AVALIAÇÃO DA EFICÁCIA DE NANOPARTÍCULAS CONTENDO PENTAMIDINA PARA O TRATAMENTO DA LEISHMANIOSE TEGUMENTAR

MAYARA GOIS DOS SANTOS

SÃO CRISTÓVÃO - SE

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Trabalho de Conclusão de Curso apresentado como exigência para obtenção do título de Bacharel em Farmácia.


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EVALUATION OF THE EFFICACY OF NANOPARTICLES CONTAINING PENTAMIDINE FOR THE TREATMENT OF LEISHMANIOSE TEGUMENTAR

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ABSTRACT

Leishmaniasis are infectious-parasitic diseases caused by protozoa of the genus Leishmania. Treatment is mainly by antimonials. Pentamidine is an alternative for treatment with antimonials, but it is administered by deep intramuscular injection and presents some adverse effects such as vomiting, headache and myalgia. Drug delivery systems such as lipid nanoparticles have shown as an interesting alternative to treatment, because they have a smaller particle size, improve bioavailability and can be administered by various routes and improve the efficacy and safety of the drug. Thus, the objective of this study was to obtain nanoparticles containing pentamidine and evaluate them in the treatment of tegumentary leishmaniasis using alternative routes of administration, such as the topical route. Nanostructured Lipid Carriers (NLC) were obtained by w/o/w double emulsion. Size, polydispersity index (IPD), Zeta Potential and encapsulation efficiency of the NLC were determined. Ex vivo cutaneous permeation study, cytotoxicity and leishmanicidal activity of the best formulation were also determined. NLC showed nanometric size from 172 to 266 nm, IPD below 0.5, and encapsulation efficiency of 14 - 75%. NLC 2 have also presented sustained release profile. However, they were not able to promote the skin permeation of the drug. The cytotoxicity of NCL 2 was similar to control (free pentamidine). The leishmanicidal activity was low, where IC$_{50}$ in promastigotes of the pentamidine from NLC was 7.2 times higher than free pentamidine. Thus, this system is not an alternative for topical treatment.

Key words: tegumentary leishmaniasis, pentamidine, nanostructured lipid carriers,
1. INTRODUCTION

Leishmaniasis is a neglected disease that is associated with parasites of the genus *Leishmania*. The lesions caused by these parasites can vary from cutaneous to mucocutaneous lesions developed at the bite site of the phlebotomine sandflies infected by the parasites. Leishmaniasis can be classified mainly in tegumentary leishmaniasis (TL) and visceral leishmaniasis (VL), mainly affecting the liver and spleen and may lead to death (Al-Kamel, 2017).

Tegumentary leishmaniasis is characterized by the presence of one or more papules, nodule or ulcers present on the skin and/or mucous membranes. The severity on the infection depends on some factors such as *Leishmania* species, number of parasites inoculated, and the nutritional and immunological status of the host (Goto and Lauletta Lindoso, 2012).

The drug of first choice for the treatment of cutaneous leishmaniasis is Meglumine Antimoniate. However, therapy with this drug has some drawbacks because the treatment is long, requires parenteral administration and presents high toxicity. Many side effects are described such as a join pain, abdominal pain, changes in renal and hepatic functions, pancreatitis and cardiovascular toxicity (Adinehbeigi et al., 2017).

Amphotericin B and Pentamidine Isethionate are recommended as second line drugs for the leishmaniasis treatment (BRASIL, 2010). Amphotericin B has high efficacy, however its use is limited, since the treatment is long, it presents high toxicity and necessity of a qualified professional for its administration, is used in the impossibility of using the Antimonial or when the treatment with this is not satisfactory. (Neves et al., 2011).

Pentamidine (PTD) is an aromatic diamine, which has been used in the treatment of tropical diseases including leishmaniasis since 1930 (Aronson, 2006). Pentamidine is available
commercially in two forms, mesylate and isethionate; it is marketed in the form of lyophilized powder, which when solubilized in sterile water can be administered by inhalation or by injection (intramuscular or intravenous) (Neves et al., 2011; Sundaramurthi et al., 2012)

Among the available treatments for tegumentary leishmaniasis, pentamidine has lower side effect and shorter treatment time (Neves et al., 2011). However, treatment for tegumentary leishmaniasis using pentamidine needs to be optimized since its parenteral administration cause discomfort and pain to the patient, and is associated to adverse effects.

Nanotechnology can be a tool for reducing or even solving these problems (Kumar, 2009). Nanostructured lipid carriers (NLC) have been widely studied as drug delivery systems (Shah et al., 2010). They are composed of a mixture of solid and liquid lipids dispersed in an aqueous solution containing surfactants. They have several advantages such as low toxicity of the compounds, ability to protect the drug of the degradation improving the stability, and nanometric size that promotes a larger contact surface and can increase the permeation of the drug through the skin or mucous membranes (Kelidari et al., 2017; Shah et al., 2010).

In view thereof comments, the present work proposes to obtain NLC containing pentamidine and evaluate its efficacy and toxicity for the treatment of tegumentary leishmaniasis, using alternative routes of administration, such as the topical route.

2. MATERIALS AND METHODS

2.1. Material

Pentamidine and Squalene were purchased from Sigma-Aldrich®, Beeswax was from GM Ceras (Brazil) and Phospolipon 80H was from Lipoid. All solvents used in HPLC
quantification were HPLC grade. Other solvents and reagents were of analytical grade and obtained from national suppliers.

### 2.2. Animals

Male Wistar rats (200–300 g) were obtained from the vivarium of the Department of Physiology of the Federal University of Sergipe after approval by the local Ethics Committee (protocol 20/2016). In the vivarium, rats were housed in individual cages at 25 °C with a 12:12 h light/dark cycle. Food and water *ad libitum* was provided and the sawdust was changed every two days. All animal experiments were performed according to the NIH Guidelines for the Care and Use of Laboratory Animals.

### 2.3. Formulation preparation

NLC was obtained by w/o/w double emulsion - solvent diffusion method associated with ultrasonication. Initially, the internal aqueous phase containing PTD in H₂O or 1% Dextran 70 (Lacrima Plus – Alcon ®) solution was prepared. After, the organic phase (ethanol, Phospholipon 80H, squalene and beeswax) and the external aqueous phase (*Tweem 80* and water) were prepared. The solid and liquid lipids with the Phospholipon 80H were heated 5 – 10 °C above the melting point of the solid lipid. Thereafter, 3mL of ethanol was added to the lipid mixture. The internal aqueous phase was then dispersed in the hot organic phase using a probe sonicator (35 Hz) (*VIBRACELL® CV 18*) for 2 minutes forming the primary emulsion, which has been dispersed into the external aqueous phase using Ultra-Turrax (*IKA® T25*) at 14000 rpm for 1 minute. Finally, the dispersion was sonicated for 5 minutes (35 hz) and cooled in an ice bath until 5° C. The formulation was then stirred under magnetic stirring for 24 hours for solvent evaporation. Table 1 shows the concentration of the components used to prepare the NLC (Kelidari et al., 2017).
Table 1: Composition of NLC obtained

<table>
<thead>
<tr>
<th>Composition</th>
<th>NLC&lt;sub&gt;inert&lt;/sub&gt; (%)</th>
<th>NLC 1 (%)</th>
<th>NLC 2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug (PTD)</td>
<td>-</td>
<td>0,2</td>
<td>0,2</td>
</tr>
<tr>
<td>Beeswax</td>
<td>0,28</td>
<td>0,28</td>
<td>0,28</td>
</tr>
<tr>
<td>Squalene</td>
<td>0,12</td>
<td>0,12</td>
<td>0,12</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Phospolipon</td>
<td>0,25</td>
<td>0,25</td>
<td>0,25</td>
</tr>
<tr>
<td>Ethanol</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>1% dextran solution</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Water (internal phase)</td>
<td>5</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Water (external phase)</td>
<td>93</td>
<td>93</td>
<td>93</td>
</tr>
</tbody>
</table>

2.4. Characterization of NLC

The determination of the particle size, and polydispersity index (IPD) was performed by the photon correlation spectroscopy method. The zeta potential (ZP) was determined by the electrophoretic mobility using the same equipment, a Zetasizer (MALVERN INSTRUMENTS®, UK). For these analysis the NLC samples were diluted in 1:20 (w/w) ultrapure water, then sonicated for 30 minutes using an ultrasound bath (n = 3).

2.5. Determination of Encapsulation Efficiency

Nanoparticles dispersions were centrifuged at 12,000 rpm for 30 minutes at 4°C using ultrafiltration tubes (Vivaspin MWCO 10,000 Da). The supernatant from formulation was collected and analyzed by high performance liquid chromatography coupled with ultraviolet detection (HPLC-UV). The encapsulation efficiency was determined through the indirect method, the amount of free drug present in the aqueous phase of dispersions by Equation 1.
\[ EE(\%) = \left( \frac{m_1 - m_2}{m_1} \right) \cdot 100 \]  

At where:

- \( EE \% \): Encapsulation efficiency
- \( m_1 \): mass of drug used in the formulation
- \( m_2 \): mass of drug present in the supernatant

2.6. **In vitro release and ex vivo skin permeation.**

2.6.1. **Preparation of formulations**

Gelified dispersions were used to facilitate the adhesion of the formulation under the membrane. NLC dispersions were mixed with a 0.4% hydroxyethylcellulose gel in the ratio 1:1 (w / w) of gel: NLC dispersion. The control was obtained by dissolving PTD in pH 7.4 phosphate buffer, and incorporated in hydroxyethylcellulose gel 1:1 (w / w).

2.6.2. **Skin preparation**

For this assay, rat skin was used as the biological membrane. The rats were euthanized under anesthesia by using an overdose of isoflurane. Dorsal hair was removed by electrical shaver carefully. Full thickness skin was excised from the shaved dorsal site. Subcutaneous fat was removed using tweezers, and the skin pieces were washed and used immediately.

2.6.3. **In vitro Release and ex vivo permeation experiments**

The **ex vivo** permeation experiments were performed using modified Franz type diffusion cells with diffusion area of 1.275 cm\(^2\) and volume of 14 mL (Carvalho et al., 2016). For the **ex vivo** permeation experiments mouse skin was used as membrane, and for the **in vitro** release studies dialysis membrane was used, these remained in contact with the receptor.
solution: phosphate isotonic buffer pH 7.4 (0.2 M). In the \textit{in vitro} release studies, dialysis membrane was used as barrier. NLC formulation or control was placed over the entire skin area or membrane (400 ± 4 µL), avoiding the formation of bubbles between the formulation and membrane. The experiments were conducted at 37 ± 1 °C and the receptor solution was under constant agitation at 300 rpm using a magnetic stirrer and maintained at 37 ± 0.5 °C. After predetermined times, aliquots (1 mL) of the receptor solution were collected for quantification of PTD. The collected samples were filtered on 0.45 µm PTFE membrane filters and analyzed by HPLC-UV for PTD quantification.

2.6.4. \textit{Data Analysis}

Fluxes (µg.cm$^{-2}$.h$^{-1}$) were calculated using the angular coefficient of the equation obtained by the linear portion of the graph of permeated drug amount (µg.cm$^{-2}$) per time. The PTD permeated amount after 24 (Q24) hours of experiment was also calculated. The permeation enhancing activities were expressed as enhancement ratios of flux (ER) as shown below (El-Kattan et al., 2000)

\[
ER = \frac{\text{PTD flux in NLC formulation}}{\text{PTD flux in control formulation}}
\]

2.7. \textit{Analytical method}

The analyses were performed by HPLC (YOUNG LIN INSTRUMENT® model YL 9100), using the fluorescence detector with excitation at 270 nm and emission at 345 nm. Chromatography separation was performed in isocratic elution mode; the methodology was previously validated. The column used was C18 column (4.6 x 15.0 mm and particle size 5µm) at the temperature of 35° C. The mobile phase consisted of a mixture of acetonitrile and sodium phosphate buffer (0.025 M pH 3.2) in the ratio 17:83 (v / v), respectively. The flow rate of
mobile phase was 1 mL/min. Under these conditions the method was accurate (CV <5%).

Linearity was achieved in the range of 0.4-10 µL / mL, with correlation coefficient $r \geq$ the minimum values required by current legislation (BRASIL, 2017) The drug retention time was 10 minutes (Poolla et al., 2003).

2.8. Cytotoxicity assay of NLC in macrophages

Macrophage cells (J774) were seeded in 96-well culture plates (2 x $10^5$ cells / well) and cultured in DMEM (Sigma- Aldrich®) medium containing NaHCO$_3$ (1.2 g / L), ampicillin (0.025 g / L), streptomycin (0.1 g / L), supplemented with 10% fetal bovine serum (FS). Two formulations were prepared, one formulation contained pentamidine (NLC), an inert formulation (NLCI) and a solution of pentamidine diluted in dimethylsulfoxide (PTD), cells were subjected to different doses of the formulations and pentamidine solution (8; 2; 0.5; 0.12 µg / mL) for 24 hours at 37° C and 5% CO$_2$. Cell viability evaluated by the colorimetric method using Methyl-thiazolyl-tetrazolium MTT (Sigma-Aldrich®). MTT is a yellow dye, which is reduced by mitochondrial and cytoplasmic enzymes to a blue compound called formazan; this compound is insoluble in aqueous solution. The reduction of MTT tetrazolium salt, mainly by the mitochondrial succinate dehydrogenase enzyme (Doktorovova et al., 2014), is widely used in cell survival and proliferation evaluation trials, since only viable cells reduce MTT (yellow) to formazan (blue), which once solubilized can be quantified spectroscopically. Thus, the amount of formation produced is proportional to the number of viable cells present (Gaspar et al., 2016). A solution of 0.025 g of MTT diluted in 50 mL of PBS was placed in contact with the cells that were then incubated at 37° C for 3 hours. After removal of the MTT, dimethylsulfoxide (DMSO) was placed for 10 minutes to solubilize the tetrazole salt crystals, and thereafter the optical density (OD) reading was performed on an automated plate reader.
(ELISA) at a wavelength of 570 nm. The tests were performed in quadruplicate and then according to Equation 2.

\[
Cell \ viability \ (\%) = \left( \frac{|treated \ cells| - |white|}{|positive \ control| - |white|} \right) \cdot 100
\]  

(2)

After the test the one-way ANOVA statistical test was performed, after Tukey test to better analyze the results.

2.7. Evaluation of leishmanicidal action of NLC on *Leishmania amazonensis* promastigotes

The promastigote forms of *L. amazonensis* (LTCP9667) were cultured in Shneider’s medium (Sigma - Aldrich®) supplemented with 10% fetal bovine serum (FBS) (Gibco®, Thermo Fisher Scientific) ampicillin 1% and gentamicin 0.1% (Sigma – Aldrich®) and maintained at 24°C in a B.O.D greenhouse (Biochemical oxygen demand). Promastigotes of *L. amazonensis* in a log phase of growth (5x10⁵ cells / well) were distributed with different concentrations of the compounds diluted in Schneider’s medium and incubated for 24 h at 24°C in a B.O.D. Promastigotes incubated in the absence of the formulation were used as a negative control and amphotericin B were used as a positive control. Wells with culture medium were used as a white. The cellular viability of the parasites were evaluated using the colorimetric method of resazurin, modified from Kulshrestha et al., (2013). To do this, after treatment time, 20 µL of resazurin (500 µM / mL in PBS, pH 7.4) (Sigma - Aldrich®) per well, the plates were incubated again for 2 hours at 24°C, and then read in spectrophotometer (Biotek Synerg H1 model) at 560 nm excitation and 590 nm emission. The obtained fluorescence measurement were used for the calculation of viability as described in Equation 3.
\[
Viability(\%) = \left( \frac{test - white}{control - branco} \right) \cdot 100
\] (3)

The IC\textsubscript{50} was obtained by non-linear regression analysis from the viability values using the program GraphPad Prism 5.0. All experiments were performed in triplicate of one experiment.

### 2.8. Statistical analysis

Analyses were done using GraphPad Prism 5.0. The one-way ANOVA test was performed and Tukey pos-test for greater reliability of results.

### 3. RESULTS AND DISCUSSION

#### 3.1. Characterization of the systems obtained

The formulations presented a homogeneous appearance without the presence of sediments. Particle size, polydispersity index (PI) and zeta potential are presented in Table 2.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Size (nm)</th>
<th>IPD</th>
<th>PZ (mV)</th>
<th>EE %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLC\textsubscript{inert}</td>
<td>172.5</td>
<td>0.48</td>
<td>-14.9</td>
<td>-</td>
</tr>
<tr>
<td>NLC 1</td>
<td>266.2</td>
<td>0.49</td>
<td>-10.8</td>
<td>14.0</td>
</tr>
<tr>
<td>NLC 2</td>
<td>206.2</td>
<td>0.44</td>
<td>-13.6</td>
<td>76.2</td>
</tr>
</tbody>
</table>

**Table 2:** Results of size, IPD, PZ and EE of the NLC obtained

NLC of nanometric size were obtained, in the range of 172 to 266 nm, with IPD below 0.5. It was observed that the incorporation of the drug promoted an increase in the size of the
nanoparticles. Hu et al. (2008) and Prombutara et al (2012) found similar results. In these studies, the incorporation of the drug also resulted in an increase in the size of nanoparticles studied. The polydispersity index (IPD) indicates the uniformity of the size of the nanoparticles in the formulation, where the values below 0.5 indicate a smaller tendency in agglomeration (Garg and Singh, 2011).

NLC 2 (obtained with dextran 70 in the internal aqueous phase) have also shown smaller particle size than NLC 1.

The zeta potential (ZP) indicates physical stability of the formulations. The potential zeta shows the electric potential present on the surface of the particle. Values above |25| promote an electric repulsion between the particle, reducing the agglomeration between them and improving the stability of the formulation (Das et al., 2012).

Zeta potential ranged from -10 to -15 mV, considered low due to the use of a nonionic surfactant, Tween 80 (Venturini et al., 2015). The incorporation of the drug also modified this parameter causing slight reduction of the surface charge of the particles.

3.2. Encapsulation efficiency

Results of encapsulation efficiency are also showed in Table 2. It was observed that NLC 1 presented lower encapsulation efficiency than NLC 2. Only 14% of the pentamidine can be encapsulated in the NLC 1 formulation, whereas in the NLC 2 formulation it presented 76% encapsulation efficiency.

Kashi et al., (2012) obtained similar problems during the encapsulation of minocycline, a hydrophilic substance. As an alternative to increase the encapsulation of the drug, they used Dextran 70 to decrease the solubility of the drug in the internal aqueous phase during the process.
of obtaining the nanoparticles. The authors observed that there was an increase in EE. Thus, we
also propose the use of dextran 70 in order to improve the encapsulation efficiency of
pentamidine. NLC 2 was obtained similarly to NLC 1, with the addition of 1% dextran 70 in
the internal aqueous phase. All tests were repeated following the same conditions already
mentioned. It was noted that incorporation of 1% dextran into the NLC 2 formulation increased
more than 5 times the encapsulation efficiency. In this way the NLC 2 formulation was used
for the following experiments.

3.3. In vitro release study

The in vitro release of the PTD incorporated into the NLC was performed by Franz cells using
dialysis membranes as a barrier. The PTD release profile was obtained based on the percentage
of drug released as a function of time (Figure 1).

![In vitro release profile of PTD](image)

**Figure 1:** In vitro release profile of PTD. The results were expressed according to the mean ±
standard deviation (n = 3)
The pentamidine did not show a sustained release profile from the control formulation, since, it allowed the rapid release of PTD. The release profile of the control formulation shows that in only 2 hours of the experiment, more than 40% of the PTD was released.

The PTD profile from NLC presented a release burst phase, during the first 2 hours of the experiment, releasing 15% of the PTD. This phase is characterized by the rapid release of the drug into the formulation (Zhai et al., 2014). However, up to 24 hours of experimentation, the formulation showed a sustained release of PTD, releasing 36%, which corresponds to 146 μg of the drug.

3.4. *Ex vivo* skin permeation study

The *ex vivo* permeation of PTD incorporated in NLC was performed in Franz type cell diffusion. The permeation profile was obtained based on the amount of permeated PTD per area as a function of time (Figure 2) diffusion cells using rat skin as a biological barrier.

**Figure 2:** *Ex vivo* permeation profile of PTD. The results were expressed according to the mean ± standard deviation (n=5) of analyzes
Table 3: In vitro permeation parameters of PTD from NLC and control through rat skin

NLC 3 showed no difference in relation to the control formulation (p > 0.05). The flow of pentamidine permeated from NLC was lower than the control formulation. The amount permeated of PTD from NLC after 24h (Q24) of experiment was slightly higher but this amount was not statistically significant.

3.5. Cytotoxicity assay of NLCs in macrophages

The cell viability (Figure 3) of the NLC 2 and pentamidine solution diluted in DMSO (PTD) was equivalent at 8 µg/mL and significantly different (p<0.001) from the viability of the cells in contact with the inert formulation. Similar results were obtained using the concentration of 2 µg/mL with a difference between NLC and NLCI (p<0.05) and PTD and NLCI (p<0.01). When lower concentrations (0.5 and 0.12 µg/mL) were tested, significant differences were not observed in cell viability. Therefore, the cytotoxic effect is caused by pentamidine at concentrations above 0.5 µg/ml.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Flux (µg/cm².h)</th>
<th>Q24 (µg/cm²)</th>
<th>ER*</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0578</td>
<td>3.005</td>
<td>1</td>
<td>0.931</td>
</tr>
<tr>
<td>NLC 3</td>
<td>0.0419</td>
<td>3.403</td>
<td>0.72</td>
<td>0.896</td>
</tr>
</tbody>
</table>

* ER: enhancement ratio for drug permeation = Js in test formulation/Js in control
Figure 3: Viability of the samples and their respective concentrations on the cell viability of J774 macrophages. 1-way ANOVA, Tukey post-test, * p < 0.05, ** p < 0.01, *** p < 0.001.

NLC inert lines.

The NLC and PTD formulations showed low IC₅₀ values (Table 4), whereas the NCLI formulation had a high IC₅₀ value, which confirms its low toxicity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Citotoxicity</th>
<th>Promastigotes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (µg/mL)</td>
<td>IC₅₀ (µg/mL)</td>
</tr>
<tr>
<td>PTD</td>
<td>10.74</td>
<td>9.86</td>
</tr>
<tr>
<td>NLC 2</td>
<td>11.31</td>
<td>71.4</td>
</tr>
<tr>
<td>NLC inert</td>
<td>59545.59</td>
<td>ND*</td>
</tr>
</tbody>
</table>

ND*: Not detected.

Table 4: Effect of cytotoxicity in Macrophage cells (J774) and leishmanicidal action of NLCs on Leishmania amazonensis promastigotes

3.6. Evaluation of leishmanicidal action of NLC on Leishmania amazonensis promastigotes

The result of the evaluation of the leishmanicidal action of the NLC is also expressed in Table 4. It was observed that the NLC 2 required a dose of pentamidine 7.2 times higher than the dose of pure pentamidine to reach IC₅₀. The leishmanicidal action of the inert formulation (NLC inert) was not detected, suggesting that the NLC lipid matrix composition does not interfere with the leishmanicidal action.
4. CONCLUSION

NLC obtained in this study presented nanometric size, high encapsulation efficiency when Dextran 70 was used in inner aqueous phase, and sustained release of the PTD. However, NLC 2 did not promote the increase of skin permeation of PTD. The leishmanicidal activity was low compared to free PTD. The drug incorporated in NLC presented a much higher IC$_{50}$ (7.2 times higher). The cytotoxicity was similar to free PTD. Thus, this system has not shown to be advantageous as delivery system for pentamidine for topical treatment.
5. REFERENCES


