Development of nanostructured lipid carriers containing finasteride

Desenvolvimento de carreadores lipídicos nanoestruturados contendo finasterida

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Abstract: Beyond its aesthetic importance, the hair distinguishes the individual and can influence the social acceptance. Under certain circumstances, the normal hair growth process is impaired by a condition called androgenetic alopecia (AGA). Among the drugs used to treat AGA, finasteride is an anti-androgenic steroid, used both orally and topically. Nevertheless, it has been reported that both forms results in side effects such as irritation, contact dermatitis and mood changes. Nanostructured lipid carriers incorporating finasteride were developed in this study, in order to evaluate its main characteristics considering size, zeta potential, polydispersity index, encapsulation efficacy and cytotoxicity. Such nanoparticles were built with lipids from vegetable source and block copolymers. They were adjusted to release the drug using specific temperature as trigger. After preparation through high shear technique, the lipid nanocarriers produced exhibited hydrodynamic diameter with average size of 119.56 ± 1.07 nm and polydispersity index of 0.186. Encapsulation efficiency reached values close to 100% after HPLC quantification. The suspension kept their characteristics through 30 days, showing good stability. In addition, the nanostructures reduced the finasteride cytotoxicity for fibroblasts and keratinocytes cells. In this way, the obtained formulation becomes a good *candida*te for future *in vivo* tests as topical agent against AGA showing high encapsulation efficiency and lower cytotoxicity when compared to finasteride alone.

Keywords: nanocarrier, finasteride, androgenetic alopecia.

Resumo: Além de sua importância estética, o cabelo distingue o indivíduo e pode influenciar sua aceitação social. Sob certas circunstâncias, o processo normal de crescimento do cabelo é prejudicado por uma condição chamada alopecia androgenética (AGA). Para tratar a AGA, um esteroide antiandrogênico chamado finasterida pode ser usado de duas formas: oral ou tópica. Porém, para ambas as formas, efeitos colaterais como irritação, dermatite de contato e alterações no humor tem sido reportados. Carreadores lipídicos nanoestruturados incorporando finasterida foram desenvolvidos neste estudo com o intuito de avaliar suas principais características, considerando o tamanho, o potencial zeta, o índice de polidisperção, a eficiência de encapsulação e a citotoxicidade. Tais nanopartículas foram construídas com lipídeos de origem natural ajustados para liberação em temperatura definida e estabilizados com copolímeros em bloco. Após preparação através de alto-cisalhamento, os nanocarreadores lipídicos produzidos possuíram diâmetro hidrodinâmico médio de 119,56 ± 1,07 nm com índice polidispersividade em 0,186. A eficiência de encapsulação atingiu valores próximos a 100%, conforme quantificação por HPLC. A suspensão manteve suas características durante 30 dias, demonstrando boa estabilidade. Em adição, as nanoestruturas produzidas reduziram a citotoxicidade da finasterida frente a células de fibroblastos e queratinócitos. Desta forma, a formulação obtida se torna um bom *candida*to para futuros testes *in vivo*, como agente tópico contra a AGA.

Palavras-chave: nanocarreadores, finasterida, alopecia androgenética.

INTRODUCTION

The formation of the hair begins in the follicle which is basically a mini- organ composed by multiple layers capsuling and producing the hair shaft that is projected through the epidermis. The internal and external root sheath envelops the hair shaft, while in the follicle base the dermal papilla is surrounded by matrix cells that proliferate and differentiate to form the hair shaft. The hair bulge is located in the middle part, where the sebaceous gland and the erector muscle are found (JI et al., 2017; NILFOROUSHZADEH et al., 2019).

Hair loss is a problem for both men and women. This condition cannot be considered only an aesthetic issue but also a clear health impairment, resulting in reduced self-esteem and anxiety that can trigger depression. One of the most common causes of hair loss is the androgenetic alopecia (AGA), a physiological imbalance that affects half of Caucasians males and nearly 100% of the same population over 80 years old. In addition, 15 to 30% of women around thirty years old also suffer from hair loss (GOMES et al., 2014; HAMISHEHKAR et al., 2016; MADHESWARAN et al., 2013; PEREIRA et al., 2018). In the case of AGA, the follicles of the scalp show high levels of 5α-reductase. This enzyme converts testosterone in di-hydrotestosterone (DHT), which binds to the androgenic receptor, stimulating genes responsible for the development of AGA, which in turn reduces the size of hair follicles. There are several genes related to AGA, but the ones that have a major relationship are the *locus* AR/EDA2R in the X chromossome and the *locus* on the short arm of the chromosome 20, 20p11 (MADHESWARAN T. et al., 2013; HAMISHEHKAR et al., 2016; LOLLI et al., 2017).

The pharmacological therapies available currently for AGA treatment include the topical or oral administration of finasteride (SCOW, NOLTE, SHAUGHNESSY, 1999). Finasteride is commonly used in the treatment of hair loss. This synthetic molecule has anti-androgenic properties that inhibit, in a competitive way, the type 2 5α-reductase enzyme, blocking the conversion of testosterone in DHT. This substance is chemically classified as 4-asa-3-oxosteroid and can be used orally for benign prostatic hyperplasia treatment and/or topically for AGA (CAON et al., 2014). Finasteride can reduce over 60% of the local levels of DHT when in contact with the scalp (PRICE, 1999; CAON et al, 2014).

The current available treatment for AGA shows topical formulations prepared with large amount of ethyl alcohol and/or propylene glycol, which can cause side effects such as irritation, dandruff, contact dermatitis and allergies. Another disadvantage of topical solution is the necessity of repeated application to assure correct dosage administration (MATOS et al., 2015; KAUL et al., 2018; PEREIRA et al., 2018; RAMEZANI et al., 2018). In addition to topical treatments, the systemic therapies with finasteride result in adverse effects such as mood changes, gynecomastia, decreased libido and erectile dysfunction or impotence (MADHESWARAN T. et al., 2013). Moreover, the results of systemic treatment can disappear as soon as the treatment stops (PRICE, 1999).

Therefore, formulations that minimize the problems in AGA treatment are of great importance. The control of materials in nanometric scale allow the construction of nano-particles that can incorporate and release the drug in specific physiologic conditions, allowing their use as drug nanocarriers. There is a growing interest in the development of nanocarriers capable of passing through the corneal extract and deliver the substance in the site of action (MADHESWARAN et al., 2014).

The objective of the use of these nanostructures in therapies is to transport molecules to specific sites and deliver them with an effective therapeutic dosage. Nanocarriers show great advantages when compared with classic technologies, because they allow drug

dissolution above their standard solubility limits. In addition, they promote higher therapeutic efficiency through prolonged drug release (BARUA; MITRAGOTRI, 2014).

Nanostructured lipid carriers (NLC) have been studied to increase the efficiency of drugs in several diseases, and it is used in medicines including products for topical application. Among the different lipid nanocarriers described, NLC can be prepared using solid lipids at room temperature, suspended in aqueous medium and stabilized by surfactants (SOUTO; NAYAK; MURTHY, 2011).

This study intends to develop NLC incorporating finasteride to achieve higher drug penetration through the skin and consequent improve therapeutic potential (CAON et al., 2014; HAMISHEHKAR e al., 2016; KAUL et al., 2018). As an alternative to the use of synthetic lipids, lipids extracted from the seeds of *Passiflora incarnata* were used due to their naturally balanced fatty acid composition, which consequently produces imperfect crystalline nuclear structure supporting increased drug encapsulation (BATTAGLIA; GALLARATE, 2012).

2. MATERIAL AND METHODS

2.1. Natural lipid characterization by gas chromatography with flame ionization detector (GC-FID)

Natural lipids samples were prepared through hydrolysis and methylation, prior to the analysis by gas chromatography with flame ionization detector (GC-FID) (Agilent). A SUPELCOWAX-10 column 15m x 0.2mm x 0.2 µm (SUPELCO) was used with gradient temperature: 60°C at time zero, then increments of 10°C per minute to 240°C; injector (split 1/50) was set at 250ºC and detector at 260ºC. Hydrogen was used as carrier gas (4.0 mL/min) with injection volume of 1mL. Peak identification was made by comparison to Supelco37 Fame mix methylated fat standards (Supelco). EZChrom Elite Compact (Agilent) program was used to acquire experimental data.

2.2. Preparation of nanostructured lipid carriers

Nanoparticles were prepared using modified high pressure homogenization technique (SOLDATI et al., 2018). Firstly, lipids extracted from *Passiflora incarnata* seeds (Lot PFB018/03, Ebpm Comercial Ltda, Brazil) and finasteride (Infinity Pharma, São Paulo, Brazil) were prepared as the organic phase (10:1 w/w). This phase was mixed and heated at 60 °C for 10 minutes. In a separate flask, the aqueous phase containing Pluronic F127 (Sigma-Aldrich) was also heated at 60 °C for 10 minutes. Afterwards, the oil phase was subjected to agitation (12.000 rpm) followed by the addition of aqueous phase to the oil phase under ultrasonic irradiation (100W). The suspension was incubated at room temperature for 24 hours to allow lipidic nucleus solidification.

2.3. Nanoparticles characterization

2.3.1. Dynamic Light Scattering (DLS) and electrophoretic mobility

Samples of NLC were characterized by dynamic light scattering and electrophoretic mobility to evaluate the hydrodynamic diameter, polydispersity index (PdI) and Zeta potential, using Zetasizer NanoZS equipment (Malvern Insruments, UK). The hydrodynamic diameter and PdI were measured using a He-Ne (633 nm) laser in detection angle of 173° degrees using quartz cell (ZEN2112) at 25°C. Zeta potential values were determined through electrophoretic mobility evaluation, by the Smoluchowski equation. Prior to characterization, samples were diluted (1:400, v/v) in distilled water to obtain the adequate condition to light scattering. Measures were performed in triplicate at room temperature soon after preparation and after 30 days upon storage.

2.3.2. Encapsulation Efficiency (EE)

Encapsulation efficiency (EE) was determined indirectly through finasteride quantification in the supernatant after centrifugation, by high pressure liquid chromatography (HPLC/ UV-Vis) using a Shimadzu® LCMS-IT-TOF. NLC was centrifuged at 15000 rpm for 15 minutes and 100 µL of supernatant was collected for analysis. Two solutions were prepared for chromatographic separation. Phase "A" was composed by acetonitrile and phase "B" by water; both phases were degassed in ultrasound prior to analysis. A column "Supelco ODS" (250 mm x 4,6 mm, 5 µm) was used. A linear gradient was performed in which phase B raised from 10% in 0,01 minute to 80% when elution time reached 15 minutes and to 100% in 20 minutes of elution. Phase B concentration was kept in 100% for 7 minutes. Flow rate was 1,0 mL/min and wavelengths analyzed were 245 nm e 254 nm respectively. Finasteride standard was solubilized in methanol, and the calibration curve was built from 0.025 to 0.5 mg/mL.

2.4. Cellular viability after treatment with NLC

Cellular viability of *Mus musculus* fibroblasts (L929) and human keratinocytes (HaCaT) was evaluated by MTT assay, after the treatment with NLC containing finasteride. Cells were cultivated in 5% CO2 atmosphere at 37 °C. Further, cells were seeded to 96 well plates with cellular density of 5 x 103 (L929) and 5 x 104 (HaCaT) cells/mL for 24 hours prior to treatment. Further, NLC containing finasteride was applied to the final concentration range of 6.2-100 µg/mL and plates were incubated for another 24 hours at 37 °C with 5% of CO2. Then medium was removed and MTT solution (5 mg/mL) diluted in medium (1:10) was added followed by an incubation period (4 h at 37 °C). The formazan crystals formed after that period were dissolved in 100 µL of DMSO and absorbance was read at 540 nm in microplate reader (Spectramax 190). Non-treated controls were considered as 100% of cell viability (MOSMANN, 1983).

2.5. Statistical analysis

All experiments were analyzed through the one way variance analysis (ANOVA), followed by Tukey's post-test for group comparison using GraphPad PRISM 6.0 software (GraphPad, EUA). Statistical significance was represented as *** for $p < 0.01$.

3. RESULTS AND DISCUSSION

Nanostructure lipid carriers containing finasteride were developed to obtain a better delivery drug system for topical administration, aiming the improvement of the vehicle. To this end, we choose to use naturally balanced lipid composition of seed butters as solid core, stabilized by copolymer.

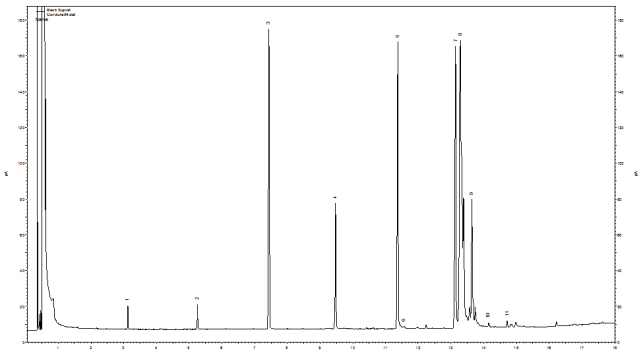

Figure 1 - Chromatogram of *Passiflora incarnata* seed butter.

3.1. Natural lipid characterization

Firstly, GC-FID analysis was carried out using lipids extracted from the seeds of *Passiflora incarnata* in order to characterize its fatty acids composition. Figure 1 shows the chromatogram obtained after analysis. Retention time and concentration of each compound are shown in Table 1. It is worth to note that the fatty acid present in highest concentration is C18:1 (38.7%).

The composition of the seed butter has direct impact on its melting point, as the number of unsaturation and

length carbon chain of the fatty acids that compose the material influence it. Thus, the less unsaturation and longer the chain, the higher the melting point (RUSTAN; DREVON, 2005). In our material, the composition of fatty acids makes it with high melting point and it is presented in the solid phase at room temperature (Table 1).

Table 1 - Percentage of fatty acids in *Passiflora incarnata* seed butter

Peak	Fatty	RT	
	Acid	Min	Area
1	C8:0	3.13	0.7
2	C10:0	5.26	0.9
3	C12:0	7.44	12.2
4	C14:0	9.47	4.9
5	C16:0	11.38	13.6
6	C16:1	11.57	0.3
7	C18:0	13.14	15.8
8	C18:1	13.28	38.7
9	C18:2	13.64	10.6
10	C18:3	14.15	0.2
11	C20:0	14.72	0.3
	Others		1.7

Legend: RT (min) = retention time in minutes; Area is related to the area of the peak obtained by GC.

3.2. Nanoparticles characterization

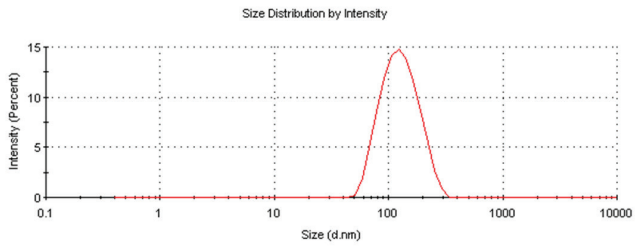

Figure 2 - Size distribution by intensity of NLC loaded with finasteride.

For NLC characterization, the size of particles and surface charge were measured and the results showed that nanoparticles loaded with finasteride has hydrodynamic diameter and polydispersity index of 119.56 ± 1.07 nm and 0.186 ± 0.02 respectively (Figure 2). According to Feng, the size obtained fits a desirable range to reach the dermis and follicle. Values of polydispersity suggest that nanoparticles in

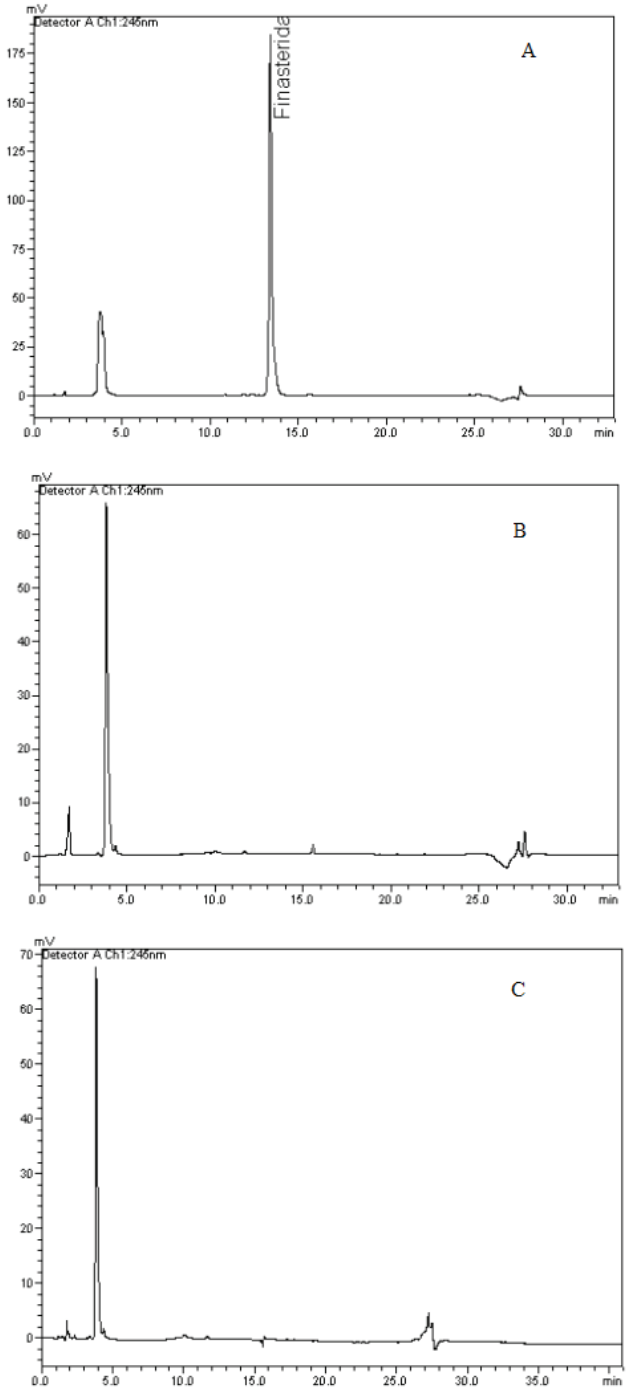

Figure 3 - Chromatograms of finasteride standard (A) and NLC at 15 days (B) and 45 days (C) of storage at 4°C.

the suspension are evenly distributed. After 30 days of storage, NLC presented hydrodynamic diameter of 132.7 nm and PdI of 0.113, suggesting formulation stability. Negative Zeta potential values (-15.36 ± 2.34) was obtained, which is probably due to the presence of fatty acids organization in the nanoparticles. This result also imply that the formulation maybe stable by ionic repulsion in addition to the steric effect of PEG in the surface of the NLC.

3.3. Encapsulation Efficiency (EE)

Encapsulation efficiency was evaluated 15 and 45 days after NLC preparation. No free finasteride was detected in the samples (Figure 3). This result suggests that the drug was incorporated into the NLC and there was no leakage during the storage period at 4°C. In addition, the nanoparticles remained stable for the whole period, as there was no change in size or PdI (data not shown).

Tripathi et al also developed stable lipid nanoparticles with drug content loss less than 1% after 120 days of storage. Another study demonstrated that there was no changes in terms of size and encapsulation efficiency after one month of storage (GRAVERINI et al., 2018). Gonçalves et al observed, as in consonacy with these priors studys, stability among parameters like entrapment efficiency, size and polydispersity after one-month storage at 5±3 °C.

3.4. Cellular viability

The NLC containing finasteride did not present cytotoxicity against fibroblasts and keratinocytes (Figure 4). Analyzing the results from L929 cells, no statistical difference was found when comparing the treatment with NLC containing finasteride and free finasteride and other controls, at several concentrations. When analyzing the results of the treatment in keratinocytes (HaCat cells) we could observe no difference in cell viability comparing NLC

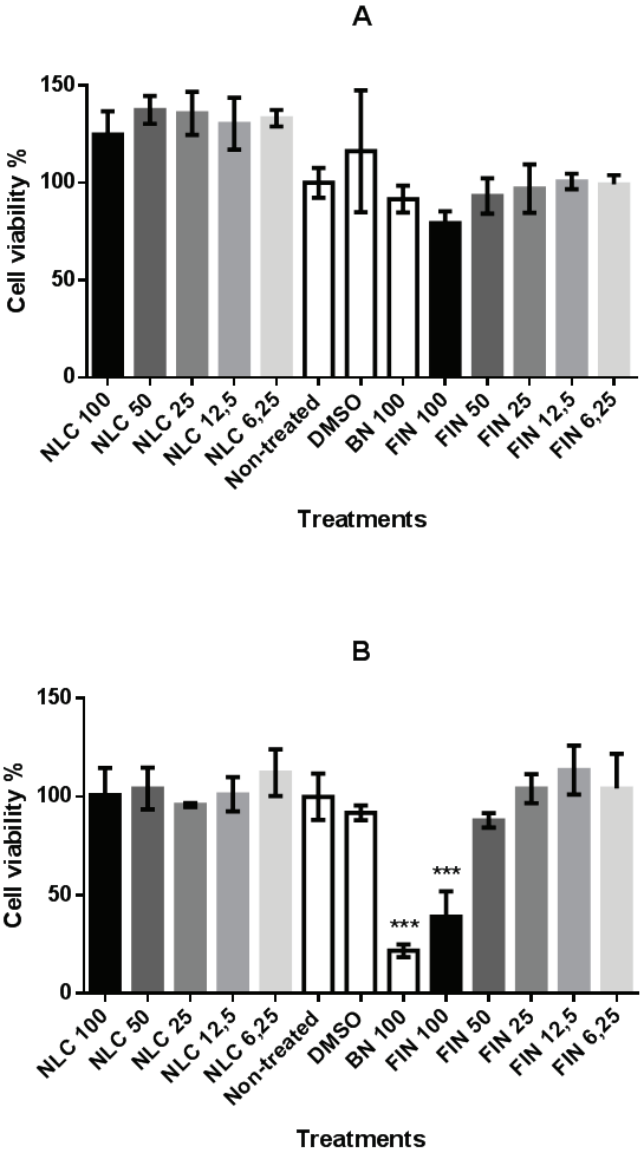

Figure 4 - Cellular viability of fibroblasts (A) and keratinocytes (B), after the treatment with NLC and the controls. NLC: nanostructured lipid carrier containing finasteride; BN: blank nanoparticle; FIN: free finasteride. Numbers indicate the concentration in µg/mL for each treatment. Data is presented as mean ± SD (*** p<0,01).

filled with drug and the non-treated cells. It´s possible to note too that free finasteride with the highest concentration is toxic and this toxicity diminishes as the concentration get lower. This strongly suggests that the new formulation maybe safe for topical use in the AGA.

4. CONCLUSIONS

This study showed that nanostructured lipid carriers filled with finasteride were successfully prepared with natural lipids, presented high encapsulation efficiency, stability upon storage and negligible cytotoxicity. Their size is adequate for biomedical application, considering the topical administration route and delivery to hair follicles. Further analysis are necessary to ensure if this formulation can reach and stay in optimal concentration on the dermis, towards controlled release of finasteride. Overall, the NLC containing finasteride are promising delivery systems for the treatment of androgenetic alopecia.

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Conflicts of Interest

None to declare