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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