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Colloidal, *in vitro* and *in vivo* anti-leishmanial properties of transfersomes containing paromomycin sulfate in susceptible BALB/c mice

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ABSTRACT

The aim of this study was to develop transfersomal formulation with respect to dermal delivery of paromomycin sulfate (PM) for possible topical therapy of cutaneous leishmaniasis (CL). PM transfersomal formulations (PMTFs) with different percent of soy phosphatidylcholine, sodium cholate (Na-Ch) and ethanol were prepared and characterized for the size, zeta potential and encapsulation efficiency. The results showed that the most stable formulations with suitable colloidal properties were obtained by 2% Na-Ch which had average size of around 200 nm. The *in vitro* permeation study using Franz diffusion cells fitted with mouse skin at 37 °C for 24 h showed that almost 23% of the PMTFs applied penetrated the mouse skin, and the amount retained in the skin was about 67% for both formulations; however, the percent of penetration and retention for PM conventional cream was 49 and 13, respectively. The 50% effective doses of PMTFs against *Leishmania major* promastigotes and amastigotes in culture were significantly less than cream and/or solution of PM. Selected PMTFs and empty transfersomes showed no cytotoxicity in J774 A.1 mouse macrophage cell line. Selected PMTFs was used topically twice a day for 4 weeks to treat *L. major* lesions on BALB/c mice, and the results showed a significantly ($P < 0.05$) smaller lesion size in the mice in the treated groups than in the mice in the control groups, which received either empty transfersomes or phosphate-buffered saline (PBS) and also PM cream. The spleen parasite burden was significantly ($P < 0.01$) lower in mice treated with selected PMTFs than in mice treated with PBS or control transfersomes, and PM cream. The results of this study showed that PMTFs prepared with 2% of Na-Ch with and without 5% ethanol might be useful as a candidate for the topical treatment of CL.

1. Introduction

Leishmaniasis is a neglected protozoan parasitic disease of the genus *Leishmania* (*L.*) with high burden especially in the least developed regions of the world. Different forms of leishmaniasis induced by the bite of an infected female sand fly (WHO, 2004). Although pentavalent antimonials are still the first-line treatment of cutaneous leishmaniasis (CL), but are associated with side effects, needs multiple injections, injection is painful and as a result compliance is poor and moreover the treatment is not always effective (WHO, 2004).

Paromomycin sulfate (PM) reported to show anti *leishmania* activity *in vitro* and since 1960s is used in clinical trials for both CL and visceral leishmaniasis (VL) (Croft et al., 2006). The results of clinical trials of topical PM in the treatment of CL are promising (Arana et al., 2001; Armijos et al., 2004; Asilian et al., 2003; Croft and Coombs, 2003; Faghihi and Tavakoli-kia, 2003; Goncalves et al., 2005; Iraji and Sadeghinia, 2005; Shazad et al., 2005).

The topical treatment provides drug activities at the desired site of action with limited or no systemic activity. A high potential for drug delivery is attributed to particulate drug carriers that obtain systems with optimized drug loading, release properties and much lower toxicity. Colloidal drug carriers generally retained at the site of administration longer than free drug and able to modify the distribution of an associated substances and increase the efficacy and/or reduce toxicity (Barratt, 2003).

Liposomes are colloidal particles and typically consist of one or more phospholipid bilayers enclosing an aqueous phase (Barratt, 2003). Liposomes were first used as topical therapy by Mezei and Gulasekharam in 1980 (Mezei and Gulasekharam, 1980) and entered the market in 1986 as cosmetic formulation and then later first therapeutic formulation of liposome antimycotic agent, econazole was commercialized (Benson, 2005; Elsayed et al., 2007). Transfersome was first introduced by Cevc (Cevc, 2001) as the first

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generation of elastic vesicles, consist of phospholipid and an edge activator that is often a single chain surfactant, which softens lipid bilayers of the vesicles and causes deformability to the bilayers so squeeze between the cells in SC in spite of the large average vesicle size and pass through intact skin under the influence of hydration gradient, carrying therapeutical agents only when applied under non-occlusive conditions (Cevc et al., 2002; Elsayed et al., 2007). Transfersomes preparation procedures are similar to the methods used for preparation of traditional liposomes (Elsayed et al., 2007). Two mechanisms are proposed for the action of transfersomes; first, vesicles act as drug carrier; intact vesicles enter the SC carrying vesicle bound drug molecules into the skin. Second, vesicles act as penetration enhancers and enter the SC and subsequently modify the inter-cellular lipid lamellae and therefore facilitates the penetration of free drug molecules into and across the SC (Elsayed et al., 2007).

Previously (Jaafari et al., 2009), reported that liposomal formulation of paromomycin (LPMF) possesses strong anti *Leishmania* activity *in vitro* and *in vivo*. LPMF was able to cure *Leishmania major* lesion in BALB/c mice and significantly reduced spleen parasite burden. Currently, the same formulation is in clinical trial. In this study, new class of liposomes (transfersomes) were prepared using fusion method (Foldvari, 1998). Encapsulation efficacy and the size of prepared vesicles were characterized and the activity of the formulation was evaluated *in vitro* against *L. major* promastigotes and amastigotes. Cytotoxicity of the preparation was measured in J 774 A.1 cell line. *In-vitro* penetration across mice skin studies were carried out and *in vivo* activity of formulations was studied in BALB/c mice infected with *L. major*.

2. Materials and methods

2.1. Ethics statement

All procedures involving animals and the proposal was approved by the Institutional Ethical Committee (Mashhad University of Medical Sciences; proposal code 85083) which has an authorization from Iranian National Ethical Committee. Animals were kept in cages and provided with food and water *ad libitum*.

2.2. Materials

Soya bean phosphatidylcholine (SPC) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Paromomycin sulfate (PM), sodium cholate (Na-Ch), propyl paraben (PP), methyl paraben (MP), propylene glycol (PG), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT, tissue culture grade) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma (USA), and vitamin E from Merck (Darmstadt, Germany). The 2,4-dinitro-1-fluorobenzene (DNFB) was purchased from fluka (Germany) and Alamar Blue (AB) from Biosource (International, Inc., USA).

2.3. Animals and parasites

Female BALB/c mice 6–8 weeks old were purchased from Pasteur Institute (Tehran, Iran). The mice were maintained in animal house of Biotechnology Research Center and fed with tap water and standard laboratory diet (Khorasan Javane Co., Mashhad, Iran). Animals were housed in a colony room 12/12 h light/dark cycle at 21 °C with free access to water and food.

L. major strain (MRHO/IR/75/ER) was used in this experiment, *Leishmania* isolate was cultured on NNN medium (Novy-MacNeal-Nicolle) and subcultured in RPMI 1640 (Sigma) containing 10% (v/v) heat inactivated FCS, 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate (RPMI-FCS) at 25 °C, *Leishmania* virulence was maintained in BALB/c mice (Jaafari et al., 2003).

Table 1
Value and code unites of 3^2 factorial design for preparation of paromomycin transfersomal formulations.

Variables	Coded units	Levels		
		1	2	3
Sodium cholate (Na-Ch)	*X*	2	4	6
Ethanol (EtOH)	*Y*	0	5	10

2.4. Preparation of transfersomes containing PM

PM transfersomal formulations (PMTF) were prepared by the fusion method (Foldvari, 1998; Jaafari et al., 2009), using factorial design in two categories; SPC 15 (A) and 20 (B) wt%. In each category, two variables were taken at its three levels as shown in Table 1. Eighteen formulations were prepared according to the experimental design shown in Table 2. Briefly, the lipid components consisted of SPC (15, 20 wt%), Na-Ch (2, 4, 6 wt%) as a bilayers softener, Chol (2 wt%), PG (7 wt%), vitamin E (0.3 wt%), MP (0.1 wt%) and PP (0.02 wt%) were melted at about 75 °C (lipid melt). HEPES buffer (10 mM, pH 7.0) containing PM (10%) (HEPES-PM 10%) were heated separately and were added up into 100% to the previously heat melted lipid and the mixture was vigorously vortexed until cooled down to room temperature. In case of formulation containing ethanol (EtOH), the lipid melt cooled down to 40 °C, and EtOH (5, 10 wt%) was added and then, HEPES-PM 10% was added and the mixture was vigorously vortexed until cooled down to room temperature. Then the final product was homogenized using homogenizer (Ultra-Turrax IKA® T10, IKA Werke GmbH & Co. KG, Staufen, Germany) for 5 min at 5000 rpm. Finally, high-pressure extrusion was performed using microfluidizer (Microfluidizer-110S, Microfluidics International Corp., Newton, MA, USA) through the 75-μ F 12 Y-type interaction chamber on PMTFs to produce homogenous products.

The PM conventional cream (10%) was prepared according to the formulation of aqueous cream BP (BP, 2007). To prepare 30 g of PM cream, the following amount was used; 5 g white paraffin, 3 g cetomacrogol emulsifying wax and 2 g mineral oil as lipid phase, 3 g PM and 20 ml water as aqueous phase. Lipid phase was melted at 70 °C, PM was dissolved in water and heated at 70 °C. Then PM solution was added to melted lipid phase and vigorously vortexed until it cooled down to room temperature. Cetomacrogol emulsifying wax was prepared by melting 90 g cetostearyl alcohol and 10 g SLS (sodium lauryl sulfate) and adding 4 ml water in the same temperature and stirred vigorously until it cooled down to room temperature.

2.5. Characterization of formulations containing PM

Physical characteristics of LPMF and PMTFs (size and polydispersity index) were determined using dynamic light scattering (Malvern, Nano-ZS, UK). Samples were diluted in HEPES buffer to a suitable concentration (fluctuation of light under 350) to minimize interference particulate matter. Each measurement was done in triplicate. The polydispersity index was determined as a measure of the homogeneity of the vesicles suspensions. Quantity of the entrapped drug in the prepared vesicles was determined directly as well as indirectly. The preparations were first centrifuged (Hettich, Universal 320 R, Germany) at 14,000 rpm for 30 min at 4 °C, washed three times using HEPES buffer and the amount of PM was measured in the supernatants as well as the purified PMTFs.

The supernatants were analyzed for PM by adding 1.5 ml DNFB (150 mM in methanol) to 0.5 ml of the sample, heated

Table 2
Experimental design of paromomycin transfersomal formulations.

A (Soy phosphatidylcholine 15 wt%)			B (Soy phosphatidylcholine 20 wt%)		
Formulation no.	*X* (Na-Ch) (wt%)	*Y* (EtOH) (wt%)	Formulation no.	*X* (Na-Ch) (wt%)	*Y* (EtOH) (wt%)
T1	2	0	T10	2	0
T2	4	0	T11	4	0
T3	6	0	T12	6	0
T4	2	5	T13	2	5
T5	4	5	T14	4	5
T6	6	5	T15	6	5
T7	2	10	T16	2	10
T8	4	10	T17	4	10
T9	6	10	T18	6	10

at 80 °C for 45 min, and made up to 25 ml with chloroform:tetrahydrofuran:water (25:28.2:0.8). Then the absorbance was measured at maximum wavelength (405 nm) after discarding the upper aqueous phase. The purified transfersomes precipitates were redispersed in 2% Triton X-100. The final clear solution was analyzed for PM content using DNFB reagent as explained (Lunn, 2000).

2.6. Effect of formulations on L. major promastigotes in-vitro

The effect of formulations on the growth of *Leishmania* promastigotes was assessed by monitoring MTT metabolism at 48 h culture period in the presence of the formulations. Parasites harvested at stationary phase and were added at 4×10^5 *Leishmania* promastigotes per well in 96-well flat-bottom plates containing different concentrations of formulations in triplicate. The plates were incubated at 25 °C for 48 h and then MTT was added (40 μl/well of a 5 mg/ml in PBS) and then the plates were incubated in the dark at 37 °C for a further 4 h. The formation of formazan was evaluated by adding 50 μl/well 20% SDS, and incubated for overnight at 37 °C and the relative absorbance was photometrically measured using ELISA reader (Statfax-2100, Awareness Technology, USA) at 545 nm. The relative absorbance was correlated to the number of promastigotes per well using a standard curve consisted of different number of promastigotes treated with MTT dye as explained above. The 50% effective dose (ED_{50}) for each formulation was calculated by Litchfield–Wilcoxon method using PCS v.4 software (Jaafari et al., 2009).

2.7. Effect of formulations on L. major amastigotes in-vitro

J774 A.1 mouse macrophage cell line (Pasteur Institute, Tehran, Iran) at 5×10^4 macrophages per 200 μl of RPMI-FCS per well were dispensed into Lab-tek (NUNC, USA) 8-well chamber slides and incubated at 37 °C, 5% CO_2 for 24 h to allow attachment of the cells. The unattached cells were washed away and infected with *L. major* promastigotes at a ratio of 5:1 and incubated for another 24 h. Then unattached parasites were removed and infected cells were incubated for an additional 24 h. The cells were exposed to formulations at different concentrations for 2 days. The experiment was terminated by methanol fixation of the slides. Then the slides were stained using Giemsa and evaluated microscopically to calculate the percentage of infected cells. The ED_{50} for each formulation was calculated using Litchfield–Wilcoxon method and PCS v.4 software (Jaafari et al., 2009).

2.8. In-vitro skin penetration and retention using cell diffusion study

Drug release studies were performed *in vitro* using jacketed Franz cells with a receiver volume of 25 ml in triplicate (Jaafari et al., 2009). The temperature of diffusion cells was kept at 37 ± 1 °C by circulating water in their jackets. The receiver compartment was stirred by magnetic bar at 1000 rpm. The penetration experiments were carried out for 24 h. Full-thickness mice dorsal skin was used for penetration experiments; the hair of the outer skin surface was removed without damaging the skin 24 h before sacrificing the animal. The skin was carefully dissected and rinsed with normal saline. The epidermal side of the mice skin was exposed to ambient conditions while the dermal side was bathed with phosphate buffer (pH 7.4) as the receptor fluid. The membranes were initially left in the Franz cells for 30 min in order to facilitate hydration. Subsequently, a weighed amount of formulations was deposited on to each membrane surface. All experiments were carried out with non-occluded donor compartments. A 250 μl samples aliquot were removed at appropriate intervals from the receiver compartment and refilled with the same volume of fresh medium (Foco et al., 2005). Amount of PM penetrated were obtained from the cumulative amount released at different time intervals and at the end of 24 h (Boinpally et al., 2003).

For the determination of the amount of PMTFs retained in the skin, which would be of benefit to the treatment of CL, at the end of the experiments (24 h), the remaining amount of formulation on the surface of membrane was collected and assayed for PM. The amount of PM retained in the skin was calculated by subtracting the sum of the amount of PM that remained on the surface and the amount of PM which was released (penetrated through the skin) from the whole amount applied (Boinpally et al., 2003).

2.9. Evaluation of leishmanicidal activity of formulations in established infection model of CL in BALB/c mice

BALB/c mice were inoculated subcutaneously with 3×10^6 *L. major* at the base of the tail. At 4 weeks post infection, lesions were measured with calipers in two dimensions, mean diameters determined and mice were distributed in groups of seven, according to an equal median lesion size. From day 28 post-inoculation, topical formulations were applied twice a day for 4 weeks. Control groups were treated with PBS, transfersomes free of drug or conventional cream containing 10% PM. Lesion size were followed weekly up to 12 weeks and analyzed with ANOVA (Jaafari et al., 2009). The *in vivo* anti-leishmania study was carried out only with T1, T4, T10 and T13 formulations which had good vesicle and *in vitro* properties.

2.10. Splenic parasite burden

The number of viable *L. major* parasites in the spleen of mice was determined by a limiting dilution assay. The mice were sacrificed at 8 and 12 weeks after treatment; the spleens were aseptically removed and homogenized in 1 ml RPMI-FCS with a sterile syringe piston. The homogenate was diluted with the same media in 8 serial 10-fold dilutions in each well of flat-bottom 96-well microtiter plates containing solid layer of rabbit blood agar in triplicate and kept at 25 °C for 7 days. The positive (presence of motile parasite)

Table 3
Particle size distribution (nm), polydispersity index (PI) and ζ-potential (mV) of liposomal formulation of paromomycin (L1) and paromomycin transfersomal formulations (mean ± SD, $n = 3$).

	Formulations SPC/Na-Ch/EtOH (%w/w ratio)	Average size ± SD	PI ± SD	ζ-potential ± SD
L1	15/0/0	89.51 ± 34.67	0.570 ± 0.05	−9.25 ± 3.44
T1	15/2/0	196.65 ± 75.80	0.588 ± 0.051	−13.3 ± 3.13
T2	15/4/0	1142.33 ± 127.36	0.310 ± 0.271	−21.8 ± 0.52
T3	15/6/0	260.38 ± 29.26	0.576 ± 0.087	−12.3 ± 3.41
T4	15/2/5	350.78 ± 57.53	0.517 ± 0.032	−13.5 ± 4.37
T5	15/4/5	183.41 ± 138.70	0.196 ± 0.189	−9.65 ± 0.72
T6	15/6/5	134.57 ± 164.05	0.775 ± 0.200	−14.1 ± 3.94
T7	15/2/10	211.38 ± 22.37	0.563 ± 0.115	−13.0 ± 4.06
T8	15/4/10	2236.93 ± 2231.78	0.151 ± 0.193	−23.3 ± 1.58
T9	15/6/10	183.48 ± 207.54	0.600 ± 0.005	−14.7 ± 4.07
T10	20/2/0	263.47 ± 44.74	0.464 ± 0.005	−12.8 ± 3.76
T11	20/4/0	1471.00 ± 43.86	0.291 ± 0.010	−20.3 ± 0.49
T12	20/6/0	255.16 ± 140.36	0.591 ± 0.076	−14.8 ± 3.89
T13	20/2/5	235.84 ± 57.89	0.443 ± 0.062	−13.9 ± 3.76
T14	20/4/5	1142.30 ± 162.46	0.398 ± 0.142	−20.7 ± 1.35
T15	20/6/5	632.23 ± 54.23	0.157 ± 0.020	−13.1 ± 3.89
T16	20/2/10	204.27 ± 16.43	0.473 ± 0.037	−12.2 ± 2.92
T17	20/4/10	1368.78 ± 937.46	0.166 ± 0.118	−22.9 ± 0.25
T18	20/6/10	363.34 ± 25.52	0.724 ± 0.239	−11.3 ± 4.70

and the negative (absence of motile parasite) wells were detected using an invert microscope. Data reported is the calculated mean and standard error of mean of the last positive well multiplied by dilution factor (Jaafari et al., 2009).

2.11. Statistical analysis

One-way ANOVA statistical test was used to assess the significance of the differences between the various groups. In the case of a significant F value, multiple comparison Tukey test was used to compare the means of different treatment groups, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of transfersomes

In order to develop topical formulation containing PM for the treatment of CL, PMTFs were prepared by the fusion method as described by Foldvari (Foldvari, 1998) and characterized for the colloidal properties.

The mean diameter of the various transfersomal suspensions was from 90 to 2200 nm with polydispersity index (PI) between 0.151 and 0.775 (Table 3). Particle diameter of vesicles containing 2% Na-Ch was between 190 and 350 nm, increasing in percent of Na-Ch to 4% led to larger particles and incorporation of 6% Na-Ch decreased the average particle diameter which could be due to the higher concentration of Na-Ch that reaches its Critical Micelle Concentration (CMC). The CMC of Na-Ch is 14 mM (6 mg/ml) (Table 3).

The zeta potential of PMTFs was more negative compared to that of the LPMF (L1) which indicates incorporation of the negatively charged Na-Ch in liposome bilayers. In general formulations containing 4% Na-Ch was more negative than 2%; however, formulation with 6% Na-Ch had almost the same zeta potential as of 2% which could be due to incomplete incorporation of Na-Ch in vesicles (Table 3). Vesicles containing 6% Na-Ch were unstable and during the storage converted to gel-like state and formulations with 2% Na-Ch provided the most stable formulations.

Encapsulation efficiency of PM was determined by both direct and indirect method after adding DNFB reagent and making a derivative compound with chromophore. The encapsulation efficiency was comparable between the two direct and indirect methods. All formulations showed encapsulation efficiency of more than 50% when the efficiency was determined using direct method (Table 4). PMTFs with 2% Na-Ch, which had proper colloidal properties, provided higher encapsulation efficiency (more than 60% in most of them).

3.2. Promastigote assay

ED_{50} of the formulations against *L. major* promastigotes was determined using MTT method, considering the percent of decrease in parasite viability in each well containing a certain amount of promastigotes in contact to different concentration of formulations. The data generated was compared with the standard concentration of promastigotes grown in the absence of drug in RPMI-FCS. ED_{50} of formulations calculated using Litchfield–Wilcoxon method PCS v.4 software are shown in Table 5. ED_{50} of liposomal and transfersomal formulations were in range of 18.60–124.67 μg/ml and that of PM solution was 205.70 ± 10.05 μg/ml. Statistically significant differences ($P < 0.05$) between PM solution and other formulations were seen. Formulations containing 6% Na-Ch and/or 10% EtOH

Table 4
Encapsulation efficiency (direct and indirect method) of liposomal formulation of paromomycin (L1) and paromomycin transfersomal formulations (mean ± SD, $n = 3$).

	Formulations	%Encapsulation	
	SPC/Na-Ch/EtOH (%w/w ratio)	Direct	Indirect
L1	15/0/0	60.30 ± 1.19	51.68 ± 0.61
T1	15/2/0	48.94 ± 0.99	60.22 ± 0.59
T2	15/4/0	70.86 ± 0.22	78.91 ± 0.18
T3	15/6/0	73.49 ± 0.05	53.05 ± 0.14
T4	15/2/5	64.80 ± 0.78	72.22 ± 0.45
T5	15/4/5	42.72 ± 1.28	59.36 ± 0.27
T6	15/6/5	68.46 ± 0.51	84.75 ± 0.86
T7	15/2/10	83.99 ± 0.04	67.56 ± 0.52
T8	15/4/10	58.67 ± 0.23	72.40 ± 0.60
T9	15/6/10	85.63 ± 0.28	81.95 ± 0.24
T10	20/2/0	44.72 ± 0.087	57.73 ± 1.07
T11	20/4/0	54.99 ± 0.37	54.50 ± 0.09
T12	20/6/0	52.93 ± 0.51	69.45 ± 0.12
T13	20/2/5	59.12 ± 0.13	74.90 ± 0.54
T14	20/4/5	66.25 ± 0.58	72.61 ± 0.17
T15	20/6/5	45.67 ± 0.32	64.54 ± 0.13
T16	20/2/10	77.85 ± 1.20	66.23 ± 0.21
T17	20/4/10	79.85 ± 0.14	84.16 ± 0.56
T18	20/6/10	77.43 ± 0.33	78.55 ± 0.53

Table 5
In vitro activity of different formulations against *Leishmania major* promastigotes (mean ± SD, $n = 3$).

Formulations	ED_{50} for *L. major* promastigotes (μg/ml)
L1	65.32 ± 7.57
T1	19.85 ± 6.72
T2	46.67 ± 15.31
T3	19.97 ± 3.96
T4	19.63 ± 4.51
T5	19.30 ± 1.81
T6	22.65 ± 0.07
T7	107.73 ± 15.62
T8	19.90 ± 2.72
T9	116.35 ± 4.45
T10	87.67 ± 40.03
T11	98.50 ± 139.30
T12	124.67 ± 19.13
T13	22.20 ± 4.36
T14	18.60 ± 9.30
T15	110.60 ± 25.13
T16	94.97 ± 6.74
T17	64.074 ± 23.70
T18	75.60 ± 39.34
PM solution	205.70 ± 10.05
Control empty liposome	Inactive

There was a significant difference between PM solution and L1, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14, T15, T16, T17 and T18 ($P < 0.05$). There was a significant difference between T12 and L1, T1, T3, T4, T5, T8, T13, T14 and PM solution ($P < 0.05$). There was no significant difference among T2, T6, T7, T9, T10, T11, T12, T15, T16, T17 and T18 ($P < 0.05$). There was no significant difference among L1, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T13, T14, T15, T16, T17 and T18 ($P < 0.05$).

were excluded from the rest of the study because of the lack of stability.

3.3. Amastigote assay

To examine the efficacy of the formulations on intracellular amastigotes, the macrophage cell line J774.1A was infected with *L. major* at a ratio of 5:1, parasite to macrophage.

ED_{50} of liposomal and transfersomal formulations were in range of 10.24–25.77 μg/ml, ED_{50} of PM solution was 83.31 ± 16.64 μg/ml and that of PM cream was 106.65 ± 8.12 μg/ml. ANOVA analysis indicated no significant difference amongst ED_{50} of PMTFs, however, a significant ($P < 0.05$) difference was seen between PMTFs and cream and solution form of 10% of PM (Table 6).

3.4. Cell diffusion study

In-vitro penetration across mice skin studies were carried out by diffusion cells and the percent of penetrated and retained PM

Table 6
In vitro activity of different formulations against *Leishmania major* amastigotes (mean ± SD, $n = 3$).

Formulations	ED_{50} for *L. major* amastigotes (μg/ml)
L1	24.64 ± 1.51[a]
T1	14.78 ± 2.37[a]
T2	10.24 ± 1.59[a]
T4	15.08 ± 3.97[a]
T5	25.77 ± 5.09[a]
T10	20.875 ± 5.55[a]
T11	11.89 ± 4.86[a]
T13	17.71 ± 4.29[a]
T14	20.18 ± 14.18[a]
PM Solution	83.31 ± 16.64
PM Cream	106.65 ± 8.12
Control empty liposome	Inactive

[a] Significant ($P < 0.05$) difference between the ED50 of liposomal formulation of paromomycin (L1) and paromomycin transfersomal formulations (PMTFs),and those of cream and solution.

Table 7
Percent of penetration and retention of paromomycin from paromomycin transfersomal formulations across the mouse skin after 24 h.

Formulation	Penetration (%)[a]	Retention (%)[b]
T1	23.99 ± 0.18	66.52 ± 0.07
T4	23.87 ± 0.31	67.24 ± 0.36
T10	22.26 ± 0.46	67.85 ± 0.74
T13	22.56 ± 0.18	69.05 ± 0.21
Cream	49.18 ± 5.05	13.24 ± 8.89

[a] The penetration percent of cream was significantly ($P < 0.05$) higher than transfersomal formulations (T1, T4, T10 and T13).
[b] The retention percent of transfersomal formulations (T1, T4, T10 and T13) was significantly ($P < 0.05$) higher than conventional cream.

in the skin was determined for PMTFs up to 24 h. This study was carried out only with T1, T4, T10 and T13 formulations which had good vesicle and *in vitro* properties. The percent of penetrated PM for PMTFs and PM cream was approximately 23 and 49% respectively; and the percent of retained PM for PMTFs and PM cream was 67 and 13%, respectively (Table 7). The penetration percent of PM cream was significantly ($P < 0.05$) higher than PMTFs. The retention percent of PMTFs was significantly ($P < 0.05$) higher than conventional PM cream. There was no significant difference in the percent of penetration and retained PM between the T1, T4, T10 and T13 formulations.

3.5. Evaluation of leishmanicidal activity of formulations in established infection model of CL in BALB/c mice.

There were no significant differences ($P > 0.05$) in lesion sizes among different groups before initiation of the treatment (Fig. 1 week 4 post-infection). The topical application of PMTFs caused significant reductions in the lesion sizes ($P < 0.05$) compared to PM cream and also PBS and control empty transfersomes (Fig. 1). When the treatment was stopped relapse was observed in the animals treated with PMTFs (weeks 9–12 of postinfection); however, at 12 week post-infection the lesion sizes for T1, T10 and T13 was still significantly ($P < 0.05$) less than PM cream and also control groups. No statistically significant differences were seen among the lesion sizes of the animals treated with the PMTFs, and no statistically significant differences were seen among the two control groups that received PBS or control empty transfersomes and also PM cream.

3.6. Effects of formulation on splenic parasite burden of mice

The number of viable *L. major* was quantified using limiting dilution assay in the spleen of different groups of mice at weeks 8 and 12 postinfection. At week 8 postinfection, the mice treated with PMTFs showed a significantly lower parasite burden than groups received PM cream and control empty transferosome or PBS ($P < 0.01$) (Fig. 2a). There was no significant difference among the groups treated by PM loaded formulations, and no significant differences were seen among the control groups and PM cream (Fig. 2a). At week 12 post infection, the mice treated with PMTFs showed a significantly lower parasite burden than groups received either control empty liposomes or conventional cream as well as PBS ($P < 0.001$) (Fig. 2b). There was no significant difference among the groups treated by PMTFs, and no significant differences were seen among the control groups and PM cream (Fig. 2b).

4. Discussion

Cutaneous leishmaniasis is the most common form of leishmaniasis, although CL is a self-healing skin lesion but healing process usually takes months and leaves a disfiguring scar (WHO, 1990). Treatment of CL is a challenging issue especially when the causative

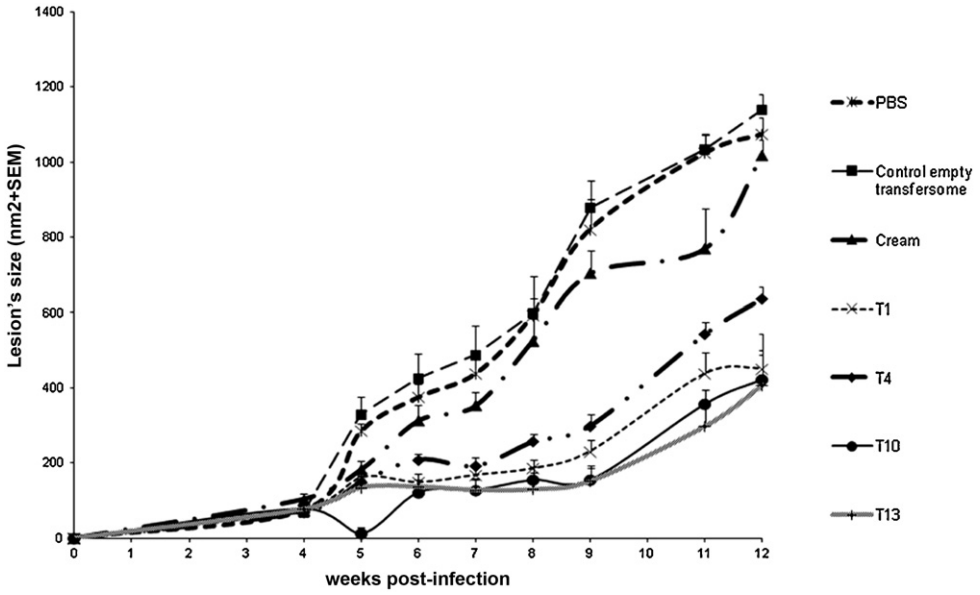

Fig. 1. Progress of lesion size in *L. major* infected BALB/c mice treated with different formulations of topical transfersomal PM. Female 6–8 weeks BALB/c mice were infected with subcutaneous injection of 3×10^6 stationary-phase *L. major* promastigotes at the base of the tail. At week 4 post infection, the lesions size were measured in two dimensions using calipers, mean diameters determined and mice were stored into groups of 7. The lesions were treated topically with 50 mg formulations twice a day for 4 weeks. The lesion size was recorded weekly during the treatment and up to 8 weeks after termination of the treatment.

agent is *Leishmania tropica* (Croft and Coombs, 2003; Firooz et al., 2006; Hadighi et al., 2006).

Interest in PM as a treatment for leishmaniasis initiated in 1960s and topical formulation for CL and a parental formulation for VL are developed (Croft et al., 2006). Different topical formulations of PM showed to be effective in some cases (Arana et al., 2001; Armijos et al., 2004; Asilian et al., 2003; Faghihi and Tavakoli-kia, 2003; Goncalves et al., 2005; Iraji and Sadeghinia, 2005; Shazad et al., 2005).

In 1984 El-On et al. (El-On et al., 1984) showed that 15% PM plus 12% methylbenzethonium chloride in soft paraffin is effective against CL and was marketed in Israel but later in clinical studies this formulation was reported to induce local toxicity and was not well tolerated (Arana et al., 2001).

Topical formulations of 15% PM and 10% urea showed to be effective and well tolerated (Bryceson and Moody, 1994) but in a clinical trial study in Iran, Faghihi and Tavakoli-kia (Faghihi and Tavakoli-kia, 2003) found this formulation much less effective than intralesional antimony with cure frequencies of 16.6% and 41.7%, respectively. Asilian (Asilian et al., 2003) reported that 4 weeks treatment still about one-third of all patients remained uncured. Shazad et al. (Shazad et al., 2005) showed that PM is as effective as intralesional meglumine antimoniate.

The formulation used by Grogl et al. (Grogl et al., 1999) is a complex cream carrier containing 15% of PM associate with 0.5% of gentamycin. However, no significant difference in cure rates was reported in clinical trial performed in New World CL of the formulations in comparison with placebo (Soto et al., 2002).

PM in the conventional formulations is a relatively large molecule with molecular mass of 713.71 with high water solubility and oligosaccharide nature, which make it difficult to penetrate through the SC of the skin (Ferreira et al., 2004), in the other hand

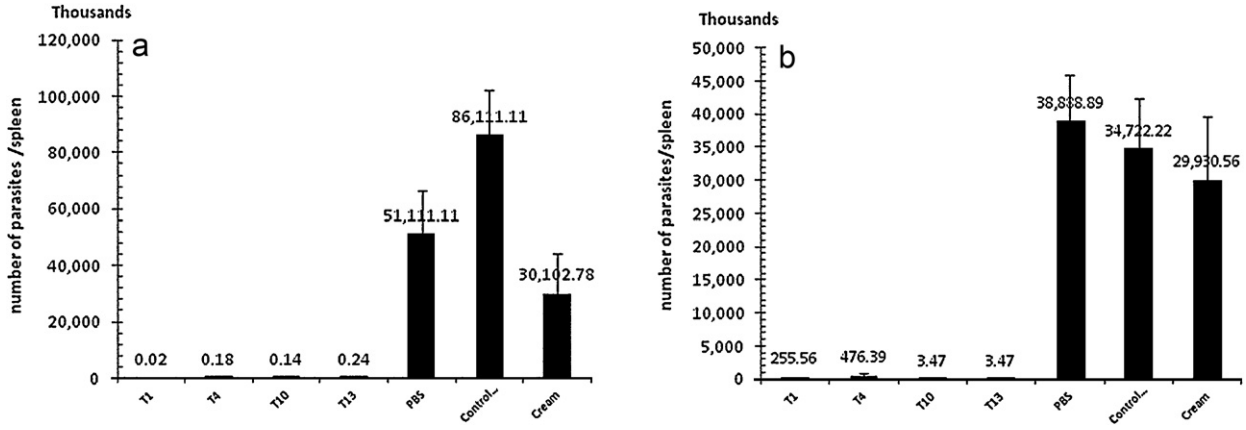

Fig. 2. Splenic parasite burden in BALB/c mice treated with topical transfersomal PM. The number of viable *L. major* was quantified using limiting dilution assay in the spleen of different groups of mice at weeks 8 (a) and 12 (b) after infection with *L. major* promastigotes. The spleen were aseptically removed and homogenized in RPMI-FCS, diluted with the same media in 8 serial 10-fold dilution in each well of flat-bottom 96-well microtiter plates contains solid layer of blood agar. The plates were kept at 25 °C for 1 week and then were read to score the number of positive wells (containing one or more promastigotes). The final titer was the last dilution for which the well contained at least one parasite. (a) $n = 3$, mean ± SEM, there were significant ($P < 0.01$) differences between PM transfersomes and groups received control empty transfersomes or PBS; (b) mean ± SEM, there were significant differences between PM transfersomes and groups received either control empty transfersomes or conventional cream ($P < 0.01$) as well as PBS ($P < 0.001$).

topical medicines need to reach the *Leishmania* parasites, which reside in the phagolysosomes of infected macrophages deep in the dermal layer of the skin. Therefore, the candidate drugs not only need to target the macrophages in the dermis but also need to cross the phagolysosomal vacuole membranes and parasite membrane to reach to the cytosol of the parasite (Mauel, 1990).

New treatment for CL especially anthroponotic form is needed (Croft et al., 2006). Topical treatment of CL is ideal for the patients and authorities and PM showed promising results in clinical trials (Asilian et al., 2003; Shazad et al., 2005). Owing to the location of this parasite, the use of carrier systems is very helpful as it is well known that macrophages internalize particulate carriers by the process of endocytosis and may act as secondary drug depot, thus helping in localized delivery of the drug at the infected site. It also allows the use of higher doses, achieving a greater and more prolonged effect (Ashan et al., 2002).

The introduction of liposomes as skin drug delivery systems, initially promoted primarily for localized effects with minimal systemic delivery. Reduction of vesicle size improves drug deposition into deeper strata and accelerates drug penetration through the skin. Electron microscopy studies confirmed the presence of intact liposomes in the epidermis and dermis (Foldvari et al., 1990). The rate of liposomes' penetration to the epidermis and dermis depends upon the kind of phospholipid, the concentration, the size of liposomes and the penetration enhancer (Ghyczy and Gareiss, 1994).

LPMF (Liposomal PM Formulation) showed to be effective against *L. major in vitro* and *in vivo* (Jaafari et al., 2009) and is currently in clinical trial against CL induced by *L. tropica* and CL caused by *L. major*.

Ferreira et al. (2004) prepared topical liposomal formulation containing paromomycin and showed improved skin permeation and retention across the intact skin with encapsulation of PM in liposomes in comparison with solution or empty liposome plus PM (Ferreira et al., 2004). Lately, Carneiro et al. (2009) studied PM liposomal formulations in murine model of leishmaniasis. In animals treated with PM liposomal gel and free PM gel, complete healing was observed 21 days after treatment initiation but gradually relapse of the lesions was seen by day 91 after treatment, at a faster rate in animals treated with free PM gel. At the end of the follow-up period, cure rate was 0 and 30% for the free and liposomal PM respectively (Carneiro et al., 2009).

A higher *in vitro* uptake of pentavalent antimony by mouse peritoneal macrophages infected with *Leishmania chagasi* was seen using negatively charged phosphatidylserin liposomes (Tempone et al., 2004). Doxorubicin-bearing immunoliposomes increase the activity of liposomes and free doxorubicin in macrophages infected by *L. donovani*, with no toxicity. Studies carried out in mice have confirmed better efficiency with spleen infections being fully eliminated (Mukherjee et al., 2004).

Recent advances and alteration in the composition and structure of vesicles result in vesicles with tailored properties. Flexible and ultradeformable liposomes are one such advance with claims of enhanced transdermal drug delivery to efficiencies.

This study is designed to prepare and characterize PMTF, using Na-Ch as an edge activator and then evaluate the effect of formulations *in vitro* and *in vivo* against *L. major*. Cytotoxicity test was also carried out for some of the formulations. In this study the fusion method was used to prepare the PMTFs (Foldvari, 1998). The fusion method is simple, efficient, and reproducible; is devoid of organic solvents like chloroform; and yields homogeneous bilayer vesicles with high encapsulation efficiencies (Foldvari, 1998). The prepared formulations showed more than 50% encapsulation efficiency when determined by direct method. Furthermore, formulations prepared with this method showed enough viscosity to apply on the skin directly with no need to be mixed with other bases.

It is shown that among different phospholipids, phospholipids with cholin head-groups interacts and hydrates skin more with stronger penetration compared to the other types of phospholipids (Ghyczy and Gareiss, 1994), therefore, SPC was used to prepare the transfersomal PM.

Different concentrations of phospholipid showed no effect in size of particles and formulations containing 6% sodium cholate and 10% ethanol resulted in instability in formulations. Also, in the formulation of PMTFs, cholesterol was included to stabilize the lipid bilayers and decrease the leakage of encapsulated PM and vesicle aggregation; vitamin E was used to prevent SPC oxidation, PP and MP as microbial preservative, and HEPES to control the pH of formulations for the maximum stability of the formulations. Incorporation of 2% Na-Ch in formulations led to stable colloidal particles with mean diameter between 190 and 350 nm. The prepared vesicles showed also higher encapsulation efficiency (most of them more than 60%). Higher percent of Na-Ch resulted in increasing the mean diameter of the particles or instability of the formulations. For these formulations the concentration of Na-Ch is much higher than its CMC (14 mM or 6 mg/ml) which might be the reason for instability of the formulations. Although Na-Ch possesses a steroidal backbone similar to cholesterol, which is used in liposomal formulations as a membrane stabilizer, the presence of the carboxylate and hydroxyl groups in cholate induces an edge activating rather than a membrane stabilizing effect and resulted in flexibility of the bilayers of vesicles which enables the vesicles to pass through pores smaller than the vesicle size under the influence of trans-epidermal water activity gradient and likely improves the penetration and diffusion of the compounds into the dermis, where intracellular amastigotes present and multiply inside the macrophage lineage (El Maghraby et al., 2004).

Colloidal drug carriers are naturally concentrated within macrophages, using colloidal drug carriers to deliver drugs to the macrophages is logical particularly when the microorganism is within the lysosomes. For example, a liposomal formulation of Amikacin (MiKasome®) is currently in clinical trials against complicated bacterial infections and showed to be more tolerable than the free antibiotic. Also increase intracellular penetration of muramyldipeptide into the macrophages is reported when the compound is associated with colloidal particles (Barratt, 2003)

Ethanol may also provide flexibility to the vesicles which allow them to penetrate easier into the deeper layers of the skin (Elsayed et al., 2007).

The leishmanicidal activities of PMTFs were tested against both the extracellular promastigotes and the intracellular amastigotes form of the parasite. ED_{50} of formulations against promastigotes and that of selected formulations against amastigotes are represented in Tables 5 and 6. As it was expected, fresh prepared PMTFs containing high percent of Na-Ch and EtOH showed a lower ED_{50} against promastigotes due to their anti-leishmanial activity by themselves. More importantly the results of promastigote assay showed that the ED_{50} of all formulations against promastigotes was significantly ($P < 0.05$) lower than PM solution which might be due to the fact that transfersomes delivers PM to the cytosol of parasites more effectively than PM-solution. This is probably due to the intrinsic ability of bilayer vesicles that might deliver the drugs into the cells by different mechanisms like adsorption, fusion or endocytosis (Kamps and Scherphof, 2003). On the other hand the presence of Na-Ch in the bilayer of PMTFs, which is a surface active agent, might have membrane disturbing effect and also give fusogenic properties to the transfersomes. No statistically difference was seen between ED_{50} of selected PMTFs against amastigotes but a significantly difference ($P < 0.05$) was shown when compared to the cream and solution form of PM. This could be explained by the fact that PM transfersome vesicles were phagocytosed by macrophages (Ashan et al., 2002) and then PM is released from

transfersomes by acidic lysosomal enzymes in the phagolysosome of the macrophage, where *Leishmania* parasites live and multiply. The assays with promastigotes and amastigotes also showed that the processes used for the preparation of PMTFs by the fusion method do not affect on the activity of the PM.

In order to evaluate the accumulation of PM into and diffusion through the skin, cell diffusion studies was carried out using dorsal mice skin and vertical Franz cells. Transfersomes showed higher percentage of PM retention in the skin compared to the conventional cream which might be attributed to the intact penetration of self-optimizing deformable liposomes (Table 7). The results suggested that deformable liposomes may be of value for the topical administration of PM in the treatment of CL since the transfersomes remained at the site of infection.

The *in vivo* results in BALB/c mice infected with *L. major* showed that using topical transfersomes containing PM twice a day for 4 weeks started at week 4 after infection induced a significantly smaller lesion size and lower spleen parasite burden than control groups and PM cream (Figs. 1 and 2). The effect of the treatment on the site of inoculation which is the initial parasite growth might be a reason of lower parasite burden in the spleen of treated mice. Furthermore, since some of the PM transfersomes are very small, they can act as transdermal vehicle, pass from the epidermis intact, and reach the blood stream in the dermis. When liposomes are in the blood, spleen macrophages and kupffer cells in the liver are the main cells that phagocyte the liposome particles (Drummond et al., 2008). It is speculated that after topical application of PM formulations, at least some of the vesicles, especially those with sizes smaller than 100 nm, pass through SC of intact skin and reach epidermis and deep dermis. In the dermis infected macrophages phagocyte PM vesicles, and then PM is released in the phagolysosome of the macrophage by acidic lysosomal enzymes where *Leishmania* parasite lives and multiply (Ashan et al., 2002; El Maghraby et al., 2008).

In this study when the treatment was discontinued, some relapse was observed in the lesion sizes of the mice treated with PMTFs (Fig. 1). This could be due to the fact that BALB/c mice are the most susceptible strain to *L. major* infection and conventional treatments are not fully effective in this strain of mice. However, the results suggest that PMTFs may be useful in the treatment of cutaneous leishmaniasis in human, since humans unlike the BALB/c mice are resistance against leishmaniasis.

Colloidal deformable drug carriers might be loaded with a wide range of different agents, such as low molecular weight drugs like 5-FU (El Maghraby et al., 2001a) as well as large molecular weight ones such as oestradio (El Maghraby et al., 2001b), triamcinolone acetonide (Cevc and Blume, 2003), cytokines such as IL-2 and IFN-α (Hofer et al., 2000), bleomycin (Lau et al., 2005), insulin (Cevc et al., 2002) and pDNA (Kim et al., 2004). Studies showed that transfersomes are superior to rigid liposomes in regard to dermal delivery of the drugs. Interactions of different formulations with SC are reported on the physical state of phospholipid bilayers at the temperature of the experiment. Since the epidermal membrane temperature is above the phase transition temperature (T_m) of transfersomes, transfersomes with smaller size are able to penetrate through skin more efficiently than conventional liposomes (Cevc et al., 2002). Transfersomes overcome the skin barrier by opening extracellular pathways between the cells in organs and then deform to fit into such passages. When a vesicle is forced into an orifice, such as an inter-corneocyte constriction in the SC, its components redistribute and accommodate to the surrounding stress and adjust its form to the shape of the pathway and cross it. Transfersomes are driven across the skin by the water activity gradient across the SC. The rate and the efficacy of the resulting motion are independent of the applied dose (Cevc and Blume, 2003). Once the very deformable vesicles loaded with drug reached the wet viable epidermis, experience no further inward water activity gradient. Consequently, any carrier motion ceases in this skin region. This role may be of great effect in improving skin deposition and stands to reason that drug delivery mediated by very deformable carrier shall reduce the side effects that are often observed with conventional therapy (El Maghraby et al., 2008). Our cell diffusion study results (Table 7) also confirms that transfersomes are accumulate in the skin region and permeate less through the skin compared to conventional PM cream.

5. Conclusion

Overall results of this study showed that PMTFs prepared by Na-Ch, improved *in vitro* activity against *L. major* promastigotes and amastigotes compared to either aqueous solution or cream formulations with no cytotoxicity. Also when applied non-occlusively, improved *in vitro* skin delivery of PM compared to the conventional cream. Incorporation of 2% Na-Ch led to formulations with acceptable characteristics regard to mean diameter, stability and physical appearance and anti-leishmanial activity. Although the results of lesion' size showed no complete cure, however, it should be considered this fact that BALB/c mice is the most susceptible strain to *L. major* infection and conventional treatments are not fully effective in this strain of mice. Accumulation of PM within the skin might help to optimize targeting the drug, creating new opportunities for well-controlled and modern topical application of PM in the treatment of CL. The results of this study showed that PMTFs prepared with 2% of Na-Ch with and without 5% ethanol might be useful as a candidate for the treatment of CL.

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