities used in the world. Ee tried to determine the parasite burden in the skin lesion using the semi quantitative PCR method described earlier (14). They described a simple PCR method that has been used for the quantitative analysis of parasites in the host cells (14). In the present study, the mentioned quantitative PCR method showed a reduction of the parasite burden in the *Leishmania*-mediated skin lesion of mice treated with different concentrations of nanoliposomal curcumin (40, 80, and 120 µM). Interestingly, in comparison to the results achieved by infected skins treated with Glucantime, the most reduction in parasite burden could be observed in *Leishmania*-infected skin lesions treated with 80 µM of nanoliposomal curcumin. In addition, a reduction in the kinetic of lesion seize development was significantly observed in the mice group treated with 80 µM nanoliposomal curcumin.

Conclusion

Curcumin could reduce the parasite burden in the skin lesion with similar effectiveness achieved with the standard drug Glucantime. Therefore, it is recommended to use curcumin in the demonstrated formulation in cutaneous leishmaniasis by other animals like dogs and with randomized trials.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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