Development of liposomes loaded with anti-leishmanial drugs for the treatment of cutaneous leishmaniasis

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Abstract

Cutaneous leishmaniasis is caused by different species of Leishmania parasites and its available treatments have not yet provided a strong consistent result. The weak response of current chemotherapeutics is due to their deficient effects on stealth parasites inside macrophages, rapid clearance from the site of action and systemic side effects in high doses. Liposomal formulation of anti-leishmanial drugs could overcome these problems. In this study, different liposomal formulations of three famous anti-leishmanial drugs: Glucantime®, miltefosine and paromomycin were prepared by a modified freeze-drying double emulsion method. Liposome size, zeta potential and encapsulation efficiency were evaluated, and their imaging was carried out by means of atomic force microscopy. Three formulations were evaluated in vivo by subcutaneous injection into skin lesions caused by Leishmania major in BALB/c mice. Encapsulation efficiency of prepared liposomes was up to 90%; however, they inherited a bimodal size distribution that caused their encapsulation efficiency to decrease to 50% during filtering sterilization. Besides, the effect of surface charge was significant on preparation procedure, size and encapsulation efficiency. All three formulations reduced amastigote counts and lesion size but only miltefosine-loaded formulations had significant therapeutic effects compared with control group (p < 0.05).

Keywords

Cutaneous leishmaniasis, freeze-drying double emulsion, Glucantime, liposome, miltefosine

Introduction

Leishmaniasis is caused by different strains of Leishmania parasites, which survive and proliferate in macrophages in the mammalian host, and are transmitted by the bite of infected sand flies. There are four kinds of leishmaniasis including visceral (Kala-Azar), mucocutaneous, diffuse cutaneous and cutaneous leishmaniasis (CL; Vandome et al., 2009; World Health Organization, 2000).

Statistically, two million new cases of leishmaniasis occur annually within which more than 1.5 million are CL (World Health Organization, 2000). Anthroponotic and Zoonotic are the two major types of CL caused by Leishmania tropica and Leishmania major parasite species, respectively. CL initiates with a red papule at the bitten site of the sand-fly transforming into a lesion with purple borders. This lesion usually heals spontaneously after several months leaving atrophic scars (Emilio, 2009; Vandome et al., 2009).

Despite self-healing, treatment is indispensable because of issues such as patient debilitation during the disease, transformation into severe forms of CL such as Lupoid, losing a body part, and disfiguring occurring on the face in half of the cases (Momeni & Aminjavaheri, 1994; Sadeghian et al., 2010).

Currently, meglumine antimoniate (Glucantime®) is the first choice treatment for both visceral and CL (Layegh et al., 2009; Vargas-Gonzalez et al., 1999). For more than half of a century, local and systemic injection of this drug has been the standard treatment of CL (Vargas-Gonzalez et al., 1999), but due to inconsistent results and significant side effects has not received FDA approval yet (PanizMondolfi et al., 2011). On the other hand, miltefosine (Impavido®) originally an anti-cancer drug, has become the center of attention due to its significant therapeutic effects on leishmaniasis in its oral and topical forms (Sindermann & Engel, 2006; Sindermann et al., 2004). Also, paromomycin, the other famous anti-leishmanial agent is being administered topically in its ointment form (Krause & Kroeger, 1994; Minodier & Parola, 2007). However, all these available treatments have not provided a strong consistent result due to the rapid clearance from the site of action, ineffectiveness against intracellular parasites and significant side effects in high doses (Aguiar et al., 2009; Al-Jaser et al., 1995; Khatami et al., 2007), resulting an urgent need for a new efficacious treatments (Modabber et al., 2007).
Liposome is a great delivery system to overcome these problems. Liposomes are bilayer lipid membranes encapsulating part of the water phase in which they are suspended (Shailesh et al., 2009). Liposomes passively target drugs to macrophages (Chellat et al., 2005; Cullis et al., 1989; Sharma & Sharma, 1997), and therefore are great candidates in the treatment of immune system diseases. Because of this unique property, liposomes have been exploited for delivery of anti-leishmanial agents to macrophages where intra-cellular parasites stealth and proliferate (Alving, 1983; Black & Watson, 1997; Carvalheiro et al., 2009; New et al., 1978). All of this research led to AmBisome®, which is the liposomal formulation of Amphotericin B (Boswell et al., 1998; Sharma & Sharma, 1997), and has received FDA approval in treatment of visceral leishmaniasis since 1999 (Meyerhoff, 1999); AmBisome® has been ineffective against CL due to the weak therapeutic effect of Amphotericin B on CL’s parasite strains (Reithinger et al., 2001; Yardley & Croft, 1997). There are countless reports on other anti-leishmanial lipid formulations but the focus of all of them is visceral leishmaniasis (Davidson et al., 1991; Reed et al., 1984; Tempone et al., 2010), and indeed, application of liposomes for CL has been neglected. In fact, a liposomal formulation for CL not only could enhance the therapeutic effects of current chemotherapeutics but also might make the topical administration of a hydrophilic drug such as Glucantime® possible. Recently, several reports have been published on the application of paromomycin-loaded liposomes for the treatment of CL (Carneiro et al., 2010; Ferreira et al., 2004), but there is still no report on other formulations include Glucantime® and specially miltefosine. Therefore, the main aim of this study was to prepare liposomal formulations of these two anti-leishmanial drugs in addition to paromomycin and evaluate them in vivo on groups of BALB/c mice that were inoculated subcutaneously with CL parasite strain.

Previous attempts to encapsulate Glucantime® into liposomes have been problematic due to the low encapsulation efficiency of this drug caused by its hydrophilic nature (Chapman et al., 1984; Frézard et al., 2000; Rao, 1986; Ribeiro et al., 2008; Schettini et al., 2005, 2006). Low encapsulation efficiency means that a very large amount of lipid is required to reach therapeutic levels, and also it mandates free agent removal. Therefore, in this study, a modified freeze-drying double emulsion (FDE) method was utilized to fabricate liposomal formulations of anti-leishmanial drugs. FDE method has been reported to produce liposomes with high encapsulation efficiencies for both hydrophilic and hydrophobic drugs (Wang et al., 2006, 2007). Besides, miltefosine is an amphiphilic molecule with a structure that is similar to phosphatidyl choline. Therefore, its ability to play both structural and therapeutic roles was also investigated.

Materials and methods

Materials

Egg yolk phosphatidyl choline (PC, P3556, purity > 99%), phosphatidyl glycerol (PG, P5531, purity > 99%), phosphatidyl ethanolamine (PE, P8068, purity > 98%), cholesterol (Ch, C8667, purity > 99%) and sucrose (Sc, S7903, purity > 99.5%) were purchased from Sigma (St. Louis, MO). Glucantime® (GL, Rorer Rhone-Poulenc Specia, Paris, France) and miltefosine (Mi, Zentaris GmbH, Frankfurt, Germany) powders were generous gifts from the Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences. Paromomycin (Pa) powder was received from Alhavi Pharmaceutical Co. (Tehran, Iran). All other solvents and chemicals were of reagent grade.

Methods

Preparation of liposomal formulations

Liposomes were prepared by a modified FDE method. The FDE method is previously reported to produce small unilamellar sterilized liposomes with high encapsulation efficiency (Wang et al., 2006, 2007). Briefly, Glucantime® and paromomycin were dissolved in the inner aqueous phase (w1). Miltefosine, PC, PE, PG and Ch were dissolved in cyclohexane to form the oil phase (o). Sucrose, the lyoprotectant, was dissolved in the inner and outer aqueous phases with an overall mass ratio that was five times of lipids.

A total of 1.5 ml of (w1) was added to 3 ml of (o), and emulsified by a Silent Crusher Shomogenizer (Heidolph, Germany) at 45 000 rpm for 1 min. This first emulsion (w1/o) was mixed with 4 ml of outer aqueous phase (w2) at 30 000 rpm for 1 min to form the second emulsion (w1/o/w2). Immediately, 4 ml of this double emulsion was transferred to 10 ml vials, and frozen in a −20°C freezer (cooling rate: ~1 °C/min).

These frozen vials were dried by an Alpha 1-2 LD freeze dryer (Christ, Germany) in three steps. First, they were kept at −52°C for 12 h, then the chamber pressure was decreased to 20 Pa, and vials were kept at this pressure for 24 h. Finally, secondary drying was carried out at the same pressure but at room temperature for 24 h.

Lyophilized powders were kept in nitrogen-filled rubber-sealed vials at 4 °C, and when needed, addition of the original volume of water produced liposomes. For sterilization, final lipid solutions were passed through a 0.22 μm BIOFIL® syringe filter.

Table 1 shows different formulations that were prepared. The molar ratios for different formulations are as follows:

Size distribution studies

The size distribution of formulations 2, 2-a and 2-b (without filtration), and all other formulations after filtration were studied in triplicate using a coulter counter (Mastersizer 2000, Malvern, Worcestershire, UK) with a 10 nm limit of detection. Particle refractive index, light absorption and obscuration values were 1.30%, 0.1% and 0.8%, respectively. The volume weighted mean diameter of liposomes and their specific surface area are reported.

Zeta potential

The zeta potential of all formulations was determined in triplicate by a zetasizer (DTS4.20, Malvern, Worcestershire,
Table 1. Composition of prepared formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Freezing method</th>
<th>PC (mg)</th>
<th>Ch (mg)</th>
<th>PE (mg)</th>
<th>PG (mg)</th>
<th>Mi (mg)</th>
<th>Pa (mg)</th>
<th>Gl (mg)</th>
<th>Sc/w1</th>
<th>Sc/w2</th>
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<td>3.13</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.67</td>
<td>15.1</td>
</tr>
<tr>
<td>2</td>
<td>SF</td>
<td>5.71</td>
<td>3.53</td>
<td>–</td>
<td>1.41</td>
<td>–</td>
<td>–</td>
<td>0.67</td>
<td>14.55</td>
<td>38.7</td>
</tr>
<tr>
<td>2-a</td>
<td>SF</td>
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<td>3.53</td>
<td>–</td>
<td>1.41</td>
<td>–</td>
<td>0.67</td>
<td>14.55</td>
<td>38.7</td>
<td></td>
</tr>
<tr>
<td>2-b</td>
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<td>3.53</td>
<td>1.36</td>
<td>–</td>
<td>–</td>
<td>0.67</td>
<td>14.55</td>
<td>38.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>SF</td>
<td>7.9</td>
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<td>–</td>
<td>–</td>
<td>1.13</td>
<td>–</td>
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<td>14.45</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>1.13</td>
<td>–</td>
<td>14.45</td>
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</tr>
<tr>
<td>6</td>
<td>SF</td>
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<td>3.53</td>
<td>1.36</td>
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<tr>
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<td>4.1</td>
<td>0.67</td>
<td>–</td>
<td>9.9</td>
<td>26.5</td>
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<td>–</td>
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<td>2.98</td>
<td>1.13</td>
<td>10.8</td>
<td>28.8</td>
<td></td>
</tr>
<tr>
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<td>SF</td>
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<td>1.13</td>
<td>10.7</td>
<td>28.6</td>
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</tr>
<tr>
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<td>3.53</td>
<td>–</td>
<td>1.41</td>
<td>2.98</td>
<td>–</td>
<td>0.67</td>
<td>10.8</td>
<td>28.8</td>
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<tr>
<td>15</td>
<td>SF</td>
<td>–</td>
<td>3.53</td>
<td>1.36</td>
<td>–</td>
<td>2.98</td>
<td>–</td>
<td>0.67</td>
<td>10.7</td>
<td>28.6</td>
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<tr>
<td>16</td>
<td>SF</td>
<td>4.28</td>
<td>2.83</td>
<td>–</td>
<td>1.41</td>
<td>1.49</td>
<td>–</td>
<td>0.67</td>
<td>13.65</td>
<td>36.4</td>
</tr>
</tbody>
</table>

RF, rapid freeze; SF, slow freeze; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; Ch, cholesterol; Mi, miltefosine; Pa, paromomycin; Gl, glucantime; Sc, sucrose; w1, inner aqueous phase; w2, outer aqueous phase.

Encapsulation efficiency and drug loading

The amount of free drug (before filtration) was measured by the UV/Vis HPLC detector (Bio-Tek, Kontron Instruments, Milan, Italy) with a reverse phase C18 column (150 × 4.6 mm², 5 μm, Waters, Milford, MA). The mobile phase was 100% deionized filtered water with a flow rate of 1 ml/min. Wavelengths were 207 nm for Glucantime, and 215 nm for paromomycin. A total of 20 μl of samples was directly injected into the HPLC. Sucrose, free Glucantime and free paromomycin passed the column early with retention times of 1.17, 1.76 and 1.93 min, respectively. Calibration curves were produced by standard solutions of both drugs in water. Correlation coefficients were 0.97 (25–200 μg/ml) for Glucantime, and 0.95 (50–300 μg/ml) for paromomycin. The encapsulation efficiency of miltefosine before filtration was assumed to be complete due to the fact that it is a structural component of liposome bilayer (Papagiannaros et al., 2006).

Filtration procedure eliminates large liposomes and their entrapped drug. So, it is essential to evaluate encapsulation efficiency once more after filtration; therefore, 300 μl of filtered formulations was added to 2 ml of chloroform and vortexed for 5 min to dissolve liposomes. This solution was centrifuged at 20 000 g for 10 min, and then 20 μl of its upper aqueous phase was injected into the HPLC to determine the drug concentration by the same procedure mentioned above. The encapsulation efficiency of miltefosine after filtration was determined based on the cholesterol studies which
determined the amount of structural components including miltefosine being lost during filtration.

Encapsulation efficiency and drug loading before (%EE, %DL), and after sterilization (%EEs, %DLS) were calculated based on the following formula:

\[
\begin{align*}
%\text{EE} &= \frac{m_0 - m_f}{m_0} \times 100, \\
%\text{DL} &= \frac{m_0 - m_f}{m_0 - m_l + m_f} \times 100, \\
%\text{EE}_s &= \frac{m_0}{m_s - m_f} \times 100, \\
%\text{DL}_s &= \frac{m_s - m_f}{m_s - m_f + m_{ls}} \times 100
\end{align*}
\]

where \(m_0\) is the original amount of drug, \(m_f\) the amount of free drug, \(m_s\) the total amount of drug after filtration and \(m_l\) and \(m_{ls}\) the original and filtered amount of lipids, respectively.

In vivo evaluation

Due to difficulties in growing Leishmania parasites in vitro and the usual lack of correlation between in vitro and in vivo results, three formulations were chosen to be directly tested in vivo. Male BALB/c mice, 6–8 weeks old with a body weight of \(\sim 20\) g were obtained from the Animal Breeding Stock Facility of Razi Institute of Iran. Experiments were carried out according to the ethical protocol in the Declaration of Helsinki, and measures were taken to protect the animals from pain and discomfort.

The animals were inoculated subcutaneously with approximately \(2 \times 10^6\) \textit{L. major} promastigotes (MRHO/IR/75/ER) at the base of the tail. In a month, a local lesion was obvious and a control group (without treatment). For treatment, the dry cake of liposomes was reconstituted with the original and a control group (without treatment). For treatment, the presence of \textit{Leishmania} protozoa was confirmed by a parasitological examination of the lesion site. Mice were randomly divided into four groups for formulations 2, 8, 16 and a control group (without treatment). For treatment, the dry cake of liposomes was reconstituted with the original amount of water, and then passed through a 0.22 \(\mu\)m filter into a sterile capped vial. From this vial, 0.2 cc of formulation was injected subcutaneously in and around the lesion site twice a week for 35 days.

Therapeutic effects of formulations were compared with the non-treatment group by the measurement of the size of the skin lesions in millimeters (before treatment, 25 and 35 days after beginning of the treatment), and observation and counting of amastigote forms at the same time by light microscopy with a high magnification (\(\times 1000\)) on a smear prepared from the lesion (Chang et al., 2004).

The statistical significance between groups was analyzed by a two tailed Student’s \(t\)-test using Microsoft Excel 2007 software (Microsoft, Redmond, WA). \(p\) Values lower than 0.05 were considered statistically significant.

Results

Preparation of liposomal formulations

Some unexpected problems were faced in the preparation of liposomes by the FDE method. In formulations without PG or PE, uncharged formulations, the second emulsion was not stable at all. Actually, in less than 5 min, the second emulsion separated into a milky layer of emulsion on top and a cloudy water phase on the bottom (Figure 1A). Higher rates of homogenizer, applying probe sonication and increase in phospholipid concentration were not successful in preventing this phase separation. However, addition of a small amount of charged phospholipids, PG or PE, stabilized the second emulsion up to 2 h (Figure 1B). These charged formulations were frozen in the freezer before freeze drying (Table 1). In contrast, in uncharged formulations, a rapid freezing method was required; freezing by liquid nitrogen.

There was another problem with the FDE method. Previously, sterilizing was reported to take place by 0.22 \(\mu\)m syringe filtration of the second emulsion before freezing (Wang et al., 2006, 2007). However, in our study, filtration at this stage cracked the filter in most cases, and sometimes led to phase separation. Similarly, extrusion of the second emulsion through a 200 \(\mu\)m filter led to phase separation. As a result, filter sterilizing was shifted to the last step after reconstitution.

This modified FDE method was employed to prepare different formulations after solving these two problems. The final product was a lyophilized white cake that was stable at 4°C for months (Wang et al., 2006; Figure 1C). In contrast, room temperature and contact with air oxidized it in less than 2 weeks leaving a black powder behind. Addition of water to this lyophilized cake immediately dissolves it and forms liposomes (Figure 1D).

As expected, formulations 10, 11, 14 and 15 were successfully prepared with miltefosine as the structural agent and without any phospholipid. Surprisingly, achieving the first emulsion was impossible for all paromomycin-loaded formulations with the exception of formulation 10. In these formulations (4, 5, 6, 12 and 13), which all contain a phospholipid with paromomycin, homogenizing produced white agglomerated particles.

Size distribution of formulations

Figure 2 profiles a bimodal size distribution of formulation 2 with a large peak at 146 nm, and a small peak at 901 nm. In formulation 2-a, probe sonication was applied to the first emulsion to achieve a more homogeneous size distribution, but it hardly had any effect. In contrast, extrusion and syringe
filtration of the final liposome solutions eliminated large or aggregated liposomes and left only the first peak.

Table 2 shows the mean diameter of all formulations after filtration. Obviously, their mean diameters are smaller than 220 nm because they have lost their large or aggregated liposomes during filtration. Student’s t-test also shows that charged formulations are significantly (p < 0.05) smaller than their corresponding uncharged formulations (e.g. formulations 2 and 3 versus 1; 8 and 9 versus 7; 14 and 15 versus 11).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Mean diameter (nm)</th>
<th>Specific surface area (m²/g)</th>
<th>Zeta potential (mV)</th>
<th>Percentage of liposome loss after filtration (%)</th>
<th>%EE</th>
<th>%DL</th>
<th>%EEs</th>
<th>%DLs</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>183 ± 25</td>
<td>48.7 ± 6.7</td>
<td>−3.17 ± 0.53</td>
<td>63.2 ± 2.6</td>
<td>74</td>
<td>4.3</td>
<td>33</td>
<td>5.2</td>
</tr>
<tr>
<td>2</td>
<td>138 ± 20</td>
<td>50.5 ± 7.3</td>
<td>−60.2 ± 0.86</td>
<td>42.8 ± 3</td>
<td>90</td>
<td>5.4</td>
<td>51</td>
<td>5.3</td>
</tr>
<tr>
<td>2-a</td>
<td>151 ± 28</td>
<td>50.9 ± 9.2</td>
<td>−58.8 ± 1.01</td>
<td>40.7 ± 2.1</td>
<td>88</td>
<td>5.2</td>
<td>52</td>
<td>5.4</td>
</tr>
<tr>
<td>2-b</td>
<td>173 ± 15</td>
<td>49.1 ± 4.1</td>
<td>−55.3 ± 0.84</td>
<td>17.2 ± 4.5</td>
<td>59</td>
<td>3.6</td>
<td>59</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>140 ± 22</td>
<td>50.3 ± 8</td>
<td>+39.6 ± 1.17</td>
<td>47.5 ± 1.3</td>
<td>91</td>
<td>5.3</td>
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<td>5.7</td>
</tr>
<tr>
<td>7</td>
<td>166 ± 20</td>
<td>49.6 ± 6.1</td>
<td>−3.69 ± 0.55</td>
<td>58.5 ± 3</td>
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<tr>
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<td>46 ± 6.2</td>
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<td>14</td>
<td>125 ± 16</td>
<td>51.6 ± 6.3</td>
<td>−64.2 ± 0.85</td>
<td>41.2 ± 2.9</td>
<td>90</td>
<td>5.7</td>
<td>57</td>
<td>6.1</td>
</tr>
</tbody>
</table>

%EE, encapsulation efficiency before filtration; %DL, drug loading before filtration; %EEs, encapsulation efficiency after filtration; %DLs, drug loading after filtration. Numbers show mean value ± SD (n = 3).

Zeta potential of formulations

Zeta potential is the surface electrostatic potential of a particle that depends on its size and surface molecules. Figure 3(A) and (B) shows the zeta potential profiles of formulations 2 and 3, respectively. The presence of PG in formulation 2 with its glycerol cap group created a negative charge of −60.2 mV that is similar to the previous report by Pavelic that has reported a zeta potential of −56.2 mV for a similar liposome formulation (PC:PG (9:1)) with the same size.
In contrast, PE with the NH₂ group generated a positive charge of +39.6 mV in formulation 3. The zeta potential of uncharged formulations (e.g. formulation 1) was close to neutral. The zeta potential of all formulations is reported in Table 2.

### Cholesterol concentration

Table 2 shows the percentage of liposome loss due to the filtration. All formulations have lost a great amount of liposomes during this process with the exception of formulation 2-b that was extruded prior to syringe filtration; this formulation has lost only 17% of its liposomes during filtration and/or extrusion.

These cholesterol-concentration results together with size distribution studies imply that in charged formulations (2, 3, 8, 9, 14, 15 and 16) more than half of liposomes are smaller than ~220 nm, while uncharged formulations such as formulation 1 are mostly constituted of large or aggregated liposomes.

### Encapsulation efficiency and drug loading

Prior to filtration, the amount of free drug was determined by HPLC. Subtracting this amount from its original amount resulted in %EE and %DL for different formulations that are presented in Table 2.

The %EE for all Glucantime®-loaded formulations (1, 2, 3, 11, 14, 15 and 16) is higher than 70%, with uncharged formulation 1 having the lowest value. Similarly, paromomycin-loaded formulation 10 displayed high %EE, but in contrast, extrusion has substantially decreased %EE in formulation 2-b. In the case of miltefosine, as described in Methods section, %EE is considered to be 100% before filtration, because it is the structural part of the liposomes.

Subsequently, sterilization of formulations by syringe filters excluded a large amount of liposomes and their drug content. Therefore, it was essential to determine %EEₐ and %DLₐ again (Table 2). Once more, charged formulations demonstrated higher %EEₐ because their original %EE was higher and plus, filtration has removed a lower amount of their liposomes.

### Atomic force microscopy

Imaging of liposomes was carried out using AFM only for formulation 2. Figure 4(A) shows the topographic image of a large number of liposomes in the height mode. White round spots are liposomes and their mean diameter and height were measured by Image Pro Plus and Dualscope™ software, respectively (Figure 4B). Based on these analyses, the mean diameter of the liposomes is 101 ± 23 nm, and their mean height is 15 ± 5 nm. This mean diameter of the liposomes is almost similar to their original size of 138 nm (formulation 2) derived from light scattering. However, their height has shrunk tremendously. It should be borne in mind that even though no rigorous fixation is required for the preparation of AFM samples, the results will be influenced by liposome spreading and flattening as they are soft and deformable, and then, drying greatly reduces their height (Jass et al., 2003; Sriamornsak et al., 2008). In other words, drying of liposomes on a mica surface is like a balloon that settles on a surface and is then depleted of air.

The other common phenomenon in the AFM study of liposomes (Jass et al., 2003) is their elongation by the cantilever that causes a white line in the direction of the cantilever movement (Figure 4A). Efforts to eliminate these lines by continuous adjustment of set points were unsuccessful probably due to the weak coupling of the liposomes to the mica surface because they are both negatively charged.

A high magnification 3D image in Figure 4(C), demonstrates a closer view of liposomes, and Figure 4(D) shows a phase image of an agglomerated particle that is clearly formed from multiple liposomes probably because during the drying process, the possibility of liposomes aggregation is high. Liposome aggregation might be the reason of bimodal size distribution observed by light scattering in our formulations which could be eliminated using processes such as extrusion and filtration.

### In vivo evaluation

From all formulations, only formulations 2, 8 and 16 were tested in vivo. These three formulations were chosen because they could make it possible to compare the therapeutic effect of miltefosine and Glucantime®-loaded liposomes. They had almost the same liposomal composition, size and zeta potential while entrapping different drugs. Furthermore, these formulations had the highest %EE. Formulation 2 was loaded with Glucantime® alone (124 µg/ml) and

![Figure 3. The zeta potential of: (A) formulation 2 with phosphatidyl glycerol and (B) formulation 5 with phosphatidyl ethanolamine.](image-url)
formulation 8 with miltefosine alone (293 μg/ml). Formulation 16 was loaded with both drugs at concentrations of 139 and 309 μg/ml for Glucantime® and miltefosine, respectively.

Table 3 shows the therapeutic effect of these formulations on the lesion size and number of amastigotes in localized CL induced by *L. major* on BALB/c mice compared with control group.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lesion size (mm) Before treatment</th>
<th>Day 25</th>
<th>Day 35</th>
<th>p Value</th>
<th>Amastigote count Before treatment</th>
<th>Day 25</th>
<th>Day 35</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.5 ± 7</td>
<td>8.3 ± 5.3</td>
<td>6.7 ± 4.4</td>
<td>&gt;0.05</td>
<td>2 ± 0.8</td>
<td>1.8 ± 1</td>
<td>1.5 ± 1.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>8</td>
<td>8.6 ± 3.5</td>
<td>5 ± 2.7</td>
<td>3.2 ± 1.6</td>
<td>0.05</td>
<td>2.5 ± 1</td>
<td>1 ± 0.5</td>
<td>0.75 ± 0.5</td>
<td>0.03</td>
</tr>
<tr>
<td>16</td>
<td>11.4 ± 4.8</td>
<td>7.2 ± 3.1</td>
<td>2.2 ± 1</td>
<td>0.03</td>
<td>3 ± 0.8</td>
<td>2.25 ± 1.5</td>
<td>0.75 ± 1.5</td>
<td>0.04</td>
</tr>
<tr>
<td>Control</td>
<td>9.3 ± 3.2</td>
<td>–</td>
<td>15 ± 5.2</td>
<td>&gt;0.05</td>
<td>1.8 ± 1.5</td>
<td>–</td>
<td>2.5 ± 2.4</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

p Values, comparing lesion size and amastigote count before and after treatment. Numbers show mean value ± SD.

Table 3 and Figure 5 show the therapeutic effect of these formulations on the lesion size and number of amastigotes. Before treatment (Figure 6A), the difference between the lesion size of the therapeutic groups and the control
non-treatment group is not significant ($p > 0.48$), but after treatment (Figure 6B), in all three treatment groups, the lesion size is significantly smaller than the control group ($p < 0.05$). This shows that all formulations have successfully reduced the lesion size, but comparing the lesion size in each therapeutic group individually (Table 3), clarifies that only the effect of miltefosine-loaded formulations (8 and 16) has been significant ($p < 0.05$).

Similarly, amastigote counts of the skin lesions decreased in all groups except for the control group, and again, only miltefosine-loaded formulations have reduced the number of amastigotes significantly (Table 3). Also, scenes of disintegrated protozoa (Figure 6C) and parasite free macrophages (Figure 6D) were common in the smear of the treatment groups.

**Discussion**

The mechanism of the FDE method has been described previously in detail (Wang et al., 2006). This method is based on the emulsifying ability of phospholipids. Briefly, homogenizing ($w_1$) with ($o$) phase in low molar values of phospholipids produces a reverse micellar emulsion phase ($L_2$) (Angelico et al., 2000). However, some dissolved phospholipids still remain in the ($o$) phase that generate the second layer of vesicles after the addition of the ($w_2$) phase to
the first emulsion. Subsequently, in the freeze drying step, sucrose as the lyoprotectant preserves these bilayer vesicle structures by replacing water molecules on their water-faced surfaces, finally, addition of water to this lyophilized product binds the two vesicle layers forming unilamellar liposomes (L₁₂) (Angelico et al., 2000).

Based on this mechanism, water soluble drugs are entrapped inside of monolayer vesicles during the first emulsion and, in the next steps, they are unable to leak out because they should diffuse through the cyclohexane oil phase, and this would lead to high %EE. As a result, in this study, %EE of Glucantime® is much more than the previous reports (Chapman et al., 1984; Frézard et al., 2000; Rao, 1986; Ribeiro et al., 2008; Schettini et al., 2005, 2006), where liposomes were prepared by dehydration–rehydration method through introduction of drug solutions to freeze-dried empty liposomes. In fact, only drugs with low partition coefficients between oil and water phases (Maiti et al., 2010; Wang et al., 2006), or fusion of bilayer vesicles/liposomes could decrease %EE. The latter has caused uncharged formulation 1 to have a significantly (p < 0.05) lower %EE than formulation 2 that is charged with PG.

Also, in uncharged formulations, the second emulsion was not stable mandating a rapid-freezing step by liquid nitrogen. However, it has been reported that freezing in boiling nitrogen results in some liposome types with enhanced leakage of encapsulated compounds (Van Widen, 2003). Therefore, slow freezing which became possible by the addition of PG or PE is superior. These charged phospholipids prevent the fusion of bilayer vesicles and phase separation by increasing the zeta potential. Stabilizing bilayer vesicles is previously reported by other charged phospholipids including PS (Wang et al., 2006) and PE-PEG (Wang et al., 2007). It is very well known that zeta potential affects liposomes stability in aqueous solutions and their clearance rate inside the body but in the FDE method, zeta potential also affects the preparation procedure and the stability of emulsions.

Despite its advantages, the major drawback of the FDE method, in contrast to the previous report (Wang et al., 2006, 2007), is its bimodal size distribution that caused a great amount of liposome loss during filtering sterilization. Phase separation of second emulsions after filtration (or extrusion), caused by the rupture of large bilayer vesicles shows that this bimodal size distribution exists right from the beginning. Applying probe sonication in the emulsion steps was not capable of solving this problem. Actually, it seems that in the FDE method, similar to all other emulsion methods, many variables affect the size of the final product and this complicates the preparation procedure extremely (Momeni & Mohammadi, 2009). In fact, only extrusion or filtration of the final liposome solution led to a narrow size distribution. The former breaks down liposomes when they are consecutively passed through a polycarbonate filter. As a result, drug leaked out and low %EE was the outcome. In contrast, based on the cholesterol concentration studies, filtration has excluded large or aggregated liposomes without breaking them down. Therefore, filtration is a better alternative than extrusion because it does not dictate an additional free agent removal procedure.

Figure 7. The chemical structures of: (A) Phosphatidyl choline; (B) Miltefosine; and (C) paromomycin.

It should be taken into the account that despite reduction in %EE during filtration, %DL almost remains constant in all formulations because of simultaneous removal of phospholipids and encapsulated drug.

Aforementioned, liposomal formulations containing a phospholipid and paromomycin were not prepared successfully. In these formulations, homogenizing produced white agglomerated particles. Comparing formulation 4 with 10, and the structure of PC with miltefosine clarifies the possible cause of agglomeration: miltefosine structure differs from that of PC in having only one group of fatty acid, and lacking the glycerol group (Figure 7A and B). Fatty acids rarely incorporate in chemical reactions; therefore, a chemical bond between O–C=O groups of phospholipids and possibly, NH₂groups of paromomycin (Figure 7C) might be the cause of agglomeration. Yet, this assumption is not conclusive requiring more analytical investigations.

In vivo studies demonstrated the therapeutic effect of liposomal drugs in much lower doses than traditional ones (Krause & Kroeger, 1994; Layegh et al., 2009; Minodier & Parola, 2007; Sindermann & Engel, 2006; Sindermann et al., 2004; Vargas-Gonzalez et al., 1999), probably because liposomes passively target intra-cellular parasites, and inhibit the rapid clearance of drugs. In previously published literatures, the therapeutic effects of Glucantime®-loaded
liposomes have been both acknowledged and criticized on visceral leishmaniasis (Chapman et al., 1984; Frézard et al., 2000; Rao, 1986; Ribeiro et al., 2008; Schettini et al., 2005, 2006); the same statement could be held for miltefosine-loaded liposomes (Papagianiaros et al., 2005). However, we could not compare them with our results owing to the completely different mode of administration (intravenously), and target (reticulo-endothelial system and Leishmania donovani) (Weldon et al., 1983). One should note that subcutaneously administered liposomes do not have access to the bloodstream as the permeability of blood capillaries is restricted to small molecules (Oussoren & Storm, 2001). Instead, based on their size, they are taken by regional lymphatic capillaries or remain at the site of injection (Oussoren & Storm, 1998, 2001; Oussoren et al., 1997). The threshold size of liposomes for restrained lymphatic delivery is 100–120 nm (Trubetskoy et al., 1998). Therefore, our large liposomes are mostly localized in the injection site, and this is a great opportunity for their phagocytosis by the skin macrophages (Oussoren & Storm, 1999), where Leishmania protozoa proliferate. Even if they are not being phagocytized by macrophages as has been suggested for miltefosine-loaded liposomes (Papagianiaros et al., 2005; Zeisig et al., 1996a,b), the liposomes could provide a sustained localized release of chemotherapeutics. There are reports that show miltefosine-loaded liposomes induce NO release from macrophages (Eue et al., 1995; Zeisig et al., 1995). This effect in addition to the direct toxic effects of miltefosine on the parasites could explain why there was a significant therapeutic effect in miltefosine-loaded liposomes. In future, further in vitro and in vivo release studies are needed to explore the release behavior of these formulations. Moreover, more comprehensive in vivo studies are required to determine the optimum therapeutic dosage of these different formulations.

Conclusion
In this study, liposomal formulations of Glucantime®, miltefosine and paromomycin were prepared by a modified FDE method for the treatment of CL. High %EE was the distinguishing characteristic of the FDE method; however, a bimodal size distribution and the necessity to sterilize by filtration weakened this quality and in the trade-off, reduced the final %EE to 50%, which is still acceptable for hydrophilic drugs. The size and %EE in different formulations were primarily dependent on their surface charge, which also plays an important role in the stability of formulations in the FDE method.

In vivo results pictured the possibility of administering liposomes for the treatment of CL. Different liposomal formulations were evaluated while administered subcutaneously. However, developing these liposomal formulations into an optimum technically and clinically feasible topical product is more preferred and requires much more investigation that hopefully this study acts as its start-up.

Declaration of interest
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References


