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NANOPARTÍCULAS DE β-SITOSTEROL ASSOCIADAS À LASERTERAPIA NO TRATAMENTO DE ALOPECIA ANDROGENÉTICA

Santa Maria, RS, Brasil
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Tese apresentada ao Programade Pós Graduação em Nanotecnologia Farmacêutica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Nanotecnologia Farmacêutica.**

Orientadora: Prof[a]. Dr[a]. Cristiane de Bona da Silva

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Se você não estiver preparado para errar, você nunca terá uma ideia original.

Ken Robinson

RESUMO

NANOPARTÍCULAS DE β-SITOSTEROL ASSOCIADAS À LASERTERAPIA NO TRATAMENTO DE ALOPECIA ANDROGENÉTICA

AUTORA: Tatiele Katzer
ORIENTADORA: Dra. Cristiane de Bona da Silva

Os cabelos são de extrema relevância para homens e mulheres, tanto do ponto de vista de saúde quanto estético. A principal doença que cursa com diminuição da densidade capilar é a alopecia androgenética (AAG), sendo a hereditariedade, a ação de hormônios androgênicos e a microinflamação fatores implicados na sua fisiopatologia. Frente ao limitado número de opções terapêuticas para contornar os sinais e sintomas da AAG e os efeitos adversos a elas associados, faz-se necessário o desenvolvimento de novas estratégias. Esta tese apresenta uma nova combinação terapêutica para o tratamento da AAG, associando a nanotecnologia, um ativo multifuncional - β-sitosterol (BS) - e a laserterapia. O BS possui propriedade anti-inflamatória e moduladora da enzima 5-α-redutase (enzima chave na fisiopatologia da AAG). Por sua vez, a nanoencapsulação pode favorecer um aumento na penetração folicular e uma liberação prolongada de substâncias e o LBP apresenta múltiplos benefícios, como o estímulo da circulação sanguínea local e da produção de ATP, efeito anti-inflamatório e mitogênico. O efeito destas nanoestruturas e da associação com o LBP foi avaliado *in vivo* e *in vitro.* As nanocápsulas (NC) e os carreadores lipídicos nanoestruturados (CLN) foram preparados pelo método de deposição interfacial de polímero pré-formado e por emulsificação seguida por ultrassonicação, respectivamente. Os nanocarreadores apresentaram distribuição de partículas exclusivamente na faixa nanométrica (111-132 nm para CLN; e 197-268 nm para NC) e baixo índice de polidispersão. Os valores de potencial zeta foram negativos (~ -6,5 mV para CLN e – 9 mV para NC) e o pH levemente ácido (~ 6,1 e 6,7 para NC e CLN, respectivamente) para todas as formulações. O teor de BS foi próximo ao teórico (1 mg/mL) para ambas nanopartículas. A irradiação das nanopartículas com LBP 660 ou 830 nm (4 J/cm^2) não induziu mudanças no tamanho médio da partícula, potencial zeta e teor. De acordo com os dados do ensaio HET-CAM, as nanoformulações não foram irritantes, independentemente da presença de BS. O tratamento das linhagens celulares 3T3 e HaCaT com as nanopartículas (com ou sem BS) não induziu citotoxicidade nem proliferação celular, bem como os tratamentos associando o LBP. A viabilidade celular diminuiu apenas para ambas as linhagens celulares quando a solução de BS foi utilizada, demonstrando a capacidade das nanoestruturas de proteger contra a citotoxicidade do BS. No ensaio *in vivo Allium cepa,* o índice mitótico não foi influenciado pelas formulações, independentemente da presença de BS e da sua nanoencapsulação. O modelo animal de AAG induzido por testosterona revelou através da histologia que o efeito de estímulo no crescimento capilar é melhor quando as NC contendo BS foram associadas ao LBP. Em conclusão, esta tese propôs, pela primeira vez, a associação de nanopartículas contendo BS com o LBP e esta combinação provou ser promissora para o tratamento da AAG.

Palavras-chave: Calvície. Citotoxicidade. Laserterapia. Nanotecnologia.

ABSTRACT

β-SITOSTEROL NANOPARTICLES ASSOCIATED WITH LASERTHERAPY IN THE TREATMENT OF ANDROGENETIC ALOPECIA

AUTHOR: Tatiele Katzer
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Hairs are of extreme relevance for men and women, both from the point of view of health and aesthetic. The main disease that occurs with decreased hair density is androgenetic alopecia (AGA), being heredity, the action of androgenic hormones and microinflammation factors involved in its pathophysiology. Given the limited number of therapeutic options to circumvent the signs and symptoms of AGA and the negative side effects associated with them, it is necessary to develop new strategies. This thesis reports a new therapeutic combination for the treatment of AGA, associating nanotechnology, a multifunctional active substance - β-sitosterol (BS) – and the lasertherapy. BS has anti-inflammatory and modulating properties of the enzyme 5-α-reductase (a key enzyme in the pathophysiology of AGA); nanoencapsulation may favor an increase in follicular penetration and prolonged release of substances, and the LLL has multiple benefits, such as stimulation of the local blood circulation and the production of ATP, anti-inflammatory and mitogenic effects. The effect of these nanostructures and the association with LLL was evaluated *in vivo* and *in vitro*. Nanocapsules (NC) and nanostructured lipid carriers (NLC) were prepared by the interfacial deposition method of preformed polymer and by emulsification followed by ultrasonication, respectively. Nanocarriers presented particle distribution exclusively in the nanometric range (111-132 nm for NLC; and 197-268 nm for NC) and low index of polydispersion. The zeta potential values were negative (~ -6.5 mV for NLC and – 9 mV for NC) and the pH slightly acidic (~ 6.1 and 6.7 for NC and NLC, respectively) for all formulations. BS content was close to the theoretical (1 mg/mL) for both nanoparticles. Irradiation of the nanoparticles with LLL 660 or 830 nm (4 J/cm^2) did not induce changes in mean particle size, zeta potential and BS content. According to the HET-CAM assay data, the nanoformulations were not irritating, regardless of the presence of BS. Treatment of the 3T3 and HaCaT cell lines with the nanoparticles (with or without BS) did not induce cytotoxicity or cell proliferation, as well as the treatments associated with LLL. Cell viability decreased for both cell lines only when the BS solution was used, demonstrating the ability of nanostructures to protect against BS cytotoxicity. In the *in vivo Allium cepa* assay, the mitotic index was not influenced by the formulations, regardless of the presence of BS and its nanoencapsulation. The animal model of testosterone-induced AGA revealed through the histology that the effect of stimulation on hair growth is best when NC containing BS were associated with LLL. In conclusion, this thesis proposed, for the first time, the association of nanoparticles containing BS with LLL and this combination proved to be promising for the treatment of AGA.

Keywords: Baldness. Cytotoxicity. Laser therapy. Nanotechnology.

SUMÁRIO

1 INTRODUÇÃO

A alopecia androgenética (AAG) é a causa mais comum de diminuição da densidade capilar (RAMOS e MIOT, 2015), afetando 30 a 50% dos homens e cerca de 30% das mulheres de meia idade (SINCLAIR, 2016). Segundo a Organização Mundial de Saúde, a definição de "saúde" vai além da ausência de afecções e enfermidades; estar saudável é um estado de bem-estar físico, mental e social. Embora a AAG não seja considerada uma condição severa, torna-se um problema à medida que sua manifestação, especialmente quando prematura, causa distúrbios psicoemocionais e um impacto importante na qualidade de vida dos acometidos (RINALDI, BUSSA e MASCARO, 2016). Uma vez que a incidência de AAG é muito alta, bem como o desconforto associado a sua manifestação, o estudo de novas estratégias terapêuticas é uma necessidade atual e de grande interesse.

Os sinais clínicos da AAG são diminuição e variabilidade na espessura, densidade e coloração capilar; produção sebácea aumentada e inflamação (CRANWELL e SINCLAIR, 2016). Há um aumento na quantidade de pelos vellus (diâmetro <0,03 mm) e uma diminuição dos pelos terminais (espessura >0,03 mm), modificando a proporção de 8:1 para menos de 4:1 (SIAH, MUIR-GREEN e SHAPIRO, 2016). Além disso, a proporção de folículos em fase anágena (fase de crescimento do ciclo capilar) e em fase telógena (fase de repouso do ciclo capilar) muda de 12:1 para 5:1 (SIAH, MUIR-GREEN e SHAPIRO, 2016).

As causas mais elucidadas para a AAG são a genética e a miniaturização folicular mediada por andrógenos. No entanto, tratamentos farmacológicos relacionados à modulação da atividade androgênica, como a finasterida, mostraram eficácia variável, além de efeitos adversos (ROSSI *et al.*, 2016). Embora a fisiopatologia da AAG esteja intimamente relacionada ao metabolismo de andrógenos, evidências científicas sugerem que a AAG está associada à desregulação na expressão de citocinas inflamatórias (RAMOS *et al.,* 2016), sendo a microinflamação crônica um fator agravante (TRÜEB, 2002).

A busca por alternativas para o tratamento da AAG leva ao emprego de plantas medicinais, como a *Serenoa repens* e a *Eclipta albain* (ROY, THAKUR e DIXIT, 2008; ROSSI *et al.,* 2012). Dentre os componentes presentes nestas e na maioria das plantas, os fitoesterois são bastante estudados. O β-sitosterol, por exemplo, apresenta múltiplas propriedades, como antioxidante, antimicrobiano, angiogênico, imunomodulatório, antidiabetogênico, hipocolesterolemiante, anti-inflamatório e modulador da enzima 5α-redutase; as duas últimas de grande interesse para o tratamento da AAG (CHEN *et al.,* 2016; SAYEED *et al.,* 2016). A disponibilidade de β-sitosterol sintético fez com que várias

fórmulas por via oral (PRAGER et al., 2002) ou tópica (REJUVIANCE PRODUTCTS CORP, 2019) fossem desenvolvidas e avaliadas para o tratamento da AAG e outras condições (FERNANDEZ e VEGA-LÓPEZ, 2005).

A nanomedicina é uma área de pesquisa, desenvolvimento e aplicação de sistemas coloidais nanoestruturados (nanopartículas) e outros elementos em escala nanométrica com finalidades terapêutica ou diagnóstica (TORCHILIN, 2012). A nanoencapsulação de fármacos pode modificar as propriedades físico-químicas da molécula incorporada e, normalmente, incrementa a distribuição de substâncias de difícil absorção (CONTRI *et al*., 2016). Há uma gama de ativos de interesse para o tratamento da AAG que já foram incorporados em sistemas nanoestruturados, como a arginina (YAZDANI-ARAZI *et al.*, 2016), a flutamida (HAMISHEHKAR *et al.*, 2016), a espironolactona (SHAMMA e ABURAHMA, 2014), a finasterida (GOMES *et al.*, 2014), a dutasterida (NOOR *et al.*, 2017) e o minoxidil (MATOS *et al.*, 2015).

Os nanocarreadores podem diferenciar-se pela morfologia, tamanho, solubilidade no meio biológico e por sua composição supramolecular (KHAN, SAEED e KHAN, 2017) e estas características, somadas ao teor de fármaco, à eficiência de encapsulação e a sua carga de superfície afetarão os efeitos biológicos dos mesmos. As nanopartículas lipídicas são as mais estudadas para aplicação tópica e, dentre estas, estão as nanoemulsões, os lipossomas, as nanopartículas lipídicas sólidas e os carreadores lipídicos nanoestruturados. Nanoemulsões constituem-se por gotículas de óleo estabilizadas por uma combinação de tensoativos (HAMEED *et al.*, 2019); os lipossomas são sistemas vesiculares compostos de bicamadas lipídicas capazes de encapsular moléculas hidrofóbicas ou hidrofílicas (HAMEED *et al.*, 2019); as nanopartículas lipídicas sólidas foram desenvolvidas pela substituição do lipídio líquido na fase coloidal da nanoemulsão por um lipídeo sólido nas temperaturas corporal e ambiente (MÜLLER; RADTKE e WISSING, 2002) e, por fim, os carreadores lipídicos nanoestruturados (CLN) são formados por uma mistura de lipídios sólidos e líquidos e, por isso, apresentam maior capacidade de nanoencapsulamento (MÜLLER *et al*., 2007).

Por sua vez, as nanopartículas poliméricas são sistemas carreadores que, conforme a organização estrutural, são denominadas como nanocápsulas (NC) ou nanoesferas (KUMARI; YADAV e YADAV, 2010). Nanocápsulas apresentam em sua estrutura um núcleo oleoso envolto por uma membrana polimérica ao passo que as nanoesferas caracterizam-se pela ausência de óleo, configurando-se como uma matriz polimérica (HAMEED *et al.*, 2019). Dentre as vantagens das nanopartículas sobre os sistemas convencionais estão a redução de

efeitos adversos (CONTRI *et al.*, 2014), a proteção dos ativos contra a degradação (WEBER *et al.*, 2016), o aumento na penetração folicular (LADEMANN *et al.*, 2015), bem como o maior tempo de permanência no local de interesse terapêutico (LADEMANN *et al.*, 2011). O núcleo oleoso das NC, bem como a composição lipídica dos CLN favorece a incorporação de substâncias lipofílicas, como é o caso do BS (log P = 9.65) (UPADHYAY; GUPTA; DIXIT, 2012), com a vantagem de apresentarem fase externa aquosa em detrimento do uso de solventes orgânicos, comumente utilizados para solubilização de substâncias com elevada lipofilia.

Substâncias convencionalmente utilizadas por via oral para o tratamento da AAG, como a finasterida, têm sido estudadas por via tópica (GOMES *et al.*, 2014). A administração de fármacos via folículos pilosos é de grande interesse na área da tricologia, principalmente se o alvo da ação é a unidade pilossebácea. A remoção das moléculas e/ou das nanopartículas do ambiente folicular acontecerá lentamente pelo crescimento do cabelo e pelo fluxo de sebo (LADEMANN *et al.*, 2007). Uma das principais razões para explorar a aplicação tópica no couro cabeludo seria proporcionar o aumento da biodisponibilidade e efeitos locais, evitando os potenciais efeitos adversos advindos do uso sistêmico de fármacos (CASERINI *et al.*, 2014).

Um grupo de pesquisadores desenvolveu lipossomas (denominados de “fitovesículas” pelos autores) contendo β-sitosterol e os utilizou para o tratamento da AAG em modelo animal (ratos Wistar) (UPADHYAY; GUPTA e DIXIT, 2012). Após 21 dias de tratamento tópico, os resultados alcançados com os lipossomas foram superiores ao da solução de β-sitosterol, provavelmente pela maior penetração folicular do sistema nanoparticulado.

Recursos não medicamentosos também são empregados para o tratamento da AAG, como por exemplo o laser de baixa potência (LBP), o microagulhamento e terapias autólogas (plasma rico em plaquetas). O LBP é o único recurso não medicamentoso aprovado pelo *Food and Drug Administration* (FDA), órgão regulamentador americano, para o tratamento da AAG (ADIL e GODWIN, 2017). A terapia com LBP consiste em expor células ou tecidos à energia luminosa, geralmente com comprimentos de onda na região do vermelho ou infravermelho (CHUNG *et al.*, 2012). Dezenas de artigos já foram publicados buscando avaliar sua eficácia e/ou compreender seu mecanismo de ação no tratamento da AAG, com resultados promissores. Uma meta-análise de 45 artigos sobre os três únicos tratamentos aprovados pelo FDA para a AAG (minoxidil, finasterida e LBP) mostrou que o LBP induz

maior crescimento capilar do que o minoxidil quando usado como monoterapia (ADIL e GODWIN, 2017).

Frente ao exposto, o presente trabalho objetivou avaliar o efeito de nanopartículas contendo β-sitosterol associadas ou não à laserterapia no tratamento da AAG. A hipótese é a de que a associação de estratégias – nanotecnologia, ativo multifuncional de interesse para o tratamento da AAG e laserterapia – possa representar uma nova abordagem no tratamento da AAG, com melhorias em relação às modalidades atuais. O β-sitosterol possui propriedade anti-inflamatória e moduladora da enzima 5-α-redutase, no entanto demonstrou toxicidade para algumas linhagens celulares – 3T3, HeLa e MCF-7 – e, por isso, sua nanoencapsulação torna-se interessante (AYAZ *et al*., 2019). Além disso, esta estratégia nanotecnológica pode favorecer um aumento na penetração folicular e o laser de baixa potência apresenta múltiplos benefícios, como o estímulo da circulação sanguínea local, efeito anti-inflamatório e mitogênico. O referencial teórico desta tese será apresentado na forma de um artigo de revisão (MANUSCRITO 1), enquanto a sessão “Materiais e Métodos” será apresentada diretamente no MANUSCRITO 2.

1.2 OBJETIVOS

1.2.1 Objetivo geral

O objetivo deste estudo foi desenvolver uma nova proposta terapêutica para o tratamento da AAG, por meio do uso de formulações nanotecnológicas contendo β-sitosterol associadas à laserterapia, bem como avaliar sua toxicidade e eficácia *in vitro* e *in vivo.*

1.2.2 Objetivos específicos

Desenvolver e caracterizar nanocápsulas poliméricas e carreadores lipídicos nanoestruturados contendo β-sitosterol para aplicação tópica;

Avaliar os efeitos da irradiação do laser de baixa potência sobre a nanoestruturas desenvolvidas;

Avaliar a interação das nanopartículas desenvolvidas com a fibra capilar;

Determinar o potencial de citotoxicidade das nanopartículas desenvolvidas;

Avaliar o potencial de irritação das nanopartículas desenvolvidas pelo método HET-CAM (membrana cório-alantoide de ovo embrionado de galinha);

Avaliar *in vivo* a eficácia das nanopartículas desenvolvidas em modelo animal com ou sem a aplicação concomitante de laserterapia.

2. MANUSCRITO 1: PHYSIOPATHOLOGY AND CURRENT TREATMENTS OF ANDROGENETIC ALOPECIA: GOING BEYOND ANDROGENS AND ANTI-ANDROGENS

Em apreciação.

PHYSIOPATHOLOGY AND CURRENT TREATMENTS OF ANDROGENETIC ALOPECIA: GOING BEYOND ANDROGENS AND ANTI-ANDROGENS

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Keywords: androgenetic alopecia; hair growth; nanotechnology; low-level laser therapy

Running title: Androgenetic alopecia: going beyond anti-androgens

Conflict of interest: Katzer; Leite Júnior, Beck and Silva declare no conflict of interest.

ABSTRACT

Physiopathology and current treatments of androgenetic alopecia: going beyond androgens and anti-androgens

Androgenetic alopecia (AGA) is the most diagnosed hair loss disfunction. Its physiopathology comprises a genetic predisposition affording an exacerbated response of the hair follicles cells to androgens, aggravated by scalp inflammation and extrinsic factors. **Objective:** To review the mechanisms and extrinsic factors involved in the AGA physiopathology as well as its conventional and emerging treatments. **Design:** The research focused on reports regarding AGA physiopathology and treatments published up to January 2018 in medical and related journals. **Results:** The most used medical treatments for AGA – minoxidil and finasteride – turned out to be non-completely satisfactory. Lately, the low-level laser therapy was recognized as a safe and effective treatment for AGA. Pharmaceutical substances with mechanisms differing from the anti-androgen activity are under current investigation and many of them have botanical origins. Moreover, formulations with better performance are required. As so, the hair follicles ability of being a drug and nanoparticle reservoir has been exploited. **Conclusions:** The association of different strategies, i.e., substances with synergic mechanisms (anti-androgenic, anti-inflammatory, vasodilator, mitogenic), non-medical resources, as low-level laser therapy, and the use of advantageous technologies associated with lifestyle changes could improve the outcomes of the treatments for AGA.

Keywords: baldness; hair growth; nanotechnology; low-level laser therapy

Introduction

Androgenetic alopecia (AGA) is the most diagnosed hair loss disfunction (Ramos & Miot, 2015). It affects 30 to 50% of men (Cranwell & Sinclair, 2016) and around 30% of middle-aged women. The incidence of AGA increases with the age advance and can affect 90% of the Caucasian population when they reach their 80s (Rossi et al., 2016). When its signs prematurely appear AGA can cause psychoemotional disturbances and a significant impact on the quality of life of many patients (Rinaldi, Bussa & Mascaro, 2016).

Although the causes are the same (Kim, Lee, Lee, Yoon & Lee, 2012), AGA manifests itself in different macroscopic patterns among men and women. Male pattern hair loss (MPHL) commonly shows a decrease in hair density and thickness in the temples

(bitemporal), vertex (radial progression) and mid frontal scalp (Cranwell & Sinclair, 2016), as represented in the Hamilton-Norwood scale; while the female pattern hair loss (FPHL) usually shows the same alterations in hair characteristics; however, in a diffuse presentation represented by the Ludwig scale (Kim et al., 2012). In a study with 26,226 Taiwanese women, 0.4% were classified as having the male pattern AGA (Su, Chen & Chen, 2013).

The clinical signs of AGA are the decreasing and variation of the hair thickness and density, lightening of hair color, increasing oily production and inflammation (redness, itchy, pain, edema) (Cranwell & Sinclair, 2016). Besides, there is an increase of vellus hairs (diameter <0.03 mm) and a decrease of terminal hairs (diameter >0.03 mm), changing the ratio of terminal/vellus from 8:1 to less than 4:1. Also, the proportion of anagen (hair follicle cycle in the growth phase) and telogen follicles (hair follicle cycle in the resting phase) changes from 12:1 to 5:1 (Siah et al., 2016).

The most elucidated AGA etiopathogenesis are the genetics and the androgen-mediated follicular miniaturization. However, medical treatments regarding the modulation of androgen activity, such as finasteride, has shown limited efficacy both in men and women with AGA. Probably, there are more to be understood regarding its physiopathology (Rossi et al., 2016). In this scenario, this review brings an updated overview about the physiopathology of AGA and the recent advances for its treatment as well. The research focused on reports regarding AGA physiopathology and treatments published up to January 2018 in medical and related journals.

Main mechanisms involved in the androgenetic alopecia development

Considering the etymology of *androgenetic alopecia*, where the suffix *andro* comes from *androgens* and the prefix *genetic* relates to heredity; it is possible to explain part of the AGA physiopathology. However, there are many more aspects to be considered about the AGA development (Fig. 1).

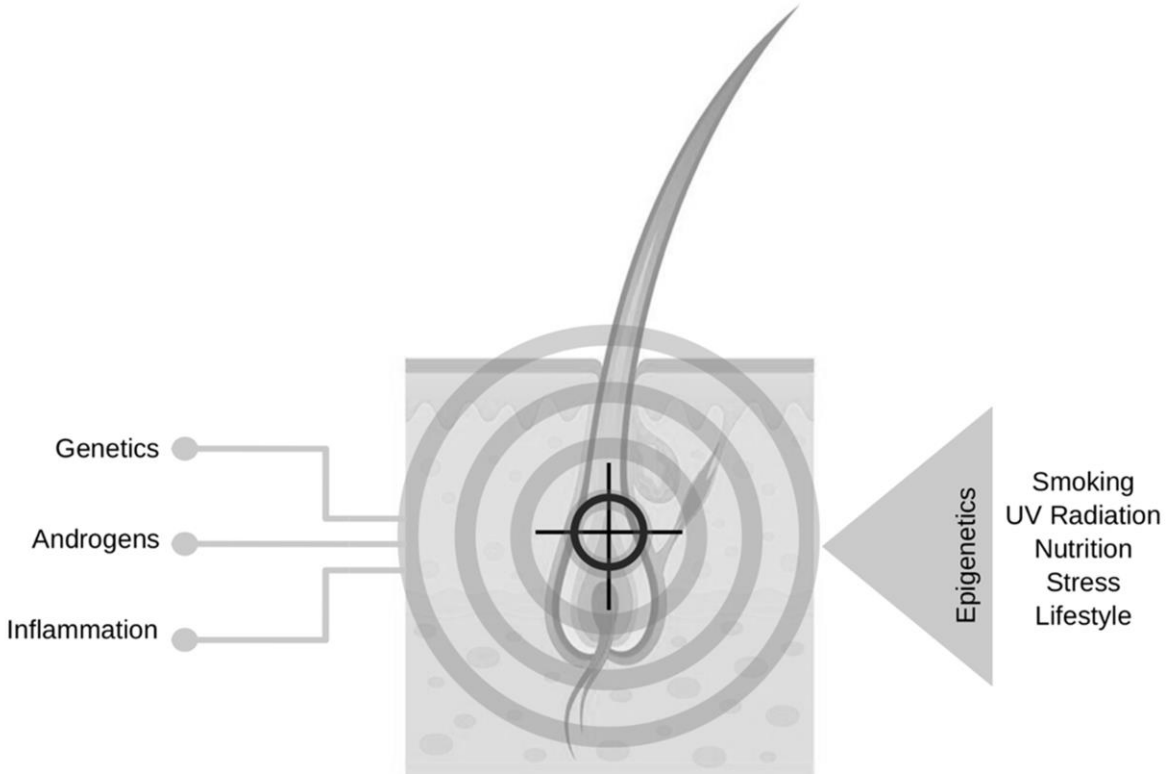

Figure 1: The most recognized causes of androgenetic alopecia. In the left of the image, the main studied causes of AGA (genetics, androgens and inflammation) are shown. In the right side of the image, the most recently studied AGA trigger factors are shown.

The genetics role

The heredity is responsible for 80% of the predisposition to baldness as shown in studies with twins (Cranwell & Sinclair, 2016). The variability of gene expression among individuals explain why some have premature hair loss while others will just show the signs of AGA near the 60s (Rossi et al., 2016). AGA has been reported in prepuberal children as young as 6 to 8 years old and in these cases the genetic predisposition is considered crucial (Siah et al., 2016). AGA is more likely to develop in white people (Caucasians) rather than other populations, specially Mongolians, Oriental, Black, Native American and African-American people (Cranwell & Sinclair, 2016). A Taiwanese study found the prevalence of 11.8% in a sample of 26.226 women aged 30 and over (Su et al., 2013).

In a study with 476 monozygotic pairs of twins and 408 dizygotic male pairs of twins confirmed that heritability is very significant for the hair loss progression (Nyholt, Gillespie, Heath & Martin, 2003). Using the data of more than 52.000 men aged between 40 and 69 from the UK Biobank it was identified more than 250 genetic loci involved with intense hair loss. The study has also generated a polygenic predictor that identify the risk for hair loss (Hagenaars et al., 2017).

The androgens role

Androgenetic alopecia develops as a response of the hair follicle cells to androgens in individuals with genetic predisposition, even though the androgen concentration in the blood is normal (Gatherwright et al., 2013).

Testosterone is metabolized into dihydrotestosterone (DHT) by the enzyme 5α-reductase (5αR). This metabolite has more affinity with androgen receptor and is considered the main responsible for androgen-mediated effects in the scalp of AGA patients . Under the androgens influence the dermal papilla of hair follicles secrets many cytokines, such as TGFβ 1, IL-1α and TNFα that can induce the anagen phase premature termination (Rossi et al., 2016).

The follicular miniaturization is a theory that explains the decreased density of hair on the scalp area affected by the AGA. This process involves the shortening of the anagen phase in the hair cycle and an ascendation of the follicle from the reticular dermis to the papillary dermis that progressively happen with each new hair cycle. The follicular units, previously larger and with terminal hairs, become smaller and with vellus hair pattern (Whiting, 2001).

Another explanation for the hair density decrease in the affected patients is the increase of the kenogen duration that can happen together with the miniaturization process. After the exogen event (when the hair falls out) a phase named kenogen takes place, i.e., the follicles are empty. This kenogen phase can last from 3 months to 1 year, but in AGA cases it can be longer (Guarrera & Rebora, 2005). An increase of the kenogen follicles can be more important for the decreased hair count than miniaturized hair follicles. The researchers concluded that after a 12-month AGA treatment the hair regrowth was initiated much more by the convertion of kenogen follicles to anagen than by an increased activity of miniaturized hair follicles (Rushton, Norris & Neste, 2016).

The microinflammation role

Although the pathophysiology of AGA is closely related to androgen metabolism, scientific evidences suggests that AGA is associated with dysregulation in the expression of inflammatory cytokines (Ramos et al., 2016), with the chronic microinflammation (slow and usually asymptomatic) being an aggravating factor.

While mild perifollicular inflammatory signs can be seen in 76% of patients with AGA and also in 30% of normal controls, more advanced levels of inflammation with deposition of concentric layers of fibrotic collagen (perifollicular fibrosis) is seen in AGA patients but not in normal controls (Nirmal et al., 2013). This inflammatory scenario can be

caused by the microbial flora, oxidative stress, aging, smoking, UV radiation and pollutants (Rossi et al., 2016) and can induce, as a final consequence, follicular atrophy with no possibility of regeneration of hair follicles (Trüeb, 2003). In a biopsy, if there are detachment of the arrector pili muscle from a miniaturized follicle this is a sign of AGA irreversibility for that follicle (Cranwell & Sinclair, 2016).

The chronic and global body inflammation present in patients with metabolic syndrome could explain why these subjects have a higher predisposition to show the signs of AGA (Su & Chen, 2010). This condition comprises abdominal obesity, dyslipidemia, high blood pressure, abnormal glucose tolerance (leading to hyperinsulinemia) and hyperaldosteronism. High insulin levels induces vasoconstriction, consequently the nutrition support of hair follicles could be compromised. Besides, insulin favors the effect of DHT over the follicles contributing to the follicular miniaturization. Therefore, the subclinic microinflammation around hair follicles could be a local manifestation of the aforementioned global inflammation (Su & Chen, 2010).

The role of environmental and lifestyle factors

Patients with a family history of AGA should be aware that their lifestyle choices and their environment can exacerbate AGA. The excessive free radicals produced by the body metabolism, the environmental exposure (ultraviolet radiation, pollutants, chemical irritants, microbes) and lifestyle (smoking, for example) are sources of oxidative stress that impact the hair during and after its production (Trüeb, 2015). This way, behavior modification can potentially decrease the extent of the clinical manifestations (Gatherwright et al., 2013).

A wide study (Su et al., 2013) evaluated the factors associated with AGA with 26,226 women aged 30 years or more. Statistically associations were noticed among an increased risk for AGA and high body mass index, high fasting glucose, earlier puberty, 3 or fewer childbirths, oral contraceptive use for 1 year or longer and ultraviolet exposure more than 16 hours per week.

Some factors such as sun radiation, pollutants, skin and intrafollicular microbiota may be responsible, in part, for the microinflammation in the upper part of the follicle in FPHL (Ramos & Miot, 2015). The UV radiation photoactivation of porphyrins produced by *Propionibacterium sp*. in the pilosebaceous duct leads to an increased free radicals productions and a consequent oxidative tissue injury and perifollicular microinflammation (Trüeb, 2003).

Scalp, even covered by a normal density of hair, suffers the impact of UV radiation (Trüeb, 2003). UV exposure can increase the severity of some hair loss disorders such as AGA. Regarding hair growth or hair cycles, some effects of UV radiation are acute telogen effluvium, perifollicular microinflammation, production of free radicals, nitric oxide and proinflammatory cytokines (Trüeb, 2003).

Epigenetics studies are proving that lifestyle can promote different response in monozygotic twins that, even with the same genetics, develop different AGA stages that can be related to differences in their life habits. In a study with 98 identical female twins it was found an association among some factors of their life and the development of FPHL in different patterns. For example, multiple marriages, longer sleep duration, higher stress severity, smoking history, diabetes mellitus, polycystic ovarian syndrome and hypertension were associated with frontal hair loss (Gatherwright et al., 2012). In another study 92 identical male twins were interviewed (Gatherwright et al., 2013). Smoking, excessive alcohol intake and increased exercise duration influenced on frontal, temporal, and vertex hair loss in a distinct spatiotemporal fashion.

The effects on hair growth cycle caused by tobacco are a point of interest. Smoking leads to the production of free radicals, which facilitates the entrance of DHT into dermal papillary cells, causing an increase in the sebaceous gland and 5αR activity (Gatherwright et al., 2013) and the release of pro-inflammatory cytokines from follicular keratinocytes inhibiting the hair growth (Trüeb, 2003). The cigarette smoking also leads to impaired circulation and eventually local ischemia, compromising the hair follicle nutrition. Finally, smoking can favor the androgen-dependent hair thinning by hydroxylation of estradiol and inhibition of the aromatase enzyme (Trüeb, 2003; Trüeb, 2015).

Current approaches in the treatment of androgenetic alopecia regarding its complex physiopathology

As a genetic condition, the currently available treatments for AGA are focused on the modulation of AGA signs and symptoms; they are not curative. The efficacy of such treatments is intrinsically related to their maintenance. The self-medication with over the counter products, as shampoos and oral supplements, is an attitude adopted by several AGA patients. It explains the gap of 4 years between the onset of hair loss and the first consultation visit to the hair clinic (Siah, Muir-Green, Shapiro, 2016).

Patients should be aware that AGA treatments can have different types of outcomes (Fig. 2), it helps them to create realistic expectations. One of them is to slow the evolution of

the balding process; another one is the prevention of further progression of hair loss; however, the most desired is the regrowth of hair (Shanshanwal & Dhurat, 2017) and the least expected is the non-responsive outcome, when the patient does not respond to the therapeutics. The association of therapeutic strategies with complementary mechanisms of action may enhance the efficacy of the treatment for a better outcome (Cranwell & Sinclair, 2016).

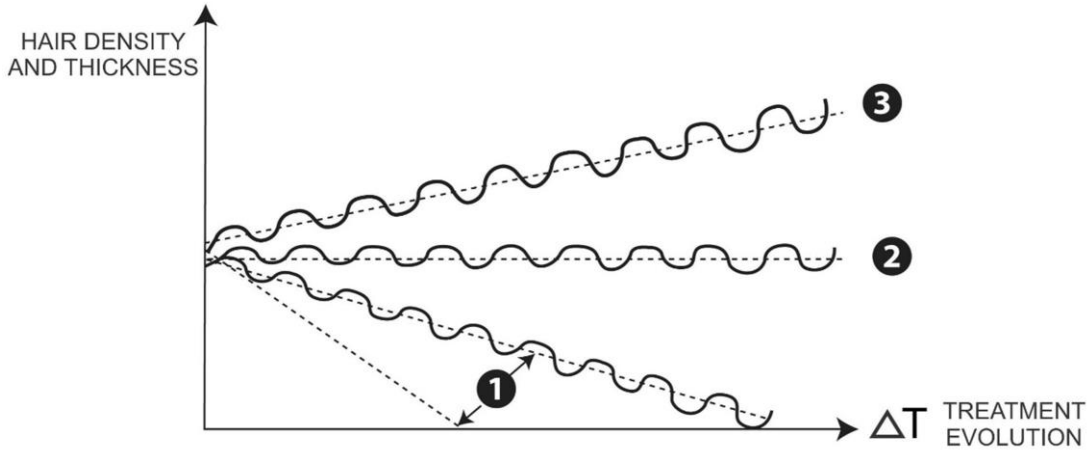

Figure 2: The image shows the three evolutionary models of an androgenetic alopecia treatment: Model 1 - Postponement of a progressive hair loss, Model 2 - Stabilization of the problem with maintenance of hair density and volume, Model 3 - Hair regrowth and improvement in thickness, promoting increased hair volume. The oscillatory lines present on the 3 models characterize the oscillatory daily hair loss of all patients.

Pharmacological treatments

The two treatments approved by the United States Food and Drug Administration (FDA) and by the European Medicines Agency for AGA is finasteride for men and minoxidil for both men and women (Cranwell & Sinclair, 2016; Rossi et al., 2016). In Brazil, the Agência Nacional de Vigilância Sanitária (http://portal.anvisa.gov.br/) approved these medicines, finasteride and minoxidil and a lotion for topical application containing 0.025% of 17α-estradiol (38). Despite the few medicines recognized and approved by the regulatory organs for treatment of AGA, doctors can prescribe off label drugs. That is the case of dutasteride, a finasteride derivative approved by ANVISA, FDA and Europe for the treatment of benign prostatic hyperplasia but its use in AGA is considered off label.

The main effects and/or mechanisms of a variety of active substances and the low-level laser therapy, the only FDA approved non-medicine treatment for the AGA, are described in Fig. 3.

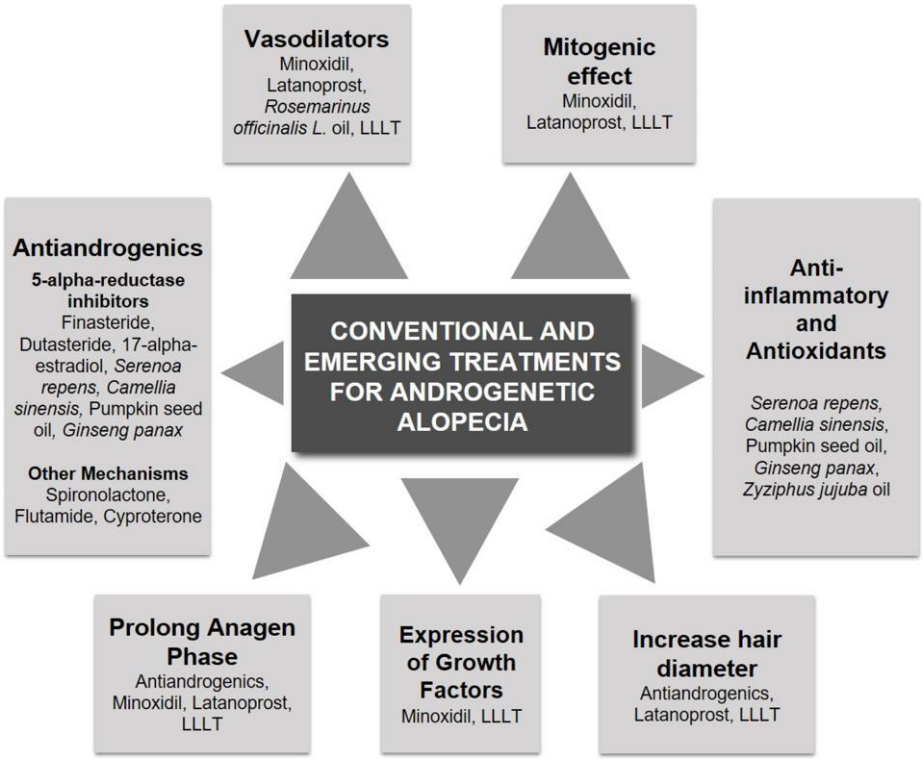

Figure 3: Conventional and emerging pharmacological treatments for androgenetic alopecia and their main mechanisms of action.

Finasteride is a type 2 5αR inhibitor. It irreversibly binds to the enzyme and because of that the conversion of testosterone into DHT is reduced (more than 60%) in serum and scalp. As DHT has 5 fold more affinity with the androgen receptor, the androgen mediated effects in the hair follicle are diminished. Besides, finasteride induces the conversion of telogen to anagen phase in the hair cycle. The results are expressive for near 50% of patients after 1 year of 1 mg oral finasteride a day (Shapiro & Kaufman, 2003). The possible adverse effects are the main reason why men decide do not undergo the treatment because it includes sexual dysfunctions, as reduced libido and erectile dysfunction (Cranwell & Sinclair, 2016). More recently, it has raised the fear by patients about the risk of persistent sexual dysfunction and depression following the use of oral finasteride, the so-called syndrome post finasteride.

The topical administration of finasteride has been evaluated and produced interesting results (Rossi et al., 2016; Caserini, Radicioni, Leuratti, Annoni & Palmieri, 2014). A 0.25% topical solution was compared to the conventional oral 1 mg a day dose. After a week of treatment, it was observed a similar inhibition of DHT production (plasma levels were diminished) for both formulations. The amount of finasteride in the plasma was lower after its topical application compared to the oral treatment (Caserini et al., 2014).

Dutasteride is capable of inhibit both type 1 and 2 of 5αR (both isoenzymes are present in hair follicles) many times more (3 and 100 times, respectively) than finasteride, so its ability to reduce scalp DHT is greater (Shanshanwal & Dhurat, 2017). A comparison between the effects of oral 0.5 mg dutasteride with 1 mg finasteride, in a daily intake for 24 weeks was performed with ninety men with AGA (Shanshanwal & Dhurat, 2017). Dutasteride was superior at the increase in total hair count per cm^2 and decrease of the thin hair count. Although reversible, the sexual dysfunction was the most related side effect for both medications. It is plausible that a reduction in the plasmatic DHT concentration may decrease the libido and/or orgasm (Caserini et al., 2014).

Minodixil is the most used topical medication to treat hair loss worldwide. In a retrospective study of female pattern hair loss, this was prescribed in the first consultation for 89% of women (Siah et al., 2016). Minoxidil has multiple functions (Cranwell & Sinclair, 2016): a) increases blood circulation because it induces vasodilatation and the overexpression of vascular endothelial growth factor; b) increases mitosis of hair matrix keratinocytes, promoting faster hair growth and thicker hair; c) prolongs the anagen phase; d) stimulates the kenogen follicles to initiate a new hair growth cycle. In an analysis of clinical trial in males, age was found to be the most important predictor of treatment success with minodixil, i.e., as younger the subjects were, better were the outcomes. Its main adverse effects in scalp are itchy, redness and dandruff and eventually facial hypertrichosis.

Monotherapy with topical minoxidil is an option when the scalp involvement in AGA is short, less than 20%. With increasing compromised areas, combined therapies should prevail, as topical minoxidil and oral finasteride, dutasteride or another active substance, as spironolactone, 17-alpha-estradiol (Kim et al., 2012) and latanoprost (Siah et al., 2016; Blume-Peytavi, Lönnfors, Hillmann & Garcia Bartels, 2012).

The efficacy and safety of a daily use of 0.025% 17-alpha-estradiol solution were investigated in Korean female patients with FPHL. At the end of 4 and 8 months, hair counts and diameter significantly increased in treated patients in comparison with baseline. The mechanisms of action of 17-alpha-estradiol in AGA is the reduction of DHT levels by suppressing the activity of 5αR. Besides, it was shown that a decrease of the testosterone production by inhibiting the action of 17β-dehydrogenase over androstenedione was achieved. It also accelerates the conversion of testosterone into estradiol by stimulating the aromatase, an enzyme considered protective in AGA patients (Kim et al., 2012).

Recent researches investigated a new possibility of treatment for AGA using prostaglandin F2α (PDF2α) analogues, as latanoprost, which has the ability to promote hair growth (Rossi et al., 2016). Latanoprost was first used to treat glaucoma and it was noticed an increased length, thickness and pigmentation of eyelashes. It is duo its ability to induce the vasodilatation, mitogenic stimulus, conversion of vellus to terminal hair and the entrance of the hair follicle in the anagen phase (Blume-Peytavi et al., 2012). A 24 weeks clinical study with 16 men with Hamilton-Norwood II-III AGA concluded that 0.1% latanoprost promoted significant increase of the hair density when compared with baseline and placebo areas (Blume-Peytavi et al., 2012).

A preliminary study about the use of cetirizine 1% in an alcoholic solution for the treatment of AGA was published in 2018 (Rossi et al.). Despite the fact of cetirizine is a second-generation histamine H1 receptor antagonist, it inhibits the prostaglandin PGD2, that plays a negative role in the hair growth. The study was realized for 6 months (1 application daily = 1 ml) and enrolled 85 patients divided in two groups, cetirizine (n=67) and control group (n=15). The authors found an increase in terminal hair density and a decrease in the vellus hair density. No side effects were reported and the compliance of the patients was good.

Valproic acid (VPA) is an anticonvulsant drug that was first studied by topical route for hair growth in 2012 by Lee and coworkers, using male murine mice. The authors noted that VPA induced some epidermal markers, such as filaggrin and loricrin, as well as the alkaline phosphatase present in dermal papilla, related to the up-regulation of the Wnt/b-catenin pathway that induces the anagen phase. A randomized, double-blind, placebo-controlled study compared the results of 27 volunteers with moderate AGA, 15 using sodium valproate 8.3% and 12 placebo, twice daily (~ 0.8 ml each time). Patients of the valproate group had a significant increased hair count in AGA areas compared with the placebo group. Adverse events were mild and self-limited in both groups.

The search for alternative therapies for the prevention or treatment of alopecia and hair loss leads to a variety of medicinal plants studied. Oral saw palmetto (*Serenoa repens*) is one of the most used and widely advertised plant to control AGA signs. *Serenoa repens* extract has properties to interfere in key processes related to the AGA pathophysiology (especially to inhibit the two isoforms of the 5αR enzyme) with the advantage of not presenting side effects like finasteride (Rossi et al., 2012).

Another phytoingredient already tested to treat AGA is pumpkin seed oil (Cho et al., 2014), *Camellia sinensis* or green tea (Amin et al., 2014), *Panax ginseng* (Murata, Takeshita, Samukawa, Tani & Matsuda, 2012) and essential oils. The composition of pumpkin seed oil comprises substances with anti-inflammatory, antioxidant and 5αR inhibition properties (Cho et al., 2014). Green tea is antioxidant and anti-inflammatory. Its properties are associated with the presence of epigallocatechin-3-gallate, a major constituent of polyphenols (Amin, Simamora, Anwar, Djajadisastra, 2014). *Panax ginseng*, the Korean ginseng, is rich in ginsenosides (Murata et al., 2012). Its anti-apoptotic and 5αR inhibition ability were studied and a recent paper showed that the mechanism of action of ginsenosides is similar to that observed with minoxidil regarding hair growth (Kim et a., 2015).

Essential oils have been studied for a big variety of medical and cosmetic application. The essential oils properties are believed to be closely related to the complexity of its composition. Nowadays, essential oils are empirically used in the treatment of hair alterations, for both scalp and fiber. There are few well-conducted scientific papers that bring the essential oils as an effective strategy for stimulating hair growth in AGA patients or for the restoration of the scalp health. A group of researchers (Yoon, Al-Reza & Kang, 2010) showed the potential of jujube (*Zizyphus jujuba*) seed essential oil, which has anti-inflammatory activity, in stimulating hair growth in mice.

The *Rosmarinus officinalis L*. essential oil, whose major components are 1,8-cineol, borneol, bornyl acetate, camphor, alpha-pinene and beta-pinene, increased the hair counts after 6 months of treatment in AGA patients (Panahi, Taghizadeh, Marzony, & Sahebkar, 2015), with effects comparable to those obtained with minoxidil. The authors suggest that the antioxidant and vasodilatation properties of rosemary essential oil may be responsible for its stimulatory effect on hair growth. It is known that the scalp of AGA patients are inflamed and, as a consequence, with a large amount of free radicals. In addition, vasodilation and the consequently increased blood circulation in the scalp capillaries could happen because of the presence of camphor, the major component of the oil.

Low-level laser therapy

Low-level laser therapy (LLLT) consists in exposing tissues to luminous energy (< 1000 mW), usually in the red (600 to 700 nm) or near infrared (700 to 1000 nm) range (Chung et al., 2012). A meta-analysis of 45 papers regarding the only three FDA-approved treatments for AGA (minoxidil, finasteride and LLLT) showed that LLLT induces more hair growth than minoxidil when used as a monotherapy and it is the only FDA-cleared device to

treat AGA (Adil & Godwin, 2017). The combination of LLLT with medicines can act synergistically in hair regrowth.

The primary photoacceptor (chromophore) for the red and near infrared light in mammalian cells is the cytochrome C oxidase, a complex of transmembrane proteins in the mitochondria (Chung et al., 2012). The energy released from this excited chromophore results in an alteration of biomolecules through photochemical reactions, commonly referred as photobiomodulation (Avram, Leonard, Epstein, Williams & Bauman, 2007). This should increase the adenosine triphosphate (ATP) production, modulate the free radicals and induce transcription factors that trigger a cascade of cellular events that lead to the synthesis of proteins involved in cell proliferation and migration, modulation of cytokine levels, growth factors and inflammatory mediators (Chung et al., 2012). Another mechanism of the LLLT is an increased blood flow at the dermal papilla (Avram et al., 2007).

Some of the LLLT applications include tissue regeneration (adjuvant in the treatment of burns, ulcers, acne, scarring processes), dandruff and other microbial-related skin disorders, reduction of inflammation and pain relief (Satino & Markou, 2004). However, one of the most commercially accepted applications of LLLT is the stimulation of hair growth in individuals with some type of alopecia. A review paper analyzed the five most relevant clinical studies using LLLT in the treatment of AGA and concluded that in all of them the LLL treated group presented a statistically significant increase in the mean hair count (Delaney & Zhang, 2018). The LLLT provides no significant side effects, which differentiates it from the other AGA treatment options in the market (Delaney & Zhang, 2018).

One of the first reports regarding the LLLT involved 35 patients with AGA, 28 male and 7 female that used every other day, for 5 to 10 minutes, the HairMax LaserComb. After six months of treatment, both sexes and all areas (vertex and temporal) did demonstrate significant improvement. The tensile strength of hair increased (Satino & Markou, 2004) as well, probably as a consequence of the increased hair diameter.

The HairMax Laser Comb® brush was first approved for the treatment of AGA in men in 2007 and in women in 2011. Jimenez and coworkers (Jimenez et al., 2014) evaluated whether there would be a difference in the final result depending on the brush model (7, 9 or 12 light-emitting sources at 635 and/or 655 nm). Depending on the model, the application time varied (15, 11 and 8 minutes, respectively) as a strategy to obtain an equal dose. All of them should be used three times a week as a monotherapy. On average, regardless the brush model, age and gender of the patient, the increase in hair density was around 20 hairs.cm^{-2} for

the LLL-treated groups. Side effects were uncommon and restricted to dry skin, itching, irritation, scalp sensitivity and heat sensation.

The LLLT effects (safety and physiology) were evaluated in men with AGA (n=44, 18–48 years, Hamilton–Norwood IIa–V). The treated group received a helmet like apparatus containing 21 lasers (5mW) and 30 LEDS, both at 655 nm, to use at home respecting the interval of one day between sessions for 4 months (60 sessions, 67 $J.cm^{-2}$/25 min treatment). Baseline hair counts increased by 39% compared to the end of the treatment in the treated group, with no side effects, while in the placebo group the hair count decreased (Lanzafame et al., 2013).

The association of LLLT with minoxidil has been reported as efficient for AGA. In a 4-month study with 45 women presenting FPHL, the efficacy and safety of topical minoxidil (twice a day) and LLLT (3 times a week, 25 min, with the helmet iGrow®) as separated treatments showed good and comparable results. The combination group (minoxidil + LLLT) induced the best results regarding the Ludwig classification and patient satisfaction (Esmat et al., 2017).

Drug-loaded nanoparticles

The nanotechnology advantages for the development of medicines and cosmetics are widely studied and include reduction of adverse effects and substance degradation (Weber et al., 2016) as well as controlled-substance release (Katzer, Chaves, Pohlmann, Guterres & Beck, 2017). In trichology, nanoparticles have been studied to treat hair fiber, scalp and hair follicles.

End products containing different types of nanocarries are commercially available for the treatment of several health and aesthetic conditions. The nanocarriers can be distinguished by their morphology, size, solubility in the biological media (Katz, Dewan & Bronaugh, 2015) and supramolecular composition, being the carbon-based, metal, ceramic, semiconductor, polymeric and lipid-based nanoparticles (NPs) the most studied. Drug delivery via hair follicles are of great interest in trichology, mainly if the target of action is the hair follicle itself. Advantages of nanoparticle carrier systems over free drugs in the trichology field comprise a deeper penetration into hair follicle as well as a prolonged storage (depot system). The drugs removal and/or the drug-loaded nanoparticles from the follicle will slowly happen by hair growth and sebum flow (Lademann et al., 2007). One of the main reasons to explore the topical application is to avoid the potential adverse side effects of wide systemic modulation of drugs (Caserini et al., 2014). The effects would preferentially happen

in the scalp (Caserini et al., 2014). Nanotechnological approaches have been explored for different substances intended for hair and scalp care, as arginine (Yazdani-Arazi, Ghanbarzadeh, Adibkia, Kouhsoltani, & Hamishehkar, 2016), spironolactone (Shamma & Aburahma, 2014), finasteride (Gomes, Martins, Ferreira, Segundo & Reis, 2014), dutasteride (Noor, Sheikh, Somavarapu & Taylor, 2017) and minoxidil (Gomes et al., 2014).

Shamma & Aburahma (2014) developed spironolactone-loaded nanostructured lipid carriers (NLC), an antiandrogen currently off label drug for the treatment of female AGA. The fluorescent-labeled nanocarriers were detected around the hair follicles and hair shafts of albino mice. The affinity of NLC to the follicular duct fulfilled with sebum could contribute to this distribution profile.

Dutasteride-loaded NLC coated with chitosan oligomer-stearic acid for topical application was developed (Noor et al., 2017). The main appeal was to target the hair follicle without exposing the whole body to undesired systemic side effects as it happens with oral administration. Unloaded NLC and free dutasteride were cytotoxic to human dermal papilla cells, while dutasteride-loaded NLC, coated or not, increased almost 20-fold the maximum non-toxic concentration, probably due to the slow drug release.

There are some studies focused on determining the ideal particle size for follicular penetration. Lademan and coworkers (Lademann et al., 2011) reviewed some papers and concluded that the optimum size should be between 320 and 750 nm. Besides, the reservoir capacity for nanoparticles is higher than for non-particle molecules (Lademann et al., 2011).

However, although the particle size plays an important role in the follicular penetration, the type of nanocarrier and consequently its molecular composition and architecture can also influence the results. Mathes and coworkers (Mathes et al., 2016) compared the follicular uptake of Rhodamin-B-labeled drug-free polymeric nanoparticles (nanospheres, 128 nm; nanocapsules, 257 nm and lipid-core nanocapsules, 197 nm - the size are representative of the mean value). A higher follicular uptake of the nanocapsules was demonstrated. It was postulated by the authors that the supramolecular structure of nanocapsules could result in a more flexible system. Interestingly, the nanocapsules were the biggest particles among the three systems evaluated.

Considering the advantages of the nanoparticulated system for the intrafollicular drug delivery, it is expected in a near future that a drug-loaded nanocarrier becomes a prescription option for the treatment of AGA.

Conclusion

The physiopathology of androgenetic alopecia comprises a genetic predisposition of the hair follicle cells to the androgen effects. Besides, a subclinical chronic inflammation and a significant oxidative stress contribute to the progression of AGA. Furthermore, the influence of environmental exposure and lifestyle (nutrition, medicines intake, sun light exposure, stress management, tabagism, …) have gained attention. The FDA approved treatments for AGA are very restrict and are not effective for all AGA patients. A repeated question about hair therapies is how to improve hair density and stimulate the hair regrowth. The answer is not as simple as patients and doctors would like it to be, but probably by combining pharmacological ingredients, new forms of drug delivery systems, instrumental technologies and changes in lifestyle, a satisfactory outcome could be reached in the treatment of AGA.

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REFERENCES

Adil, A. & Godwin, M. (2017). The effectiveness of treatments for androgenetic alopecia: a systematic review and meta-analysis. *Journal of the American Academy of Dermatology, 77*(1), 136-141. https://doi.org/10.1016/j.jaad.2017.02.054

Amin, J., Simamora, E. L. P., Anwar, E. & Djajadisastra, J. (2014). Green tea (Camellia sinensis, l.) ethanolic extract as hair tonic in nutraceutical: physical stability, hair growth activity on rats, and safety test. *International Journal of Pharmacy and Pharmaceutical Sciences, 6*(5), 94-99. https://innovareacademics.in/journal/ijpps/Vol6Issue5/8946.pdf

Avram, M. R., Leonard, R. T. Jr, Epstein, E. S., Williams, J. L. & Bauman, A. J. (2007). The current role of laser/light sources in the treatment of male and female pattern hair loss. *Journal of Cosmetic and Laser Therapy, 9*(1) 27-28. https://doi.org/10.1080/14764170601134479

Blume-Peytavi, U., Lönnfors, S., Hillmann, K. & Garcia Bartels N. (2012). A randomized double-blind placebo-controlled pilot study to assess the efficacy of a 24-week topical treatment by latanoprost 0.1% on hair growth and pigmentation in healthy volunteers with androgenetic alopecia. *Journal of the American Academy of Dermatology, 66*(5), 794-800. https://doi.org/10.1016/j.jaad.2011.05.026

Caserini, M., Radicioni, M., Leuratti, C., Annoni, O. & Palmieri, R. (2014). A novel finasteride 0.25% topical solution for androgenetic alopecia: pharmacokinetics and effects on plasma androgen levels in healthy male volunteers. *International Journal of Clinical Pharmacology and Therapeutics, 52*(10), 842-849. https://doi.org/10.5414/CP202119

Cho, Y. H., Lee, S. Y., Jeong, D. W., Choi, E. J., Kim, Y. J., Lee, J. G. …, Cha, H. S. (2014). Effect of pumpkin seed oil on hair growth in men with androgenetic alopecia: a randomized, double-blind, placebo-controlled trial. *Evidence-Based Complementary and Alternative Medicine*: *eCAM,* 549721. http://dx.doi.org/10.1155/2014/549721

Chung, H., Dai, T., Sharma, S. K., Huang, Y. Y., Carroll, J. D. & Hamblin, M. R. (2012). The nuts and bolts of low-level laser (light) therapy. *Annals of Biomedical Engineering*, *40*(2), 516-533. https://doi.org/10.1007/s10439-011-0454-7

Cranwell, W & Sinclair R. (2016). Male Androgenetic Alopecia. In: De Groot, L. J., Chrousos, G., Dungan, K., Feingold, K. R., Grossman, A., Hershman, J. M., … Vinik, A. (Eds.), *Endotext.* Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK278957/

Delaney, S. W. & Zhang, P. (2018). Systematic review of low-level laser therapy for adult androgenic alopecia. *Journal of Cosmetic and Laser Therapy, 20*(4), 229-236. https://doi.org/10.1080/14764172.2017.1400170

Esmat, S. M., Hegazy, R. A., Gawdat, H. I., Hay, R. M. A., Allam, R. S., Naggar, R. E. & Moneib, H. (2017). Low Level Light-Minoxidil 5% Combination Versus. Either Therapeutic Modality Alone in Management of Female Patterned Hair Loss: A Randomized Controlled Study. *Lasers in Surgery and Medicine*. *49*(9), 835-843. https://doi.org/10.1002/lsm.22684

Gatherwright, J., Liu, M. T., Amirlak, B., Gliniak, C., Totonchi, A. & Guyuron, B. (2012). The contribution of endogenous and exogenous factors to female alopecia: a study of identical twins. *Plastic and Reconstructive Surgery, 130*(6), 1219-1226. https://doi.org/10.1097/PRS.0b013e31826d104f

Gatherwright, J., Liu, M. T., Amirlak, B., Gliniak, C., Totonchi, A. & Guyuron, B. (2013). The contribution of endogenous and exogenous factors to male alopecia: a study of identical twins. *Plastic and Reconstructive Surgery, 131*(5), 794e-801. https://doi.org/10.1097/PRS.0b013e3182865ca9
Gomes, M. J., Martins, S, Ferreira, D., Segundo, M. A. & Reis, S. (2014). Lipid nanoparticles for topical and transdermal application for alopecia treatment: development, physicochemical characterization, and in vitro release and penetration studies. *International Journal of Nanomedicine,* 9, 1231-1242. https://doi.org/*10.2147/IJN.S45561*

Guarrera, M. & Rebora, A. (2005). Kenogen in female androgenetic alopecia. A longitudinal study. *Dermatology, 210*(1), 18-20. https://doi.org/10.1159/000081477

Hagenaars, S. P., Hill, W. D., Harris, S. E., Ritchie, S. J., Davies, G., Liewald, D. C., … Marioni, R. E. (2017). Genetic prediction of male pattern baldness. *PLoS Genetics, 13*(2), e1006594. https://doi.org/10.1371/journal.pgen.1006594

Jimenez, J. J., Wikramanayake, T. C., Bergfeld, W., Hordinsky, M., Hickman, J. G., Hamblin, M. R. & Schachner, L. A. (2014). Efficacy and safety of a low-level laser device in the treatment of male and female pattern hair loss: A multicenter, randomized, sham device-controlled, double-blind study. *American Journal of Clinical Dermatology, 15*(2), 115-127. https://doi.org/10.1007/s40257-013-0060-6

Katz, L. M., Dewan, K. & Bronaugh, R. L. (2015). Nanotechnology in cosmetics. *Food and Chemical Toxicology*. *85,* 127-137. https://doi.org/10.1016/j.fct.2015.06.020

Katzer, T., Chaves, P. S., Pohlmann, A. R., Guterres, S. S. & Beck, R. C. R. (2017). Loading a drug on contact lenses using polymeric nanocapsules: effects on drug release, transparency, and ion permeability. *Journal of Nanoscience and Nanotechnology*. *17*(12), 9286-9294. https://doi.org/10.1166/jnn.2017.13879

Kim, J. H., Lee, S. Y., Lee, H. J., Yoon, N. Y. & Lee, W. S. (2012). The efficacy and safety of 17α-estradiol (Ell-Cranell® alpha 0.025%) solution on female pattern hair loss: single center, open-label, non-comparative, phase IV study. *Annals of Dermatology, 24*(3), 295-305. https://doi.org/10.5021/ad.2012.24.3.295

Kim, S. N., Kim, S., Hong, Y. D., Park, H., Shin, S. H., Kim, A. R. ..., Park, W. S. (2015). The ginsenosides of Panax ginseng promote hair growth via similar mechanism of minoxidil. *Journal of Dermatological Science, 77*(2), 132-134. https://doi.org/10.1016/j.jdermsci.2014.12.007

Lademann, J., Richter, H., Teichmann, A., Otberg, N., Blume-Peytavi, U., Luengo, J. …, Sterry, W. (2007). Nanoparticles - An efficient carrier for drug delivery into the hair follicles. *European Journal of Pharmaceutics and Biopharmaceutics*, *66*(2), 159-164. https://doi.org/10.1016/j.ejpb.2006.10.019

Lademann, J., Richter, H., Schanzer, S., Knorr, F., Meinke, M., Sterry, W. & Patzelt, A. (2011). Penetration and storage of particles in human skin: Perspectives and safety aspects. *European Journal of Pharmaceutics and Biopharmaceutics*. *77*(3), 465-468. https://doi.org/10.1016/j.ejpb.2010.10.015

Lanzafame, R. J., Blanche, R. R., Bodian, A. B., Chiacchierini, R. P., Fernandez-Obregon, A., Kazmirek, E. R. (2013). The growth of human scalp hair mediated by visible red light laser and LED sources in males. *Lasers in Surgery and Medicine, 45*(8), 487-495. https://doi.org/10.1002/lsm.22173

Lee S.-H., Yoon J., Shin S. H., Zahoor M., Kim H. J., Park P. J. …, Choi K.-Y. (2012). Valproic acid induces hair regeneration in murine model and activates alkaline phosphatase activity in human dermal papilla cells. *PLoS ONE*, *7*(4):e34152. https://doi.org/10.1371/journal.pone.0034152

Mathes, C., Melero, A., Conrad, P., Vogt, T., Rigo, L., Selzer, D. ..., Schaefer, U. F. (2016). Nanocarriers for optimizing the balance between interfollicular permeation and follicular uptake of topically applied clobetasol to minimize adverse effects. *Journal of Controlled Release*, *223*, 207-214. https://doi.org/10.1016/j.jconrel.2015.12.010

Murata, K., Takeshita, F., Samukawa, K., Tani, T. & Matsuda, H. (2012). Effects of Ginseng rhizome and ginsenoside Ro on testosterone 5α-reductase and hair re-growth in testosterone-treated mice. *Phytotherapy Research*, *26*(1), 48-53. https://doi.org/10.1002/ptr.3511

Nirmal, B., Somiah, S., Sarvajnamurthy, S. A., Biligi, D. S. & Palo, S. (2013). Evaluation of perifollicular inflammation of donor area during hair transplantation in androgenetic alopecia

and its comparison with controls. *International Journal of Trichology*, *5*(2), 73-76. https://doi.org/10.4103/0974-7753.122963

Noor, N. M., Sheikh, K., Somavarapu S. & Taylor, K. M. G. (2017). Preparation and characterization of dutasteride-loaded nanostructured lipid carriers coated with stearic acid-chitosan oligomer for topical delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, *117*, 372-384. https://linkinghub.elsevier.com/retrieve/pii/S0939641117304575

Nyholt, D. R., Gillespie, N. A., Heath, A. C. & Martin, N. G. (2003). Genetic basis of male pattern baldness. *Journal of Investigative Dermatology, 121*(6), 1561-1564. https://doi.org/10.1111/j.1523-1747.2003.12615.x

Panahi, Y., Taghizadeh, M., Marzony, E. T. & Sahebkar, A. (2015). Rosemary oil vs minoxidil 2% for the treatment of androgenetic alopecia: a randomized comparative trial. *SKINmed Dermatology for the Clinician, 13*(1), 15-21. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25842469

Ramos, P. M. & Miot, H. A. (2015). Female Pattern Hair Loss: a clinical and pathophysiological review. *Anais Brasileiros de Dermatologia, 90*(4), 529-543. http://dx.doi.org/10.1590/abd1806-4841.20153370

Ramos, P. M., Brianezi, G., Martins, A. C. P., Silva, M. G., Marques, M. E. A. & Miot, H. A. (2016). Apoptosis in follicles of individuals with female pattern hair loss is associated with perifollicular microinflammation. *International Journal of Cosmetic Science*. *38*(6), 651-654. https://doi.org/10.1111/ics.12341

Rinaldi, S., Bussa, M. & Mascaro, A. (2016). Update on the treatment of androgenetic alopecia. *European Review for Medical and Pharmacological Sciences, 20*(1), 54-58. Retrieved from https://www.europeanreview.org/article/10128

Rossi, A., Mari, E., Scarno, M., Garelli, V., Maxia, C., Scali, E. …, Carlesimo, M. (2012). Comparative effectiveness of finasteride vs Serenoa repens in male androgenetic alopecia: a two-year study. *International Journal of Immunopathology and Pharmacology, 25*(4), 1167-1173. https://doi.org/10.1177/039463201202500435

Rossi, A., Anzalone, A., Fortuna, M. C., Caro, G., Garelli, V., Pranteda, G. & Carlesimo, M. (2016). Multi-therapies in androgenetic alopecia: review and clinical experiences. *Dermatologic Therapy*, *29*(6), 424-432. https://doi.org/10.1111/dth.12390

Rossi, A., Campo, D., Fortuna, M.C., Garelli, V., Pranteda, G., De Vita, G. ..., Carlesimo, M. (2018). A preliminary study on topical cetirizine in the therapeutic management of androgenetic alopecia. *The Journal of Dermatological Treatment*, 29(2), 149-151. https://doi.org/10.1080/09546634.2017.134161

Rushton, D. H., Norris, M. J. & Neste, D. V. (2016). Hair regrowth in male and female pattern hair loss does not involve the conversion of vellus hair to terminal hair. *Experimental Dermatology*, *25*(6), 482-484. https://doi.org/10.1111/exd.12945

Satino, J. L. & Markou, M. (2004). Hair regrowth and increased hair tensile strength using the Hairmax Lasercomb for low-level laser therapy. *International Journal of Cosmetic Surgery and Aesthetic Dermatology, 5*(2), 113-117. https://doi.org/10.1089/153082003769591209

Siah, T. W., Muir-Green, L. & Shapiro, J. (2016). Female pattern hair loss: a retrospective study in a tertiary referral center. *International Journal of Trichology, 8*(2), 57–61. https://doi.org/10.4103/0974-7753.188033

Shamma, R. N. & Aburahma, M. H. (2014). Follicular delivery of spironolactone via nanostructured lipid carriers for management of alopecia. *International Journal of Nanomedicine, 9*, 5449-5460. https://doi.org/10.2147/IJN.S73010

Shanshanwal, S. J. & Dhurat, R. S. (2017). Superiority of dutasteride over finasteride in hair regrowth and reversal of miniaturization in men with androgenetic alopecia: A randomized controlled open-label, evaluator-blinded study. *Indian Journal of Dermatology, 83*(1), 47-54. https://doi.org/10.4103/0378-6323.188652

Shapiro, J. & Kaufman, K. D. (2003). Use of finasteride in the treatment of men with androgenetic alopecia (male pattern hair loss). *Journal of Investigative Dermatology Symposium Proceedings, 8*(1), 20-23. https://doi.org/10.1046/j.1523-1747.2003.12167.x

Su, L. H. & Chen, T. H. (2010). Association of androgenetic alopecia with metabolic syndrome in men: a community-based survey. *British Journal of Dermatology*, *163*(2), 371-377. https://doi.org/10.1111/j.1365-2133.2010.09816.x

Su, L. H., Chen, L. S. & Chen, H. H. (2013). Factors associated with female pattern hair loss and its prevalence in Taiwanese women: a community-based survey. *Journal of the American Academy of Dermatology, 69*(2), 69-77. https://doi.org/10.1016/j.jaad.2012.09.046

Trüeb, R. M. (2003). Is androgenetic alopecia a photoaggravated dermatosis? *Dermatology*. *207*(4), 343-348. https://doi.org/10.1159/000074111

Trüeb, R. M. (2015). The impact of oxidative stress on hair. *International Journal of Cosmetic Science*. *37*(2 suppl), 25–30. https://doi.org/10.1111/ics.12286

Whiting, D. A. (2001). Possible mechanisms of miniaturization during androgenetic alopecia or pattern hair loss. *Journal of American Academy of Dermatology, 45*(3 suppl), S81-86. https://doi.org/10.1067/mjd.2001.117428

Weber, J., Funk, N. L., Motta, M. H., Guedes, A. M., Visintainer, A. P., Tedesco, S. B. & da Silva, C. B. (2016). Association of borage oil and betamethasone dipropionate in lipid-core nanocapsules: characterization, photostability and in vitro irritation test. *Journal of Nanoscience and Nanotechnology*, *16*(2), 1354-1362. https://doi.org/10.1166/jnn.2016.11674

Yazdani-Arazi, S. N., Ghanbarzadeh, S., Adibkia, K., Kouhsoltani, M. & Hamishehkar, H. (2016). Histological evaluation of follicular delivery of arginine via nanostructured lipid carriers: a novel potential approach for the treatment of alopecia. *Artificial Cells, Nanomedicine and Biotechnology, 45*(7), 1379-1387. https://doi.org/10.1080/21691401.2016.1241794

Yoon, J. I., Al-Reza, S. M. & Kang, S. C. (2010). Hair growth promoting effect of Zizyphus jujuba essential oil. *Food and Chemical Toxicology, 48*(5), 1350-1354. https://doi.org/10.1016/j.fct.2010.02.036

3. MANUSCRITO 2: β-SITOSTEROL-LOADED NANOPARTICLES AND LOW-LEVEL LASER THERAPY FOR THE TREATMENT OF ANDROGENETIC ALOPECIA: *in vitro* and *in vivo* studies

ABSTRACT

This paper reports the development and characterization of β-sitosterol-loaded nanostructured delivery systems associated with the low-level laser (LLL) intended for androgenetic alopecia (AGA) treatment, the main cause of hair loss. β-sitosterol has anti-inflammatory and 5-α-reductase inhibition properties and as so is a good candidate to treat AGA. β-sitosterol-loaded nanocapsules (BS-NC) and nanostrucutured lipid carriers (BS-NLC) were prepared by interfacial deposition of preformed polymer and emulsification followed by ultrasonication, respectively. The effect of LLL irradiation over nanocarriers was investigated. Irritancy potential of the nanoformulations was verified *in vitro* on the chorioallantoic membrane of hen's egg. Their citotoxicity were evaluated *in vitro,* with 3T3 (fibroblasts) and HaCaT (keratinocytes) cell lines, and *in vivo,* through the *Allium cepa* assay, with or without association with LLL. To study the effect of BS-NC on AGA, the testosterone-induced alopecia model was performed with albino Swiss rats. The developed nanoparticles showed mean particle sizes between 115 and 230 nm, BS encapsulation efficiency of 100%, with drug content near the theoretical value (1 $mg.ml^{-1}$). No chances in particle size, zeta potential and drug content were detected after LLL irradiation. Both formulations were found to be non-irritant in the chorioallantoic membrane test independent of BS presence. Treatment of the 3T3 and HaCaT cell lines with BS-NC and BS-LNC and the respective blank formulations did not induce cytotoxicity neither cell proliferation, as well as the LLL treatments. Cell viability just decreased for both cell lines when BS-solution was used, demonstrating the ability of nanostructures to protect against BS cytotoxicity. The mitotic index was not influenced by the formulations (blank and BS-loaded NLC and NC, as well as BS-solution) when compared to water in the *Allium cepa* bioassay. The *in vivo* testosterone-induced AGA model revealed through the histology that the effect on alopecia is better when BS-NC is associated with LLL, rather than BS-NC alone or finasteride. In conclusion, this paper proposed for the first time the association of nanoparticles with LLL and this was proven to be a promisor association to treat AGA.

Keywords: Nanotechnology. Cytotoxicity. *Allium cepa.* Testosterone-induced alopecia model.

INTRODUCTION

Androgenetic alopecia (AGA) also referred as male or female pattern hair loss is the most common cause of progressive reduction in hair density (HAJHEYDARI et al., 2009; OTBERG; FINNER; SHAPIRO, 2007; TRÜEB, 2002), with an incidence of 70% of men and 30 to 40% of women at some point in their lives (CHEN et al., 2016, LEVY; EMER, 2013). The level of severity, the initial age of manifestation and the location of hair rarefaction on the scalp are variable (CRABTREE et al., 2010; TRÜEB, 2002; FIURÁSKOVÁ; KUCEROVÁ; KOLÁR, 2003; LEVY; EMER, 2003). AGA is a disease of polygenic origin (HEILMANN et al., 2013), which is hereditary and, once manifested, it is considered chronic. Genetic predisposition contributes about 80% to the onset of the disease (CRANWELL; SINCLAIR, 2016).

The pathophysiology of AGA involves several possibilities, being a central question the metabolism and the exacerbated activity of androgens in pilosebaceous units genetically sensitive to them (TABBAKHIAN et al., 2006). Androgens seem to impact hair growth by decreasing the duration of the anagen phase. This process is progressive and referred as the follicular miniaturization (PARK et al., 2003). While the anagen phase decreases, the kenogen phase, defined by the period between hair loss and the growth of another hair, is increasingly long (CRANWELL; SINCLAIR, 2000; FIURÁSKOVÁ; KUCEROVÁ; KOLÁR, 2003), to the point where the hair is no longer produced (CRABTREE et al., 2010).

Although the pathophysiology of AGA is closely related to androgen metabolism, new scientific evidence suggests that AAG is associated with a dysregulation in the expression of inflammatory cytokines (CHITTUR et al., 2011), with chronic micro inflammation of the hair follicles being an aggravating factor (TRÜEB, 2002). This possibility is reiterated since modulation of androgenic action does not result in reversal of miniaturized follicles in advanced AGA cases, as well as the by the variability of effectiveness of androgen modulation treatments. In addition, histological scalp biopsy studies have shown that miniaturization of terminal hair is often associated with perifollicular lymphocytic infiltrate and, eventually, fibrosis (TRÜEB, 2002).

Several drugs and natural ingredients are being studied for the treatment of AGA, however the only drugs recognized by the Food and Drug Administration (FDA) for its treatment are oral finasteride (Propecia®, Merck, 1 mg per day, oral use, just for men) and topical minoxidil (Rogaine®, Pharmacia, 2 and 5%, solution or foam, topically applied, for both men and women) (HAJHEYDARI et al., 2009; PRAGER et al., 2002). In addition to

drug therapy, LLL is the only FDA-cleared device to treat AGA (ADIL; GODWIN, 2017; AVCI et al., 2014). LLL therapy consists of exposing cells or tissues at low levels of luminous energy (<1000 mW), usually in the red (600 - 650 nm) or near infrared (950 - 1200 nm) range (MANDEL; HAMBLIN, 2012). Some of the applications of LLL therapy include tissue regeneration, reduction of inflammation and pain relief (CHUNG et al., 2012), but the most commercially accepted application of LLL is the hair growth stimulation. When used as a monotherapy, LLLT induces more hair growth than minoxidil (ADIL; GODWIN, 2017).

The search for therapeutic alternatives for the prevention or treatment of AGA leads to the meeting of a variety of phytoingredients (REUTER; MERFORT; SCHEMPP, 2010). β-sitosterol is a sterol present in vegetable oils and structurally very similar to cholesterol (UPADHYAY; GUPTA; DIXIT, 2012). Its anti-inflammatory activity (NIRMAL et al., 2012), ability to inhibits tumor cell growth (LOMENICK, et al., 2015; AWAD; BURR; FINK, 2005) and the inhibition of 5α-reductase (a key enzyme the development of AGA) (CHITTUR; PARR; MARCOVICI, 2012; CHEN et al., 2016) have already been demonstrated. By oral route, Prager et al. (2002) evaluated a combination of *Serenoa repens* 200 mg and BS 50 mg (with bioavailability increasers: lecithin 50 mg, inositol 100 mg, phosphatidyl choline 25 mg, niacin 15 mg, biotin 100 μg) used twice a day for 24 weeks, in 26 male subjects with AGA. The treatment was effective for 60% of the patients compared to 11% of placebo, with some gastrointestinal adverse effects in three subjects of the treated group. More recently, Nichols and co-workers (2017) showed that a 24-week treatment with Forti5® (twice a day), an oral supplement containing green tea extract, omega 3 and 6 fatty acids, cholecalciferol, melatonin, soy isoflavones and BS (50 mg, standardized to 45% of BS) improved the hair count and hair mass index of AGA patients (n=6) with no major side effects.

Another strategy to increase the effectiveness of substances against AGA is their incorporation in nanostructured systems. In the pharmaceutical field, formulations containing nanoencapsulated actives offer many advantages over conventional ones (PALOMBO et al., 2014), as a reduction of side effects (CONTRI et al., 2014), slow and prolonged release of nanoencapsulated substances (CONTRI et al., 2010; CONTRI et al., 2014; KATZER et al., 2014) and the protection of photosensitive agents against degradation (SCHAFFAZICK et al., 2003; OURIQUE et al., 2011; SAVIAN et al., 2014), among others.

Nanoparticles containing drug conventionally used to treat AGA, as minoxidil (MATOS et al., 2015; GOMES et al., 2014; UPRIT et al., 2013; PADOIS et al., 2011),

finasteride (GOMES et al., 2014; POHLMANN; JORNADA; GUTERRES, 2012; ROQUE et al., 2014), dutasteride (NOOR et al., 2017), spironolactone (SHAMMA; ABURAHMA, 2014) and flutamide (HAMISHKEKAR et al., 2016) were developed. The hair follicles may act as a reservoir for the nanoparticles (LADEMANN et al., 2006) in a size-dependent manner (PATZELT et al., 2011), prolonging the residence time of the drugs into the follicular medium. There are several types of nanoparticles described in the literature, being the lipid and polymeric nanoparticles of great interest for topical application (MÜLLER; STAUFENBIEL; KECK, 2014).

Since the pathophysiology of AGA involves inflammation and androgenic effects, the main goal of this paper is to evaluate *in vitro* and *in vivo* effects of β-sitosterol-loaded nanostructured delivery systems associated with the low-level laser for AGA treatment. The hypothesis is that the association of such strategies – nanotechnology, β-sitosterol with dual function and laser therapy – may represent a new approach in the treatment of AGA, with improvements in relation to the current modalities. β-sitosterol has an anti-inflammatory and modulating property of the 5-α-reductase enzyme; the nanoencapsulation may favor an increase in follicular penetration and the low level laser has multiple benefits, such as local blood circulation stimulation, anti-inflammatory and mitogenic effects.

MATERIAL AND METHODS

Material

β-sitosterol (BS, 79.7% purity) and poly(ε-caprolactona) (PCL) were obtained from Sigma Aldrich® (São Paulo, Brazil); mango butter from Pharma Special® (Santana de Parnaíba, Brazil); medium chain triglycerides and polysorbate 80 (Tween® 80) from Delaware® (Porto Alegre, Brasil); sorbitan monostearate (Span® 60) from Fluka (São Paulo, Brazil); methanol and ethanol HPLC grade from Tedia® (Rio de Janeiro, Brazil). A 10% solution of sodium laureth sulfate was obtained from a local pharmacy. For the *in vitro* cell experiments the following reagents were used: dimethyl sulfoxide (DMSO); 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), phosphate buffered saline (PBS) and trypsin-EDTA solution (170,000 U/L trypsin and 0.2 $g.L^{-1}$ EDTA) were obtained from Sigma-Aldrich (St. Louis, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM), supplemented with L-glutamine (584 $mg.L^{-1}$) and antibiotic/antimicotic (50 $mg.L^{-1}$ gentamicin sulphate and 2 $mg.L^{-1}$ amphotericin B) were purchased from Vitrocell (Campinas, Brazil).

Preparation of polymeric nanocapsules suspensions

The BS-loaded and unloaded nanocapsules (BS-NC and B-NC, respectively) were prepared (n = 3) by the interfacial deposition of the preformed polymer (FESSI; PUISIEUX; DEVISSAGUET, 1989). The organic solution consisted of BS (1 $mg.mL^{-1}$), oil (medium chain triglycerides, 1.65% w/w), polymer (poly-ε-caprolactone, 1% w/w) and acetone. This organic phase, after reaching total solubilization of its components (40 °C for 1 h with magnetic stirrer), was added under moderate magnetic stirring to an aqueous solution containing the high hydrophilic-lipophilic balance surfactant (polysorbate 80, 0.77% w/w). The magnetic stirring was maintained for 10 minutes. Thus, the acetone was eliminated and the aqueous phase concentrated by evaporation under reduced pressure (Rotavapor R-114, Büchi, Switerzland) until the final required volume, to keep BS concentration at 1 $mg.mL^{-1}$. Blank formulations were prepared by the same way just omitting the presence of BS. The formulations were stored in dark glass containers at room temperature.

Preparation of nanostructured lipid carriers

Nanostructured lipid carriers (NLC) containing β-sitosterol (n=3) were prepared using high speed homogenization (HSH) and ultrasonic probe (US) after adaptation of previously reported methodologies (ROSLI et al., 2015; SOUTO; MÜLLER, 2005; TSAI et al., 2012). The oil phase was composed of a solid lipid (mango butter, 3.5% w/w), a liquid lipid (medium chain triglycerides, 1,5% w/w), a low hydrophilic-lipophilic balance surfactant (sorbitan monostearate, 2.5% w/w) and β-sitosterol (0.1% w/w). The oily phase was heated in a water bath at 60 °C under constant stirring until complete melting of the solid lipid and solubilization of β-sitosterol. The aqueous phase comprised a surfactant (polysorbate 80, 2.5% w/w) and purified water and was heated at the same temperature and condition before pouring it into the melted oily phase. This mixture remained under stirring for 5 minutes. This preformulation was subjected to the high speed homogenizer for 5 minutes at 19,000 rpm (Polytron® PT-MR 3100D, Kinematica, Switzerland) and subsequently to ultrasonic probe (Sonics & Materials INC, Newtown, USA), with a power of 750W and a 30% amplitude. Blank NLC (B-NLC, n=3) were prepared the same way β-sitosterol-loaded NLC (BS-NLC) just omitting BS from the oily phase. The formulations were stored in dark glass containers at room temperature.

Characterization of the nanoparticles

BS loaded nanoparticles were characterized regarding size distribution, mean particle size, zeta potential, pH, drug content, encapsulation efficiency and morphology (the correspondent black formulations were also analyzed). Particle size distribution, mean particle size, zeta potential and pH were also evaluated after 15 and 30 days of storage at room temperature.

Size distribution and mean particle size

The particle size distribution was determined by laser light diffraction (Mastersizer® 3000E, Malvern Instruments, Worcestershire, England). The mean diameter over the volume distribution ($d_{4.3}$) was used as a particle size distribution parameter and the Span value as an indication of homogeneity in size distribution. For nanocapsules, the refraction index of PLC was used (1.59); while for NLC the refraction index of the mango butter was applied (1.457).

The measurements were performed in distilled water without any previous treatment of the samples. For a more specific determination of the particle size as well as to determine the polydispersity index, formulations were analyzed by photon correlation spectroscopy (PCS) (Zetasizer® Nanoseries ZEN3600, Malvern Instruments, Worcestershire, England) after adequate dilution (1:500, v/v) of an aliquot of the suspension in filtered purified water.

Nanoparticles morphology by transmission electron microscopy (TEM)

BS-loaded and unloaded-NC and NLC formulations were analyzed by transmission electron microscopy (TEM, Jeol, JEM 1200-ExII, Tokyo, Japan) to evaluate their morphology. The samples were diluted in purified water (1:10) and put (12 µL) on formvar-carbon grids (400 mesh). The excess of formulations was removed by soothingly touching a filter paper near one extremity of the grid. Uranil acetate (2%, 12 µL) was used as the contrast substance. Its excess was removed the same way described above. The microscope operated at a voltage of 80 kV. Analyses were carried out at the Electron Microscopy Center (Centro de Microscopia Eletrônica, UFRGS, Brazil).

Zeta potential

The zeta potential (ζ-potential) (Zetasizer® Nanoseries ZEN3600, Malvern Instruments, Worcestershire, England) measurements were performed according to the electrophoretic mobility principle after diluting (1:500, v/v) the samples with a filtered (0.45 µm) 10 mM NaCl aqueous solution, at 25 °C.

Drug content and encapsulation efficiency

The BS content of BS-NC and BS-NLC right after the preparation and after 30 days of storage at room temperature was determined by high performance liquid chromatography with ultraviolet detection (HPLC-UV, Shimadzu® LC-20A system, Kyoto, Japan), following a previously validated methodology (BEDNER et al., 2008). The system consisted of a pump (LC-20AT), an auto-injector (SIL-20 AC), a diode array detector (SPD-M20A) and a controller (CBM-20a). Data were processed by the software LC solution (Version 1.24SP1, Shimadzu, Kyoto, Japan).

To determine the concentration of BS in the formulations 300 µL of the nanoparticle suspensions were diluted with methanol to a theoretic concentration of 30 $\mu g.mL^{-1}$ and submitted to ultrasound for 10 min. The use of methanol and ultrassound was necessary in order to release BS from the nanoparticles. The resulting solution was filtered through a 0.45 µm membrane and injected in the HPLC-UV system ($n = 2$). The stock solution of β-sitosterol (1 $mg.mL^{-1}$) was prepared using analytical grade ethanol. To evaluate the specificity of the method, blank formulations (B-NC and B-NLC) were diluted and treated the same way and analyzed by HPLC-UV.

The mobile phase was composed of methanol (100%), with flow of 1.2 $mL.min^{-1}$, detection at 202 nm, injection volume of 20 µL. A Nanoseparation® C18 column (180 mm x 4.6 mm, 5 µm, Nanoseparations®, Nieuwkoop, Netherland) and a pre-column (4.0 mm x 2.0 mm, 5 µm, Phenomenex®, Torrance, USA) were used. The retention time of BS was near 10 min. The method was validated according to the following characteristics: linearity ($y = 4391.2x + 2351.9$, $n=3$, r=0.9987, range between 10 - 50 $\mu g.mL^{-1}$), and precision (repeatability: RSD 3.82%; intermediate precision: RSD 4.29%). Specificity (blank formulations) was demonstrated for B-NC, showing that adjuvants did not alter the BS assay. However, for B-NLC, a small peak at the same retention time of BS was detected, probably due the mango butter in its composition that contain BS as a phystosterol (DHARA; BHATTACHA; GHOSH, 2010). Data analysis by ANOVA showed a linear regression ($F_{calculated} = 12511.82 > F_{table} = 4.96$) with no deviation from linearity ($F_{calculated} = 10.80 > F_{table} = 3.71$), with a 95% confidence. The limits of detection (LD) and quantification (LQ) of the method were calculated using the following equations (1) and (2), respectively:

$$LD = 3.3 \cdot \sigma \cdot S^{-1} \qquad (1)$$

$$LQ = 10 \cdot \sigma \cdot S^{-1} \qquad (2)$$

where σ is the standard deviation of the response (peak areas) and S is the slope of the calibration curve. The LD and LQ were 0.725 and 2.179 $\mu g \cdot mL^{-1}$, respectively.

BS entrapment to the NC and CLN formulations were determined by means of ultrafiltration-centrifugation technique (20 minutes, 2250 xG), using filters (Amicon® 10000 Da, Millipore, Bedford, USA) which allow the separation of the non encapsulated amount of drug. The ultrafiltrate was collected and directly injected in the HPLC-UV system to detect the amount of non-encapsulated BS. The encapsulation efficiency was calculated by the difference between the total BS content determined in the formulation and the free amount of BS that was able to pass the filter. To assure that the BS does not interact with the ultrafilter membrane, which could lead to a misinterpretation of the results, the same ultrafiltration-centrifugation protocol was carried out with an ethanolic solution at the same BS concentration (1 $mg.mL^{-1}$).

pH

The pH of all formulations was determined by potenciometry (Seven Easy pH®, Mettler Toledo, Brazil) without additional treatment. The equipment was previously calibrated with pH 4 and 7 buffer solutions. Three consecutive measurements were performed for each formulation.

Effect of low-level laser irradiation over nanoparticles

The following experiment aimed to evaluate if the irradiation of LLL over the nanoparticles would influence somehow in their characteristics (mean particle size, zeta potential and drug content), which were characterized before and after low-level laser irradiation.

An amount of 500 µL of nanoformulations (B-NC, BS-NC, B-NLC, BS-NLC, *n=3*) was added to the wells of a twenty-four wells plate as used for cell culture. The LLL equipment (Omnia, Ecco Fibras, Campinas, Brazil) was positioned as close as possible over the formulations in a perpendicular angle. Two wavelengths were irradiated, at a fluency of 4 $J.cm^{-2}$, one in the red (660 nm, 100 mW) and another in the near infrared range (830 nm, 120 mW) in distinct wells, so the effect of each of them could be determined.

Cell viability experiments

This experiment was performed to investigate the cell viability (cell lines 3T3 - murine Swiss albino fibroblasts; and HaCaT - human immortalized keratinocytes) after different treatments (n=3): a) low-level laser irradiation (660 or 830 nm); b) nanoparticle treatment

(BS-NC, B-NC, BS-NLC and B-NLC); c) BS-solution and d) combined treatment (nanoparticle treatment followed by low-level laser irradiation). The proliferative effects of each treatment were evaluated in 3T3 and HaCaT cells by MTT viability assay.

Cell culture

For the test, the cell lines 3T3 and HaCaT were grown in DMEM medium supplemented with 10% (v/v) FBS (v/v), L-glutamine (584 $mg.L^{-1}$) and antibiotic/antimicotic (50 $mg.ml^{-1}$ gentamicin sulfate and 2 $mg.L^{-1}$ amphotericin B), at 37 °C, 5% CO_2. The cells were routinely cultured in 75 cm^2 culture flasks and were harvested using trypsin-EDTA when the cells reached approximately 80% confluence.

MTT viability assay

The MTT assay is based on the protocol first described by Mosmann (1983), in which the endpoint is a measurement of cell metabolic activity. Initially, the 3T3 and HaCaT cells were seeded into a 24 well plate at a density of 5 x 10^4 $cells.mL^{-1}$ and incubated for 24 h, under 5% CO_2, at 37 °C. After this period, the different treatments regimens were made to evaluate the formulations cytotoxicity, the spent medium was replaced by 500 μL of fresh medium supplemented with 5% (v/v) FBS containing the nanoformulations or the free drug (BS ethanolic solution) at the concentrations of 0,5 μM. The cytotoxicity of the blank formulations was evaluated using the same volume used to test the formulations containing BS, as well as the positive control (dimethyl sulfoxide - DMSO) and the ethanol (vehicle of BS-solution). Another treatment consisted of nanoformulation associated with low-level irradiation with near infrared (830 nm) or red laser (660 nm) using a dose of 2 $J.cm^{-2}$. The effect of LLL irradiation were also evaluated alone.

Twenty-four hours after each one of the treatments, the effects on the cell culture were investigated by evaluating cell metabolism (MTT assay). Five hundred μL of MTT tetrazolium salt (0.5 $mg.mL^{-1}$ dissolved in FBS-free medium) were added to each well. Plates were further incubated for 3 h to allow the formation of purple formazan crystals. Thereafter, these crystals were solubilized by adding 500 μL of DMSO to each well, followed by stirring the plate for 10 min at room temperature. The absorbance of the solutions was measured at 550 nm in a microplate reader (Thermo Scientific™ Multiskan™ FC Microplate Photometer). The cell viability was finally calculated as the percentage reduction of the tetrazolium salt by viable cells for each sample. The viability values were normalized to control cells. The 24-well plates containing the control cells (containing medium only with 5% FBS, v/v) were

maintained at room temperature for the same irradiation times used in the respective irradiated groups, although without activating the laser beam.

In vitro determination of irritancy potential of nanoformulations

The irritation potential of BS-loaded and unloaded-NC or NLC was examined using the Hen's Egg Test - Chorioallantoic Membrane (HET-CAM), as an *in vitro* alternative. HET-CAM uses CAM, a fetal vascularized respiratory membrane, of fertilized chicken eggs (Cobb) on the tenth day of incubation (37.5 ºC, 60 % relative humidity) to evaluate the irritation potential of substances. The assay is based on the observation of some endpoints: hemorrhage (blood from a ruptured vessel), vasoconstriction (narrowing of the vessels) and coagulation (presence of blood clots) to presume the irritation potential of the applied substances or formulations, resulting in a scale that considers the observed phenomena (LUEPKE, 1985; SPIELMANN et al., 1993). Egg shells were opened at the air cell, a white membrane was removed and 0.3 mL of the nanoformulations was applied to the CAM (*n=6*). As NC and NLC are opaque, the CAM was rinsed with sterile physiologic solution 20 seconds after the application and the time points of first occurrence of each of the three reactions were monitored until 300 seconds (sec). BS-ethanolic solution was used to evaluate the irritancy potential of BS.

Positive controls (0.1 N NaOH and 1% sodium lauryl sulfate – SLS, *n=3* each) and a negative control (0.9% NaCl sterile solution, *n=3*) were included in the experiment aiming to demonstrate the functional adequacy of the test method and detect any nonspecific changes in the test system, respectively. The irritation score (IS) was determined according to the following equation (3):

$$IS = \frac{5 \times (301 - \text{hemorrhage time})}{300} + \frac{7 \times (301 - \text{vasoconstriction time})}{300} + \frac{9 \times (301 - \text{coagulation time})}{300} \quad (3)$$

The lesions are classified by means of IS, as non-irritant (0 – 0.9), slightly (1 - 4.9), moderate (5 – 8.9) and extremely irritant (9 -21) (KALWEIT et al., 1990).

Evaluation of toxicity of polymeric nanocapsules by bioassay with *Allium cepa*

The *Allium cepa* test is a well studied *in vivo* model to evaluate the toxicity of substances, as well as nanoparticles (RAJESHWARI *et al.*, 2016), as their roots grow in direct contact with the solution or suspension of interest (TEDESCO; LAUGHINGHOUSE, 2012) and have high tolerance to different growing conditions. Furthermore, its cellular kinetic proliferation characteristics, the clear mitotic phases, the large number of dividing cells, the

rare occurrence of spontaneous chromosomal damages and its availability throughout the year justify its wide use (CARITÁ; MARIN-MORALES, 2008).

The bioassay was conducted with *A. cepa* bulbs commercially available. The bulbs rooted in distilled water for a period of 72 h previously to the treatment. Samples of water and undiluted formulations (B-NC, BS-NC, BS-Solution, B-Solution) were used as the exposure media (n=3 onion bulbs per treatment) for 24 h. The low-level laser was used isolated (water as media, two wavelengths, 660 or 830 nm) and associated with the aforementioned mediums (water + 660 nm LLL, water + 830 nm LLL, B-NC + 660 nm LLL, B-NC + 830 nm LLL, BS-NC + 660 nm LLL and BS-NC + 830 nm LLL). The LLL was applied to the tip of two roots per bulb using a fluency of 1 $J.cm^{-2}$. After 24 h, two root tips were collected from each onion bulb and fixed in Carnoy 3:1 for 24 h at room temperature and stored in 70% ethanolic solution. Each root tip was then crushed and stained with 2% acetic orcein, placed over a slide and analyzed by optical microscopy. Five hundred cells were analyzed per slide, totaling 3000 cells per treatment. The mitotic index was calculated as percentage of cells undergoing mitosis in comparison to the total cells examined per each bulb.

Testosterone-induced alopecia model

Ethical aspects and animals

This *in vivo* experiment was initiated after been approved (protocol number 5005090818) by the Ethic Committee on Animal Use of the Federal University of Santa Maria, in accordance with the rules issued by the National Council for Control of Animal Experimentation.

Wistar albino rats (2 – 3 months old) were used to evaluate the effects of nanocapsules containing beta-sitosterol associated or not to laser therapy on hair growth. The animals were maintained in a room with 50 – 60% humidity, air exhaustion (10 – 15 air changes per hour), at 22 ^{o}C and with a dark/light cycle of 12h each. They were housed in solid polypropylene cages (2 to 3 animals per cage), with free access to food and water.

Treatments

The animals were be randomly distributed in different experimental groups, with 5 animals per group, similar to those performed by Upadhyay and co-workers (2012). The groups were divided as follows: Group I (Control - treated only with testosterone), Group II (Testosterone + BS-NC), Group III (Testosterone + BS-NC + LLL) and Group IV (Testosterone + finasteride).

A commercially available preparation of testosterone (Deposteron®) was diluted with peanut oil to a concentration of 1 $mg.ml^{-1}$. The alopecia was induced in all animals by a daily injection of testosterone, for 21 days, at a dose of 0.5 mg/Kg/day. The treatments of groups II, III e IV were performed in parallel, since day one of testosterone administration, by a topical application of 0.2 ml of the respective formulation at the upper dorsum of the animals. In the group III, the low-level laser was applied at 8 points at the dorsum of the animals, with approximately 2 cm of distance one another, using both 660 nm and 830 nm, simultaneously, at a fluency of 2 $J.cm^{-2}$ total.

Histological studies

All animals were euthanized after 21 days of treatment through deep anesthesia with isoflurane. After the euthanasia, a fragment of the skin of the upper dorsal area was dissected, fixed in 10% formaldehyde and submitted to histologic routine processes, embedded in paraffin. Next, 4 μm thick histological sections were cut and stained with hematoxylin and eosin. The slides were observed under a microscope by two blinded histologists.

Statistical analysis

Samples were analyzed in triplicate and the results obtained were demonstrated -as the mean ± standard deviation and analyzed for statistical significance through analysis of variance (ANOVA) at a significance level of 5%, followed by post-doc Tukey`s test.

RESULTS

Physicochemical characterization of nanoparticles

Macroscopically, BS-loaded and unloaded NC and NLC were homogeneous and presented the Tyndall effect. The first parameter evaluated of all formulations was the particle size distribution by laser diffraction. After preparation, the BS-NLC and BS-NC, showed particle distribution exclusively in the nanometric range, as well as the unloaded correspondent formulations (data not shown). By PCS analysis, BS-NC presented mean particle size of 231 nm at time zero, which slightly decreased after 30 days (211 nm) ($p<0.05$). The polydispersity index was below 0.2 for all times. The zeta potential was slightly negative and kept constant during storage time. The pH values were slightly acid and decreased after 30 days of storage ($p<0.05$). These results are showed in table 1. The BS-NC drug content was very close to the theoretical (0.987 ± 0.002 $mg.mL^{-1}$) at time zero which no

change at time 30 days. Since BS was not identified in the ultra-filtrated, the encapsulation efficiency is considered 100%.

With regard to the NLC, they presented smaller mean particle size than NC at time zero (117 nm versus 231 nm, respectively). The pH values right after preparation were proximal to the neutrality. The formulations presented negative zeta potential values. The BS content of BS-NLC was (1.02 $mg.mL^{-1}$), indicating a value close to the expected (1 $mg.mL^{-1}$). As well as happened with nanocapsules, the amount of BS in the ultrafiltrate was not detectable by the HPLC-UV method, so the encapsulation efficiency was considered 100%. In the retention test with the membrane of the ultra-filtration dispositive, the total amount of BS was recovered in the ultra-filtrate after centrifugation, indicating that there was no interaction with the membrane. It reinforces that the BS encapsulation efficiency is not influenced by the retention of BS in the filtration membrane. No further physicochemical characterization of NLC was carried out, since after 15 days of storage at room temperature visual signs of instability were noted, such as precipitation, phase's separation and the presence of macroscopic particles. No differences were found between BS-loaded and unloaded nanocapsules or nanostructured lipid carriers for all analyzed parameters.

Table 1. Physicochemical characteristics of nanoparticles at time zero and after 30 days of storage at room temperature

Formulation	Particle size (nm)	PDI[a]	Zeta potential (mV)	pH
Time zero				
BS-NC	231 ± 34	0.18 ± 0.08	-9.1 ± 1.2	6.09 ± 0.17
B-NC	250 ± 18	0.21 ± 0.07	-9.2 ± 0.5	6.16 ± 0.04
BS-NLC	117 ± 6	0.20 ± 0.02	-6.5 ± 0.1	6.72 ± 0.01
B-NLC	124 ± 8	0.21 ± 0.01	-6.6 ± 0.6	6.69 ± 0.04
After 30 days of storage				
BS-NC	211 ± 8	0.14 ± 0.01	-9.7 ± 2.3	5.27 ± 0.11
B-NC	218 ± 10	0.15 ± 0.03	-8.8 ± 1.0	5.21 ± 0.08

Mean ± standard deviation; [a]: polidispersity index

Nanoparticles morphological analysis by TEM showed spherical particles (Fig. 1) both for BS-loaded and unloaded NC and NLC.

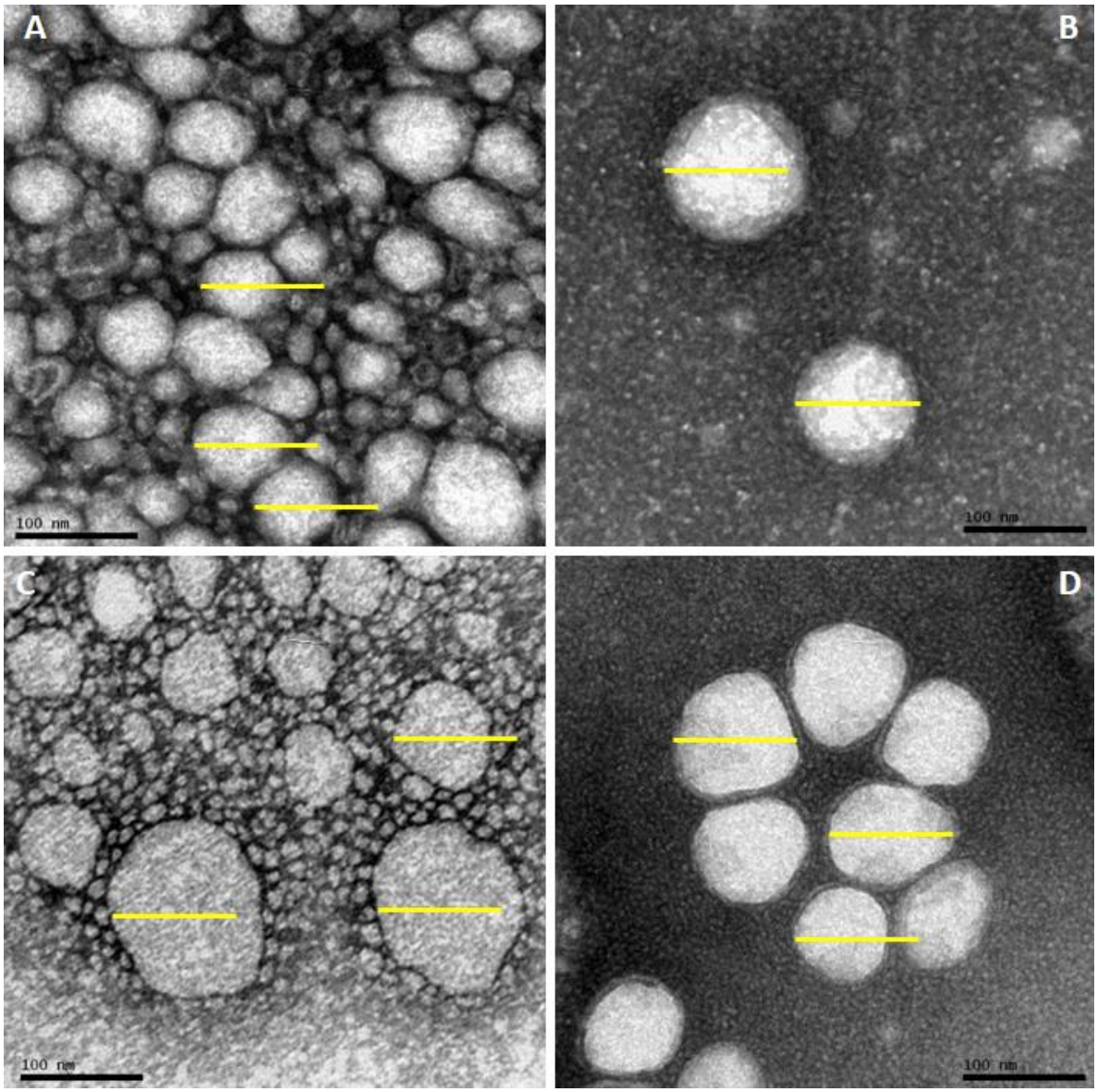

Figure 1. Transmission electron microscopy images of (A) B-NC, (B) BS-NC, (C) B-NLC and (D) BS-NLC. (300,000 X). Yellow and black bars are representative of 100 nm.

Effect of low-level laser irradiation over nanoparticles

To investigate whether the LLL irradiation over nanoparticles could affect their characteristics, their mean particle size, zeta potential and drug content were evaluated before and after red or near infrared laser irradiation. It is possible to observe that the values before and after exposition of the nanoparticles to LLL are very similar, independent of the wavelength (660 or 830 nm). The results are expressed in Table 2. Drug content of BS-NC and BS-NLC was not modified after laser irradiation (data not shown), independent of the laser type.

Table 2. Physicochemical characteristics of nanoparticles before and after low-level laser irradiation at fluency of 4 J.cm^{-2}

Formulation	Particle size (nm)			Zeta potential (mV)		
	Before	After		Before	After	
		660 nm	830 nm		660 nm	830 nm
B-NC	228 ± 2	225 ± 2	226 ± 1	-5.3 ± 0.2	-6.6 ± 0.5	-5.4 ± 0.3
BS-NC	203 ± 2	203 ± 0.5	205 ± 1	-6.4 ± 0.4	-7.4 ± 0.3	-8.1 ± 0.1
B-NLC	116 ± 1	115 ± 1	115 ± 3	-6.3 ± 0.5	-8.2 ± 0.2	-8.9 ± 0.6
BS-NLC	115 ± 2	112 ± 1	112 ± 2	-5.6 ± 0.7	-8.4 ± 1.5	-7.2 ± 0.4

Mean ± standard deviation (represents the variation among one batch, divided in three samples and treated apart)

Cell viability experiments

Analysis of the MTT results (Fig. 3) showed that, after 24 h of treatment, for both cell lines (HaCaT and 3T3) the formulations (mean values of cell viability of HaCaT: 88% BS-NC; 96% B-NC; 87% BS-NLC; 90 B-NLC and 3T3: 82% BS-NC; 97% B-NC; 99% BS-NLC; 98 B-NLC) and the low-level laser irradiation (mean values of cell viability of HaCat: 98% 660 nm; 98% 830 nm; 3T3: 97% 660 nm and 92% 830 nm) did not interfere in the cell viability when compared to control (non-treated cells) ($p>0.05$). Also, the association of LLL and NC (mean values of cell viability of HaCaT: 93% BS-NC+660nm-LLL; 98% BS-NC+830nm-LLL and 3T3: 94% BS-NC+660nm-LLL; 96% BS-NC+830nm-LLL) or NLC (mean values of cell viability of HaCaT: 93% BS-NLC+660nm-LLL; 95% BS-NLC+830nm-LLL and 3T3: 119% BS-NLC+660nm-LLL; 105% BS-NLC+830nm-LLL) did not induce cytotoxicity, regardless of the BS presence (B-NC and B-NLC). However, the BS-solution reduced the viability of both 3T3 (21%) and HaCaT (45%) cell lines ($p<0.05$), also when associated with LLL (3T3: 23% BS-Sol+660m-LLL and 21% BS-Sol+830nm-LLL; HaCaT: 44% BS-Sol+660m-LLL and 48% BS-Sol+830nm-LLL). Ethanol, the BS-solution vehicle, did not induce cytotoxicity for HaCaT (98%) and 3T3 (102%).

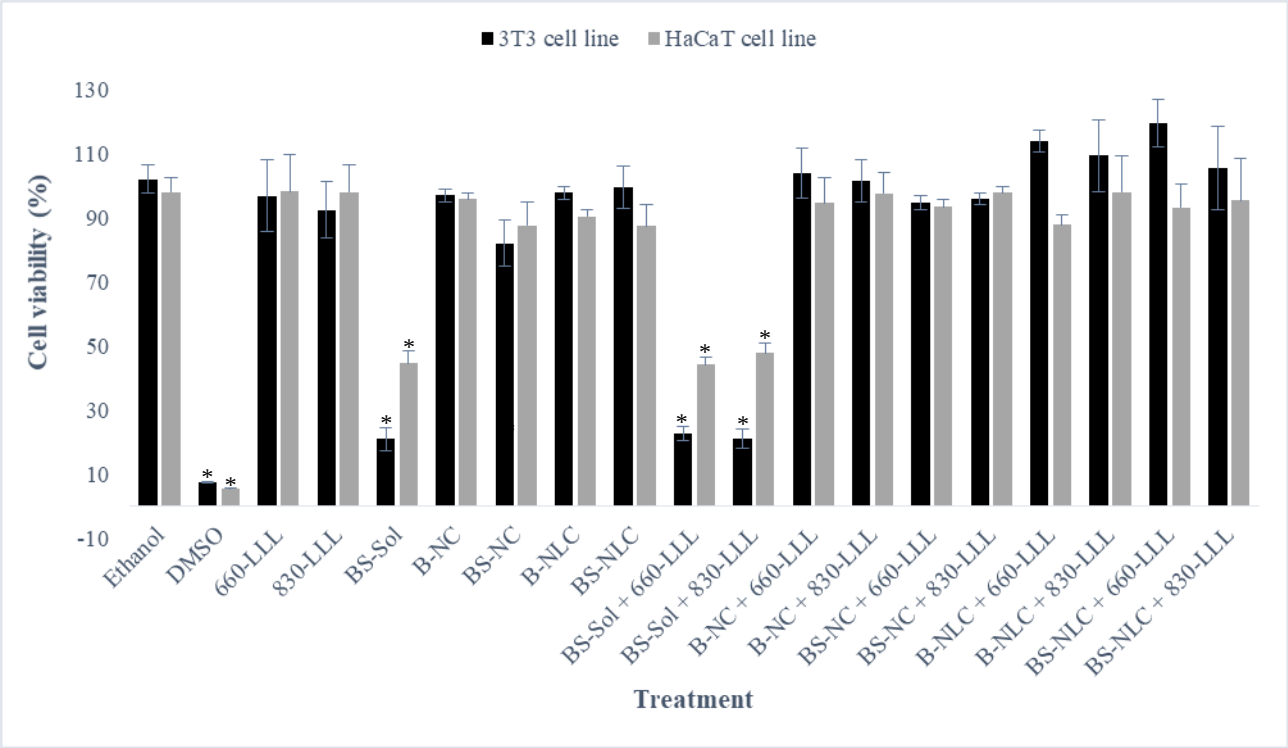

Figure 3. Cell viability (%) of 3T3 and HaCaT cells after 24 h of treatment with 0.5 µM of β-sitosterol solution (BS-Sol), BS-NC, BS-NLC and the respective blank formulations (B-NC and B-NLC). The LLL was used at two different wavelengths, 660nm (660-LLL) or 830 nm (830-LLL), both with a fluence of 4 $J.cm^{-2}$. Data represents the mean ± standard deviation of three independent experiments. * = $p<0.05$, there are statistical difference.

Irritancy potential of nanoformulations by HET-CAM assay

The irritation potential of the formulations was examined using HET-CAM assay, as an *in vitro* alternative. The following were applied directly to the egg's CAM: 0.3 mL of the positive (0.1N NaOH and 1% SLS) and negative (physiological solution) controls, the BS-loaded, the blank NC and NLC and the BS-solution (100% ethylic alcohol). Positive controls were classified as extremely irritant (1% SLS IS = 9.45 ± 1.06 and 0.1N NaOH IS = 13.47 ± 0.24). BS-ethanolic solution was classified as moderate irritant (IS = 6.95 ± 2.67). On the other hand, the negative control and all of the nanoformulations did not show any of the possible reactions (hemorrhage, coagulation and vasoconstriction).

Allium cepa bioassay

The results obtained in this experiment show that the exposure of the onion bulbs to the nanoformulations, associated or not with the LLL, did not interfere in the mitotic index (Fig. 4) when compared to the control (water) ($p>0.05$).

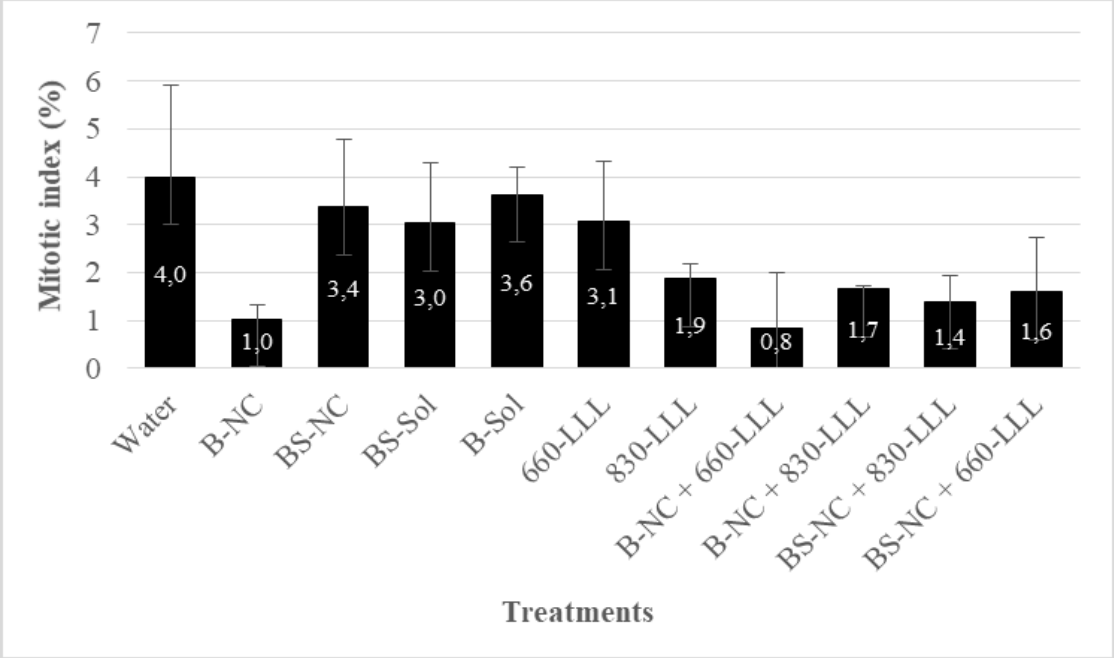

Figure 4. Mitotic index of roots of *Allium cepa* treated with BS-Sol, BS-NC, BS-NLC and the respective blank formulations (B-NC and B-NLC). The LLL was used at two different wavelengths, 660nm (660-LLL) or 830 nm (830-LLL), both with a fluence of 4 J.cm^{-2}. Data represents the mean ± standard deviation of a triplicate.

Animal model of testosterone-induced alopecia

Animals were observed carefully during the whole treatment. A hair loss was noticed for all groups. The histological sections showed a very distinct hair follicle distribution among the groups (Fig. 5). The control group (treated only with testosterone) showed the miniaturization of hair follicles (a large number of small follicles in the dermis and a reduced amount in the subcutaneous tissue), validating the testosterone-induced alopecia model. In the group treated with BS-NC it was observed predominance of small follicles in the dermis (smaller than in the control group) and a small amount of large follicles in the subcutaneous, but in greater quantity than the control group.

The group treated with BS-NC associated with LLL irradiation showed the best results. In the dermis, there is a greater quantity of small follicles than in the previous groups, whereas in the subcutaneous it was observed abundance of follicles with great variation of sizes than in the previous groups. The group treated with finasteride presented, in the dermis, a predominance of small follicles with similar amount to the group treated with BS-NS and LLL, but greater than that observed in the control group, as well as in the group treated only with BS-NC. In this group, the subcutaneous tissue presented characteristics similar to the group treated with the combination BS-NC and LLL.

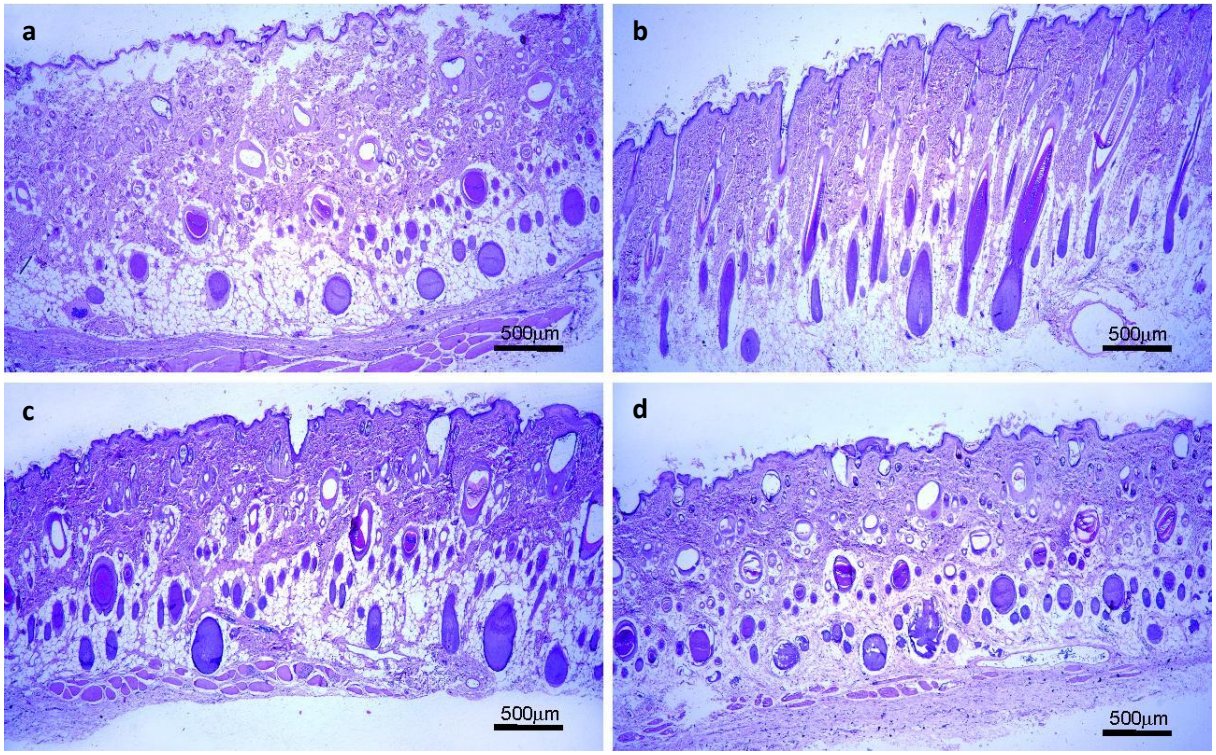

Figure 5. Photomicrographs of rats skin showing the effect on hair follicle of different treatments/groups (a-d) in a transverse section. a: negative control (treated only with testosterone); b: treated with β-sitosterol-loaded nanocaspules; c: treated β-sitosterol-loaded nanocaspules associated with 660 nm + 830 nm low-level laser and d: treated with finasteride.

DISCUSSION

BS is a phytosterol present in several plants as a minor component, even though it is considered one of the main responsible by the hair growth claims of *Serenoa repens* (ROSSI et al., 2012). Its anti-inflammatory, antioxidant, antimicrobial, angiogenic, immunomodulatory, antidiabetic and 5-α-reductase inhibition properties have been established (CHEN et al., 2016; SAYEED et al., 2016). Furthermore, BS is a hypocholesterolemic agent (WANG; NG, 1999) and, as consequence, it may inhibit the steroidal hormone biosynthesis, therefore reducing the testosterone amount in the local of treatment (PRAGER et al., 2002). Less testosterone means less substrate to 5-α-reductase and, finally, less dihydrotestosterone, the main hormone involved in follicular miniaturization.

Nowadays it is possible to acquire synthetic BS with different levels of purity. By topical route, there are also some scalp lotions commercially available. Nutrisol®-Rm is a formula containing panthenol, retinol, *Serenoa serrulata* (Saw palmetto) fruit extract, cysteine, biotin and BS as active ingredients. It is recommended for men with AGA in a daily use basis and in combination with other topical and oral products of the brand (SCALPMED, 2019). ReJuviance® Super Strength DHT Blocker is the only hair care product to promote hair

growth containing 5% BS ester. The manufacturer claims that it was developed a new technology that allowed the combination of a high concentration of BS, Saw Palmetto extract and hair follicle penetration enhancers (REJUVIANCE PRODUCTS CORP, 2019). No published *in vivo* or clinical trials of these two lotions were found.

Although finasteride is approved by the FDA and other regulatory agencies to treat AGA through oral administration, there are many attempts to use it, as well as dutasteride, by topical route (NOOR et al., 2017). This way, the inhibition of 5αR would happen preferentially in the scalp (CASERINI, 2014), avoiding the exposure of the whole body to undesired systemic side effects as it happens with oral administration (NOOR et al., 2017; SONTHALIA; DAULATABAD; TOSTI, 2016). A topical formulation of finasteride 0.25% solution was studied in 12 men with AGA (twice a day). Plasma finasteride, testosterone and DHT concentrations were determined in this group as well in the group (n=12) using oral 1 mg finasteride once a day. Plasma DHT was reduced by ~ 68 – 75% with the topical solution and by ~ 62 – 72% with the tablet; no relevant changes occurred for plasma testosterone with either treatment and no clinically significant adverse events occurred, showing the similar findings for both administration routes and probably a similar efficacy. The study did not show the DHT concentration in the scalp (CASERINI, 2014).

The penetration of active substances intended for hair growth stimuli into hair follicle are variable and suffer the influence of the composition of the formulation. The poor skin and hair follicle penetration is a known limitation of topically applied formulations and, as so, improved preparations containing penetration enhancers (GOODMAN, 2001) or new drug delivery systems are under study. A 0.25% finasteride-loaded nanocapsules of PCL or 0.25% loaded-nanoemulsion for the treatment of AGA are examples of formulations developed and patented by Pohlmann, Jornada and Guterres (2012). Using the *in vivo* B6CBAF1 androgenetic-alopecia mice model it was shown that the 0.25% finasteride-loaded nanocapsules induced hair growth while the nanoemulsion did not. Another nanocapsule formulation, incorporating 0.05% of finasteride and using an additional oil mixture (linalool + farnesol) to increase the solubilization of finasteride and, hence, its encapsulation, was developed. The hair growth potential of this formulation was evaluated using the same animal model and protocol and the results showed its superiority over the first formulation. Both finasteride-loaded nanocapsules were incorporated in gel or lotions intended to be pharmaceutical product.

Another interesting approach was developed by Chandrashekar and co-workers (2015) to access the efficacy a 5% topical minoxidil fortified with 0.1% finasteride in patients (n=45) with AGA after interruption of the conventional treatment (5% topical minoxidil and oral finasteride for two years). The change from oral finasteride + topical minoxidil to topical minoxidil-finasteride combination was able to maintain 84.44% of the patients with good hair density, thereby creating an alternative to the undesired indefinite use of oral finasteride. This shows that the topical medication alone is beneficial in maintaining hair density as well as improving hair growth.

The combination of both minoxidil and finasteride in the same formulation and using different types of nanoparticles, such as nanostructured lipid carriers (GOMES et al., 2014) and nanocapsules (POHLMANN; JORNADA; GUTERRES, 2012) have been already studied. Pohlmann and coworkers (2012) developed and patented 0.25% finasteride loaded-nanocapsules of PCL and 0.20% finasteride loaded-nanocapsules of PCL with minoxidil (0.20%), named by authors as nanocapsoids. The formulations were evaluated *in vivo* regarding their effects over hair growth in B6CBAF1 mice, an androgenetic alopecia model. The results showed that the formulation containing both finasteride and minoxidil promoted a better recovery of hairs when compared to the finasteride-loaded nanocapsules. The performance of these two formulations were superior to a commercially available product (Pilexil®, Valeant, containing *Serenoa serrulata* 22%). These formulations are examples of concretizations of the patent, even though a wide variety of compositions is cited within the document, as well as the incorporation of the aforementioned nanoparticles in pharmaceutical compositions for topical use.

Advantages of nanoparticle carrier systems in the trichology field comprise a deeper penetration into hair follicle as well as a prolonged storage when compared with conventional formulations (LADEMANN et al., 2007; LADEMANN et al., 2015). The lipid nanocarriers, mainly solid lipid nanoparticles and the nanostructured lipid carriers, are the most often chosen type of nanoparticle. Gomes and co-workers (2014) developed a nanostructured lipid carrier by the ultrasonication method, containing minoxidil and finasteride, intended for topical application.

The development and characterization of BS-loaded PCL NC and NLC are here reported. As NLC requires the use of a solid lipid, in this work, we chose mango butter, a source of bioactive components, with antioxidant and anti-inflammatory properties (MANDAWGADE; PATRAVALE, 2008). Mango butter is widely used in cosmetics because

of its emollient and moisturizing properties. Moreover, it has been increasingly used in the preparation of sunscreens because, in addition to possessing numerous antioxidants and minerals (Se, Cu, Zn), it also has properties against sunburn (NADEEM; IMRAN; KHALIQUE, 2016), which could be beneficial for the patient with AGA. Besides, Dhara, Bhattacha and Ghosh (2010) analyzed the mango butter through gas chromatography with mass spectrometer and detected the presence of sterols, of which BS represents 58.63%.

Two different techniques (laser diffraction and photon correlation spectroscopy) were used to evaluate the particle size distribution and mean size of the nanoparticles. Both results confirmed their unimodal particle size distribution within nanoscale range, with mean particle sizes near 115 nm for NLC formulations and 220 nm for NC formulations. The mean particles size of the developed NC are in agreement with previous results using the method of interfacial deposition of a preformed polymer (SCHAFFAZICK et al., 2003). The same can be said about the mean particle sizes of NLC, which are usually obtained at the range of 100 nm by the ultrasonication technique (ROSLI et al., 2015) and high-pressure homogenization techniques (FLORES et al., 2016) to 250 nm with other methods of preparation (NOOR et al., 2017; SHAMMA; ABURAHMA, 2014). TEM micrographs confirmed the NLC sizes, but showed smaller particles for NC formulations. During the sample preparation for microscopic analysis the removal of solvent may cause changes in the particle size and morphology (DAS et al., 2011). The Span values (<1) obtained from laser diffraction as well as the PDI (<0.20) obtained from PCS denote a narrow and monomodal distribution of particle size, which favors the stability of formulations (ROSLI et al., 2015).

Upadhyay and coworkers (2012) prepared round shaped phyto-vesicles containing BS complexed with phosphatidyl choline. The main goal of the authors with this complexation was to increase BS bioavailability, since it is poorly absorbed. The mean particle size of such phyto-vesicles (489 nm) was bigger than here reported. These particles were used to treat AGA induced in Wistar rats. After 21 days of treatment, the best result was reached when the phyto-vesicles were used in detriment of the physical or complex mixture of the two main substances (BS and phosphatidylcholine). In addition, BS administered in solution did not present satisfactory results. It may be due the increased drug delivery via hair follicles that has been reported with the use of nanoparticulate systems.

BS-loaded nanostructured lipid carrier was formulated using propolis wax alone or in the mixture (1:1 w/w) with glyceryl behenate, and pomegranate seed oil stabilized with Tween 80 and lecithin (SOLEIMANIAN et al., 2019). This NLC was intended for food

fortification because of BS many valuable physiological functions. As the incorporation of BS in food is challenging since it is almost insoluble in water and only slightly soluble in oils, this BS-loaded NLC carrier was developed. NLC was obtained with some similarity with our work, subjecting intermediate emulsions to a hot high shear homogenization (14 000 rpm for 10 min) and a probe-type sonicator (amplitude 50%; power 100W, for 8 min) while maintaining the temperature around the melting point of the lipids. The obtained emulsion was cooled down in an ice bath for 30 min to recrystallize lipid and form NLC. At pH 6, the mean size of both types of NLC was around 100 nm with narrow distribution (PDI < 0.25) and zeta potential negative (-5 to – 27 mW, according to NaCl concentration).

The zeta potential was slightly negative for both types of nanoparticles. For nanocapsules it can be explained by the presence of poly(ε-caprolactone) and the surfactant polysorbate 80. The first, because of the presence of ester groups in the polymer structure (MÜLLER et al., 2001); the second due to the presence of oxygen atoms in its molecules (FONTANA et al., 2009). A similar value of zeta potential was found for finasteride-loaded PLGA nanoparticles (mean size of 316 nm) (ROQUE et al., 2017) (which were incorporated in three different pharmaceutical forms, a shampoo, a lotion and a solution) and prednisolone-loaded PCL nanocapasules (KATZER et al., 2014). For NLC, the negative zeta potential, besides the aforementioned contribution of polysorbate 80, can suffer influence of the free fatty acids present in the mango butter, as shown by Flores and coworkers (2016).

pH values of BS-NC and BS-NLC at time zero were slightly acid but very proximal to the neutrality, in the range of 5.9 and 6.8. After 30 days of storage, the pH of BS-NC and B-NC decreased, probably as a consequence of hydrolysis of the polymer. No differences were found between BS-loaded and blank formulations for all analyzed parameters. The BS content of BS-NLC at time zero was 1.02 $mg.mL^{-1}$, indicating a value close to the expected 1 $mg.mL^{-1}$. The same was observed for BS-NC, with no decrease after 30 days of storage. For both formulations, the amount of BS in the ultrafiltrate was not detectable by the HPLC-UV method, so the encapsulation efficiency was considered 100%. In the retention test with the membrane of the ultra-filtration dispositive, the total amount of BS was recovered in the ultra-filtrate after centrifugation, indicating that there was no interaction with the membrane. It reinforces that the BS encapsulation efficiency is not influenced by the retention of BS in the filtration membrane. BS molecule is very similar to the cholesterol. It has a hydrophobic steroid skeleton moiety with only one hydroxyl group, which makes the whole steroid molecule weakly polar (WEI et al., 2010). Its partition coefficient is high (log P = 9.65) and

BS is insoluble in water (UPADHYAY; GUPTA; DIXIT, 2012), thus its affinity for the oily core of NC or the lipid matrix of NLC is the plausible.

BS-NLC was characterized at time zero and no further analyzes were performed, since after 15 days of storage at room temperature visual signs of instability were noted, such as precipitation, phase's separation and the presence of macroscopic particles. The short stability of NLC can be a consequence of various causes. The low zeta potential value at time zero is a predictive of such low stability; however, NC formulations presented similar zeta potential values and were stable for at least 30 days. Rosli and coworkers (2015) developed a 90 days-stable NLC containing *Zingiber zerumbet* and coconut oil, besides glyceryl monostearate as solid lipid, using the same techniques employed in our study. However, besides the particle composition, some differences were found in the methodology. They homogenized the pre-emulsion using 11,000 rpm for one minute (we used at 19,000 rpm for 5 minutes) and the following step was a 20 minutes ultrasonication with a probe at different amplitudes (the potency and the exact value of the amplitude was not informed), while in our study formulations were sonicated for 2 min by a probe of 750 W at 30% amplitude. Yet, the NLC dispersion developed by Rosli was cooled right after sonication in ice water bath to room temperature to avoid further aggregation of particles. For the same reason, formulations were stored at 4 °C. It is possible that the fact that our NLC was allowed to cool naturally the particle aggregation during the storage time was favored.

Flores and coworkers (2016) developed tioconazole-loaded NLC containing mango butter (7%, twice the amount used in our work), tea tree oil (*Melaleuca alternifolia* essential oil, 3%, while we used 1.5% of TCM), the same amount of polysorbate 80 (2.5%) and a different type and concentration of the low hydrophilic-lipophilic balance surfactant (sorbitan monooleate, 1.5%). Besides, it was added BHT as an antioxidant and the preparation of such NLC was by high-pressure homogenization. Hydrogels were obtained with this NLC and remained stable for 30 days, regarding pH, mean particle size and drug content. This suggests that the method of preparation, the components and their concentration may influence in the physicochemical properties of formulations.

To the best of our knowledge, there are no reports about the association of low-level laser and nanostructured carrier systems for the treatment of androgenetic alopecia. As so, the influence of the irradiation of red and near infrared LLL over nanoparticles properties was studied. It did not induce changes in the evaluated parameters (mean particle size, zeta

potential and drug content), demonstrating that the interaction of LLL with nanoparticles would not alter such conditions.

Safety evaluation of new formulations is necessary in order to assess the suitability of such formulations for human topical use. Therefore, *in vitro* HET-CAM and cytotoxicity and the *in vivo A. cepa* and the testosterone-induce alopecia animal model were performed. According to the data from HET-CAM assay, all nanoformulations were classified as non-irritant, independent of the presence of BS. BS-ethanolic solution was classified as moderate irritant, probably an effect related to the solvent (ethanol), as previously shown by Gilleron and coworkers (1996) in a previous study, when the irritation score of ethanol was 11.3 (considered extremely irritant). The negative (non-irritant) and positive controls (extremely irritant) validated our experiment.

Dermal papilla keratinocytes from hair follicles (HaCaT cell line) are commonly used to evaluate the response of treatments intended for hair growth stimulus. Chittur and coworkers (2011) showed that a formulation containing Saw palmetto (a plant rich in phytosterols), l-carnitine and α-lipoic acid was able to reduce the gene expression related to inflammation in those cells. In this paper, the cell viability of HaCaT and 3T3 (murine fibroblasts) were determined by MTT after different regimens of treatment: nanoformulations; LLL; nanoformulations + LLL and BS-solution. Analysis of the MTT results showed that, after 24 h of treatment, for both cell types, no cytotoxicity was found for nanoformulations (NC or NLC), either blank or BS-loaded, when compared to the control (untreated cells). The only cytotoxic treatment was the BS-ethanolic solution, even when associated with LLL, no matter the wavelength. The possibility that the ethanol used as solvent for BS could be toxic to cells was discarded, since no statistical difference in cell viability was found after treating the cells with it. This brings to light the toxicity of BS for HaCaT and 3T3 cells and the reduction in toxicity following nano-incorporation of BS.

There are many research papers that show the effects of BS on different cancer cell lines (SAYEED et al., 2016), and ultimately these cell lines undergo apoptosis or have a decrease of cell proliferation. In a study with human colon cancer cell line (HT-29), BS presented noticeable cytotoxic effect even in small doses (0.63 µM, 24h of incubation), similar to that used in this paper (0.50 µM, 24h incubation). Using higher doses, BS exhibited considerable toxicity against 3T3 (mouse embryonic fibroblast cells), HeLa (cervical cancer cells) and MCF-7 (breast cancer cell) cells (AYAZ et al., 2019). In another publication, BS

had no cytotoxic effect on noncancerous cells (COS-1 – fibroblast cell line) (JAYAPRAKASHA et al., 2007).

In trichology, LLLT is used as a tool to increase blood circulation in the scalp, ATP production, modulation of free radicals and to reduce inflammation (KARU, 1989; CHUNG et al., 2012). There are at least two dozen of relevant studies about LLLT and hair regrowth based in evidences (ZAREI et al., 2015). In our work, we irradiated 3T3 and HaCaT cells with LLL with two different wavelengths (660 and 830 nm) at a fluency of 2 $J.cm^{-2}$, so we could distinguish which combination (nanoparticle – NC or NLC + LLL – 660 or 830 nm) would provide the best results regarding cell proliferation. LLL irradiation, alone or in association with nanoformulations, did not alter de viability of 3T3 and HaCaT cell lines when compared to control. Furthermore, the association of LLL with BS-solution was not able to prevent the cytotoxicity of BS. However, although no cell proliferation was found, also no cytotoxic effects were observed, which is a positive outcome.

The effects of LLL irradiation (632, 670, 785 and 830 nm) was studied *in vivo* on hair growth of Sprague-Dawley rats on alternating days over a 2-week period (1.27 $J.cm^{-2}$ for the four first treatments and 1.91 $J.cm^{-2}$ for the last four). The results indicated that LLL with 830 nm resulted in greater stimulation of hair growth, followed by 785 nm and finally by the two wavelengths in the red range. Although we were not able to distinguish a better wavelength in this paper, these data reinforces that the effects of LLL are wavelength- and dose-dependent (KIM; KIM; YOUN, 2015)

The next step would be to evaluate the cytotoxicity of the formulations associated or not with the LLL using an *in vivo* assay, the *A. cepa*. Mitotic index of meristem cells can be used as indicative of cell proliferation (GADANO et al., 2002), showing the proliferative or cytotoxic effect of substances. Since no statistical differences were found between control (water) and all the treatments tested, they can be considered no cytotoxic. The cytotoxicity and genotoxicity of many types of nanoparticles have been studied using this model. For example, blank nanocapsules made of the same polymer here used (PCL) provided a slightly increase in the mitotic index, suggesting a possible effect of PCL in the stimulus of cell division. Also, a reduction in toxicity of an herbicide was observed following its nanoencapsulation (GRILLO et al., 2012). On the other hand, when nanoencapsulated in PCL nanocapsules, tretinoin induced a significant decrease in the mitotic index when compared to the free drug (FACCHINETTO et al., 2008). No previous reports on BS effects in *Allium*

cepa model were found. However, an *in vivo* study with mice showed that BS was not genotoxic and cytotoxic (PANIAGUA-PÉREZ et al., 2005).

When using *in vivo* animal models, a basic rule is to follow the 3R's (replace, reduce and refine). Considering this, NC formulation was chosen instead of NLC because of its superior stability. Although the LLL was studied separately at two different wavelengths in the other experiments of this article, for the *in vivo* experiment both red and near infrared wavelenghts were used concomitantly because they act at different levels of the biological tissue. Shorter wavelengths (red band, 660nm) are used to treat superficial conditions and longer wavelengths (near infrared band, 830nm) are used to treat deeper-seated tissues, as it is longer and penetrates deeper (CHUNG et al., 2012). In addition, no statistically significant differences were found between 660nm and 830 nm-LLL in the performed experiments.

To study the effect of BS-NC and the combination of BS-NC with LLL on hair growth, the testosterone-induced animal model was used. A noticeable hair loss was observed after 12 days of testosterone subcutaneous injection for all groups, but no alopecia area was detected until the end of the experiment (21 days), as it was as previously described (UPADHYAY, 2012). The photomicrographs of skin sections showed a greater amount of follicles in the groups treated with the combination of BS-NC and LLL, comparable with the finasteride-treated group and superior than the BS-NC alone or the control (animals only treated with testosterone). The number of hair follicles visualized in the histology allows classifying the results of the treatments as follows, BS-NC+LLL = finasteride<BS-NC<control, showing that the combination of BS-NC with LLL is beneficial for hair growth. Fushimi and colleagues (2011) used a LED device in the red range (638 nm, 1.5 $J.cm^{-2}$) on a culture of human dermal papilla cells. It increased the expression of growth factors (accelerates hair growth and retards entry to catagen in hair cycling) and leptin (induces anagen phase). Besides, the new hair may be darker and thicker (CHUNG et al., 2012). These findings could be a possible explanation for the results we obtained in the animal experiment.

An optimized flutamide-loaded solid lipid nanoparticles showed higher skin drug deposition (1.75 times) compared to flutamide hydroalcoholic solution in an *in vitro* experiment with skin of male Wistar rats. The *in vivo* results using male hamsters showed more anagen follicles (representative of new hair follicle growth) by utilizing flutamide-loaded SLNs, which could be a consequence of the intrafollicular accumulation of SLNs which promotes a slowly and continuous release of the antiandrogenic drug (HAMISHEHKAR, 2016). NLC and SLN generally interact with skin lipids, forming a film

on the skin surface that promotes an occlusive effect. This may increase the drug penetration in the stratum corneum (ROQUE et al., 2017).

Hinokitiol, a natural product obtained from essential oils of some trees, was loaded in PCL nanocapsules and these nanoparticles were incorporated in two products, a shampoo and a hair tonic. Their potential to stimulate hair growth was evaluated in C57BL/6 mice, an androgen responsive animal model, in comparison with a commercially available minoxidil solution. The *in vivo* hair growth promoting effects of all formulations were significant and both the hair tonic and the shampoo containing hinokitiol-nanocapsules were comparable to those of minoxidil solution. More hair follicles and longer hair fibers were found for all treatments compared to negative control (saline) (HWANG; KIM, 2008). This result may be a consequence of the properties of the drugs, as well as, the accumulation of the nanocapsules into hair follicles, which can act as a long-term reservoir (ROQUE et al., 2017).

CONCLUSION

The proposed combination of nanosystems and low-level laser is innovative and presents a great potential for the treatment of androgenetic alopecia subjects, once they are non-irritant and non-cytotoxic. Furthermore, the *in vivo* results in the testosterone-induced alopecia animal model showed that the combination of β-sitosterol loaded-nanocapsules and LLL induced the better outcome on hair growth.

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REFERENCES

ADIL, A.; GODWIN, M. The effectiveness of treatments for androgenetic alopecia: a systematic review and meta-analysis. *Journal of the American Academy of Dermatology*, v. 77, n. 1, doi: 10.1016/j.jaad.2017.02.054, 2017.

AVCI, P. et al. Low-level laser (light) therapy (LLLT) for treatment of hair loss. *Lasers in Surgery and Medicine*, v. 46, p. 144-51, 2014.

AWAD, A. B.; BURR, A. T.; FINK, C. R. Effect of resveratrol and β-sitosterol in combination on reactive oxygen species and prostaglandin release by PC-3 cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, v. 72, p. 219-26, 2005.

AYAZ, M. et al. Cytotoxicity and molecular docking studies on phytosterols isolated from Polygonum hydropiper L. *Steroids*, v. 141, p. 30-5, 2019.

BEDNER, M. et al. Development of liquid chromatographic methods for the determination of phytosterols in Standard Reference Materials containing saw palmetto. *Journal of Chromatography A*, v. 1192, p. 74-80, 2008.

BHUSHAN, B. Nanoscale characterization of human hair and hair conditioners. *Progress in Materials Science*, v. 53, p. 585-710, 2008.

CASERINI, M. et al. A novel finasteride 0.25% topical solution for androgenetic alopecia: pharmacokinetics and effects on plasma androgen levels in healthy male volunteers. *International Journal of Clinical Pharmacology and Therapeutics*, v. 52, p. 842-849, 2014.

CARITÁ, R.; MARIN-MORALES, M. A. Induction of chromosome aberrations in the Allium cepa test system caused by the exposure of seeds to industrial effluents contaminated with azo dyes. *Chemosphere*, v. 72, n. 5, p. 722-5, 2008.

CHANDRASHEKAR, B. S. et al. Topical minoxidil fortified with finasteride: An account of maintenance of hair density after replacing oral finasteride. *Indian Dermatology Online Journal*, v. 6, p. 17-20, 2015.

CHEN, L. et al. blockade of androgen markers using a novel betasitosterol, thioctic acid and carnitine-containing compound in prostate and hair follicle cell-based assays. *Phytotherapy Research*, v. 30, p. 1016-20, 2016.

CHITTUR, S.; PARR, B.; MARCOVICI, G. inhibition of inflammatory gene expression in keratinocytes using a composition containing carnitine, thioctic acid and *Saw palmetto* extract. *Evidence-based Complementary and Alternative Medicine: eCAM*, v. 2011, p. 985345, 2011.

CHUNG, H. et al. The nuts and bolts of low-level laser (light) therapy. *Annals of Biomedical Engineering*, v. 40, n. 2, p. 516-33, 2012.

CONTRI, R. V. et al. Chitosan Hydrogel Containing Capsaicinoids-Loaded Nanocapsules: An Innovative Formulation for Topical Delivery. *Soft Materials*, v. 8, n. 4, p. 370-85, 2010.

CONTRI, R. V. et al. The use of nanoencapsulation to decrease human skin irritation caused by capsaicinoids. *International Journal of Nanomedicine*, v. 9, p. 951-62, 2014.

CRABTREE, J. S. et al. A mouse model of androgenetic alopecia. *Endocrinology*, v. 151, n. 5, p. 2373-80, 2010.

CRANWELL, W.; SINCLAIR, R. Male Androgenetic Alopecia. In: De Groot, L. J.; Chrousos, G.; Dungan, K. et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2016.

DAS, S. et al. Formulation design, preparation and physicochemical characterizations of solid lipid nanoparticles containing a hydrophobic drug: Effects of process variables. *Colloids and Surfaces B: Biointerfaces*, v. 88, p. 483-89, 2011.

DHARA, R.; BHATTACHA, D. K.; GHOSH, M. Analysis of sterol and other components presents in unsaponifiable matters of Mahua, Sal and Mango Kernel oil. *Journal of Oleo Science*, v. 59, p. 169-76, 2010.

FACCHINETTO, J. M. et al. Tretinoin-loaded Polymeric Nanocapsules: Evaluation of the Potential to Improve the Antiproliferative Activities on Allium cepa root-tip Compared to the Free Drug. *Latin American Journal of Pharmacy*, v. 27, p. 668-73, 2008.

FESSI, H. ; PUISIEUX, F.; DEVISSAGUET, J.P. Procédé de préparation dês systèmes coloïdaux d'une substance sous forme de nanocapsules. *European Patent* 0274961 A1, 1989.

FIURÁSKOVÁ, M.; KUCEROVÁ, R.; KOLÁR, Z. Pathobiology of androgenetic alopecia. *Biomedical Papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia Republic*, v. 147, n. 1, p. 37-41, 2003.

FLORES, F. C. Lipid Nanoparticles Obtained with Innovative Natural Materials for Topical Delivery of Tioconazole: Mangospheres. *Journal of Nanoscienceand Nanotechnology*, v. 16, p. 1-9, 2016.

FONTANA, M. C. et al. Nanoencapsulation as a way to control the release and to increase the photostability of clobetasol propionate: influence of the nanosctructured system. *Journal of Biomedical Nanotechnology*, v. 5, p. 1-10, 2009.

FUSHIMI, T. et al. Narrow-band red LED light promotes mouse hair growth through paracrine growth factors from dermall papilla. *Journal of Dermatological Science*, v. 64, p. 237-48, 2011.

GADANO, A. et al. In vitro genotoxic evaluation of the medicinal plant Chenopodium ambrosioides L. *Journal of Ethnopharmacology*, v. 81, p. 11-6, 2002.

GILLERON, L. et al. Evaluation of a modified HET-CAM assay as a screening test for eye irritancy. *Toxicology in Vitro*, v. 10, p. 431-46, 1996.

GOMES, M. J. et al. Lipid nanoparticles for topical and transdermal application for alopecia treatment: development, physicochemical characterization, and in vitro release and penetration studies. *International Journal of Nanomedicine*, v. 9, p. 1231-42, 2014.

GOODMAN, D. S. Topical preparations for the treatment of hair loss. *US Patent.* WO 0182880 A2, 8 Nov., 2001, 21 p.

GRILLO, R. et al. Poly(ε-caprolactone)nanocapsules as carrier systems for herbicides: Physico-chemical characterization and genotoxicity evaluation. *Journal of Hazardous Materials*, v. 231-232, p. 1-9, 2012.

HAJHEYDARI, Z. et al. Comparing the therapeutic effects of finasteride gel and tablet in treatment of the androgenetic alopecia. *Indian Journal of Dermatology,Venereology and Leprology*, v. 75, n. 1, p. 47-51, 2009.

HAMISHEHKAR, H. et al. Histological assessment of follicular delivery of flutamide by solid lipid nanoparticles: potential tool for the treatment of androgenic alopecia. *Drug Development* and *Industrial Pharmacy*, v. 42, n. 6, p. 846-53, 2016.

HEILMANN, S. *et al.* Evidence for a polygenic contribution to androgenetic alopecia. *British Journal of Dermatology*, v. 169, p. 927-930, 2013.

HWANG, S. L.; KIM, J.-C. In vivo hair growth promotion effects of cosmetic preparations containing hinokitiol-loaded poly(e-caprolacton) nanocapsules. *Journal of Microencapsulation*, v. 25, p. 351-6, 2008.

JAYAPRAKASHA, G. K. et al. Inhibition of colon cancer cell growth and antioxidant activity of bioactive compounds from *Poncirus trifoliate* (L.) Raf. *Bioorganic & Medicinal Chemistry*, v. 15, p. 4923-32, 2007.

KALIYADAN, F. et al. Scanning electron microscopy study of hair damage secondary to cosmetic treatment of the hair. *International Journal of Trichology,* v. 8, n. 2, p. 94-8, 2016.

KALWEIT, S. et al. A national validation project of alternative methods to the Draize rabbit eye test. *Toxicology in Vitro*, v. 4, n. 4-5, p. 702-06, 1990.

KARU, T. Photobiology of low-power laser effects. *Health Physics*, v. 56, n. 5, p. 691-704, 1989.

KATZER, T. et al. Prednisolone-loaded nanocapsules as ocular drug delivery system: development, in vitro drug release and eye toxicity. *Journal of Microencapsulation*, v. 31, n. 6, p. 519-28, 2014.

KIM, T. H.; KIM, N. J.; YOUN, J. I. Evaluation of wavelength-dependent hair growth effects on low-level laser therapy: an experimental animal study. *Lasers and Medical Science*, v. 30, n. 6, p. 1703-9, 2015.

LADEMANN, J. et al. Hair follicles – A long-term reservoir for drug delivery. *Skin Pharmacology and Physiology*, v. 19, p. 232-36, 2006.

LADEMANN, J. et al. Nanoparticles – An efficient carrier for drug delivery into the hair follicles. *European Journal of Pharmaceutics and Biopharmaceutics*, v. 66, p. 159-64, 2007.

LADEMANN, J. et al. Hair follicles as a target structure for nanoparticles. *Journal of Innovative Optical Health Sciences*, v. 8, p. 1530004 (8 pages), 2015.

LEVY, L. L.; EMER, J. L. Female pattern alopecia: current perspectives. *International Journal of Women's Health,* v. 5, p. 541-56, 2013.

LOMENICK, B. et al. Identification and characterization of β-sitosterol target proteins. *Bioorganics & Medicinal Chemistry Letters,* v. 25, p. 4976-79, 2015.

LUEPKE, N. P. Hen's eggs chorioallantoic membrane test for irritation potential. *Food and Chemical Toxicology*, v. 23, p. 287-91, 1985.

MANDAWGADE, S. D.; PATRAVALE, V. B. Development of SLNs from natural lipids: Application to topical delivery of tretinoin. *International Journal of Pharmaceutics*, v. 363, p. 132-38, 2008.

MANDEL, A.; HAMBLIN, M. R. A renaissance in low-level laser (light) therapy – LLLT. *Photonics & Lasers in Medicine*, v. 1, n. 4, p. 231-34, 2012.

MARCOVICI, G. Hair loss treatment shows anti-inflammatory activity *in vitro. Hair: Therapy & Transplantation*, v. 2, n. 1, p. 103, 2012.

MATOS, B. N. et al. Chitosan nanoparticles for targeting and sustaining minoxidil sulphate delivery to hair follicles. *International Journal of Biological Macromolecules*, v. 75, p. 225-9, 2015.

MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological methods*, v. 65, v. 1-2, p. 55-63, 1983.

MÜLLER, C. R. et al. Spray-dried diclofenac poly(epsilon-caprolactone) nanocapsules and nanospheres: preparation and physicochemical characterization. *Pharmazie*, v. 56, p. 864-7, 2001.

MÜLLER, R. H.; STAUFENBIEL, S.; KECK, C. M. Lipid Nanoparticles (SLN, NLC) for innovative consumer care & household products. *Transdermal delivery*, v. 9, n. 2, 18-24, 2014.

NADEEM, M.; IMRAN, M.; KHALIQUE, A. Promising features of mango (*Mangifera indica* L)kernel oil: a review. *Journal of Food and Science Technology*, v. 53, p. 2185-95, 2016.

NICHOLS, A. J. *et al.* An open-label evaluator blinded study of the efficacy and safety of a new nutritional supplement in androgenetic alopecia: a pilot study. *Journal of Clinical and Aesthetic Dermatology*, v. 10, n. 2, p. 52-6, 2017.

-

NIRMAL, S. A. et al. Analgesic and anti-inflammatory activity of β-sitosterol isolated from Nyctanthes arbortristis leaves. *Inflammopharmacology*, v. 20, n. 4, p. 219-24, 2012.

NOOR, N. M. et al. Preparation and characterization of dutasteride-loaded nanostructured lipid carriers coated with stearic acid-chitosan oligomer for topical delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, v. 117, p. 372-84, 2017.

OTBERG, N.; FINNER, A. M.; SHAPIRO, J. Androgenic alopecia. *Endocrinology and Metabolism Clinics of North America*, v. 36, p. 379-98, 2007.

OURIQUE, A .F. et al. Improved photostability and reduced skin permeation of tretinoin: Development of a semisolid nanomedicine. *European Journal of Pharmaceutics and Biopharmaceutics*, v. 79, p. 95-101, 2011.

PADOIS, K. et al. Solid lipid nanoparticle suspension versus commercial solutions for dermal delivery of minoxidil. *International Journal of Pharmaceutics*, v. 416, p. 300-04, 2011.

PALOMBO, M. et al. Pharmaceutical and Toxicological Properties of Engineered Nanomaterials for Drug Delivery. *Annual review of Pharmacology and Toxicology*, v. 54, p. 581–98, 2014.

PANIAGUA-PÉREZ, R. et al. Genotoxic and cytotoxic studies of beta-sitosterol and pteropodine in mouse. *Journal of Biomedicine and Biotechnology*, v. 3, p. 242-7, 2005.

PARK, W-S. et al. The extract of *Thujae occidentalis* semen inhibited 5a-reductase and androchronogenetic alopecia of B6CBAF1/j hybrid mouse. *Journal of Dermatological Science*, v. 31, p. 91-98, 2003.

PATZELT, A. et al. Selective follicular targeting by modification of the particle sizes. *Journal of Controlled Release*, v. 150, p. 45-48, 2011.

POHLMANN, A. R. et al. Nanopartícula polimérica de finasterida e minoxidil, processo de sua preparação, suspensão aquosa contendo a mesma, composição farmacêutica, e seu uso. *Bras Patent.* BR 10 2012 022036-9 A2, 31 ago, 2012. 53 p.

POHLMANN, A. R.; JORNADA, D. S.; GUTERRES, S. S. Nanopartícula polimérica de finasterida, suspensão aquosa contendo a mesma, composição para tratamento de alopecia processo de preparação de dita composição, e seu uso. *Bras Patent*. BR 10 2012 022034-2 A2, 31 ago, 2012. 67 p.

PRAGER, N. et al. A Randomized, Double-Blind, Placebo-Controlled Trial to Determine the Effectiveness of Botanically Derived Inhibitors of 5AR in the Treatment of Androgenetic Alopecia. *Journal of Alternative and Complementary Medicine*, v.8., n.2, p. 143-152, 2002.

RAJESHWARI, A. *et al.* Toxicity evaluation of gold nanoparticles using *Allium cepa* bioassay. *RSC Advances*, v. 6, p. 2400-24009, 2016.

REJUVIANCE PRODUTCTS CORP. Super Strength DHT Blocker. Disponível em: <http://rejuviance.com/Super-Strength-DHT-blocker--5-beta-Sitosterol_p_53.html>.

REUTER, J.; MERFORT, I.; SCHEMPP, C. M. Botanicals in Dermatology: An evidence-based review. *American Journal of Clinical Dermatology*, v. 11, n. 4, p. 247-67, 2010.

ROQUE, L. V. *et al.* Design of finasteride-loaded nanoparticles for potential treatment of alopecia. *Skin Pharmacology and Physiology*, v. 30, p. 197-204, 2017.

ROSLI, N. A. et al. Formulation and characterization of nanostructured lipid carrier encapsulated *Zingiber zerumbet* oil using ultrasonication technique. *Journal of Advanced Research*, v. 11, n. 1, p. 16-23, 2015.

ROSSI, A. et al. Comparative effectiveness of finasteride vs Serenoa repens in male androgenetic alopecia: a two-year study. *International Journal of Immunopathology* and *Pharmacology*, v. 25, n. 4, p. 1167-73, 2012.

ROY, R. K.; THAKUR, M.; DIXIT, V. K. Hair growth promoting effect activity of *Eclipta alba* in male albino rats. *Archives of Dermatological Research,* v. 300, p. 357-64, 2008.

SAYEED, M. S. B. *et al.* Critical Analysis on Characterization, Systemic Effect, and Therapeutic Potential of Beta-Sitosterol: A Plant-Derived Orphan Phytosterol. *Medicines (Basel)*, v. 3, p. E29, 2016.

SAVIAN, A. L. et al. Dithranol-loaded lipid-core nanocapsules improve the photostability and reduce the in vitro irritation potential of this drug. *Materials Science* and *Engineering*, v. 46, p. 69-76, 2014.

SCALPMED. Nutrisol®-Rm. Disponível em: < https://www.scalpmed.com/>. Acesso em: 07 fev. 2019.

SCHAFFAZICK, S. R. et al. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. *Química Nova*, v. 26, n. 5, p. 726-37, 2003.

SHAMMA, R. N.; ABURAHMA, M. H. Follicular delivery of spironolactone via nanostructured lipid carriers for management of alopecia. *International Journal of Nanomedicine*, v. 9, p. 5449-60, 2014.

SOLEIMANIAN, Y. et al. β-sitosterol lipid nano carrier based on propolis wax and pomegranate seed oil: effect of thermal processing, pH, and ionic strength on stability and structure. *European Journal of Lipid Science and Technology*, v. 121, p. 1800347, 2019.

SONTHALIA, S.; DAULATABAD, D.; TOSTI, A. Hair Restoration in Androgenetic Alopecia: Looking Beyond Minoxidil, Finasteride and Hair Transplantation. *Journal of Cosmetology & Trichology*, v. 2, p. 1000105, 2016.

SOUTO, E. B.; MÜLLER, R. H. SLN and NLC for topical delivery of ketoconazole. *Journal of Microencapsulation*, v. 22, n. 5, p. 501-10, 2005.

SPIELMANN, H. et al. Validation study of alternatives to the Draize eye irritation test in Germany: cytotoxicity testing and HET-CAM test with 136 industrial chemicals. *Toxicology In Vitro*, v. 4, p. 505-10, 1993.

TABBAKHIAN, M. et al. Enhancement of follicular delivery of finasteride by liposomes and niosomes. 1. *In vitro* permeation and *in vivo* deposition studies using hamster flank and ear models. *International Journal of Pharmaceutics*, v. 323, p. 1-10, 2006.

TEDESCO, S.; LAUGHINGHOUSE, I.V. Bioindicator of genotoxicity: the *Allium cepa* test. In: J.K. Srivastava (Ed.), *Environmental Contamination*, InTech Publisher, Rijeka (2012), p. 137-156.

TRÜEB, R. M. Molecular mechanisms of androgenic alopecia. *Experimental Gerontology*, v. 37, p. 981-90, 2002.

TSAI, M.-J. et al. Baicalein loaded in tocol nanostructured lipid carriers (tocol NLCs) for enhanced stability and brain targeting. *International Journal of Pharmaceutics*, v. 423, p. 461-70, 2012.

UPADHYAY, K.; GUPTA, N. K.; DIXIT, V. K. Development and characterization of phyto-vesicles of β-sitosterol for the treatment of androgenetic alopecia. *Archives of Dermatological Research*, v. 304, p. 511–19, 2012.

UPRIT, S. et al. Preparation and characterization of minoxidil loaded nanostructured lipid carrier gel for effective treatment of alopecia. *Saudi Pharmaceutical Journal*, v. 21, p. 379-85, 2013.

WANG, H. X.; NG, T. B. Natural products with hypoglycemic, hypotensive, hypocholesterolemic, antiatherosclerotic and antithrombotic activities. *Life Sciences*, v. 65, n. 2, p. 2663-77, 1999.

WEI, D. et al. β-sitosterol solubility in selected organic solvents. *Journal of Chemical Engineering Data*, v. 55, p. 2917-19, 2010.

WEI, G.; BHUSHAN, B.; TORGERSON, P. M. Nanomechanical characterization of human hair using nanoindentation and SEM. *Ultramicroscopy*, v. 105, n. 1-4, p. 248-66, 2005.

ZAREI, M. et al. Low level laser therapy and hair regrowth: an evidence-based review. *Lasers and Medical Science*, v. 31, n. 2, p. 363-71, 2015.

DISCUSSÃO

A AAG é a principal causa de rarefação capilar em homens e mulheres. De origem poligênica (HEILMANN *et al*., 2013), a fisiopatologia da AAG envolve a hereditariedade, a ação de hormônios androgênicos sobre as células do folículo piloso e a microinflamação, entre outros fatores. A testosterona é o principal hormônio androgênico que, sob a ação das enzimas de membrana 5-α-redutase I ou II, é convertida irreversivelmente à di-hidrotestosterona (DHT), seu metabólito mais potente, com afinidade cerca de cinco vezes maior aos receptores androgênicos (KAUFMAN, 2002). A ligação do DHT a um receptor induz o processo de miniaturização (PARK *et al.*, 2003), com diminuição da fase de crescimento do ciclo capilar (anágena) (HAJHEYDARI *et al*., 2009).

Embora a fisiopatologia da AAG esteja estreitamente relacionada ao metabolismo androgênico, evidências científicas sugerem que a AAG está associada com uma desregulação na expressão de citocinas inflamatórias (CHITTUR; PARR e MARCOVICI, 2011), sendo a microinflamação crônica perifolicular um fator agravante na AAG (TRÜEB, 2002). O nível de severidade, a idade inicial de acometimento e a localização da rarefação capilar no couro cabeludo são variáveis (CRABTREE *et al*., 2010).

Apesar de não ser considerada uma doença que ofereça risco de vida, seus efeitos sobre a qualidade de vida dos acometidos podem ser devastadores e têm sido amplamente estudados (GONUL *et al*., 2018). Um estudo prospectivo e multicêntrico realizado com 998 homens com AAG demonstrou que a doença afeta negativamente a sua qualidade de vida e que seu impacto é tão maior quanto mais avançada estiver a alopecia, maior o tempo de manifestação e menor a idade do indivíduo (HAN *et al*., 2012).

Frente à ampla revisão de literatura apresentada no Manuscrito1, é possível afirmar que os tratamentos existentes variam conforme o país, são restritos e apresentam limitações de eficácia, além de efeitos adversos frequentes. No Brasil, apenas minoxidil e 17-α-estradiol são utilizados por via tópica e finasterida por via oral, este último apenas para homens; nos Estados Unidos, minoxidil, finasterida e o laser de baixa potência e, em alguns países, como Coreia e México, a dutasterida (0,5 mg por dia) também foi aprovada para o tratamento da AAG em homens (KELLY; BLANCO e TOSTI, 2016).

Por isso, a procura por novas abordagens visando ao aumento da eficácia terapêutica é uma necessidade. O β-sitosterol é um fitoesterol presente em várias plantas (ROSSI *et al*., 2012; ROY; THAKUR e DIXIT, 2008). Embora seja um componente minoritário, é considerado um dos principais responsáveis pelo estímulo de crescimento capilar por elas

promovido. Inúmeras funções biológicas já foram demonstradas para esta molécula, sendo suas propriedades anti-inflamatórias, antioxidantes, angiogênicas, hipocolesterolemiantes e de inibição da 5-α-redutase (CHEN *et al*., 2016; SAYEED *et al*., 2016) interessantes para o tratamento da alopecia androgenética. O BS está disponível comercialmente sob a forma de loções para uso tópico e suplementos de uso oral, no entanto, não existem estudos clínicos realizados com estes produtos.

Explorar os benefícios do BS para o tratamento da AAG a uma forma diferenciada de entrega deste ativo por via tópica foi uma das ideias centrais da presente tese. Além disso, associar as nanopartículas desenvolvidas ao LBP, um recurso seguro e eficaz para o tratamento da AAG é uma proposta inédita. Neste sentido, nanocápsulas (NC) e carreadores lipídicos nanoestruturados (CLN) contendo BS foram desenvolvidos. A nanoencapsulação de fármacos pode modificar as propriedades físico-químicas da molécula incorporada e, normalmente, incrementa a distribuição de substâncias de difícil absorção (CONTRI *et al*., 2016).

As NC e CLN foram preparados pelo método de deposição interfacial de polímero pré-formado e por emulsificação seguida por ultrassonicação, respectivamente e apresentaram distribuição de partículas exclusivamente na faixa nanométrica. O tamanho médio de partícula foi de 231 e 117 nm para BS-NC e BS-NLC, respectivamente, com baixo índice de polidispersão. Os valores de potencial zeta foram negativos, o pH levemente ácido e o teor de BS foi próximo ao teórico (1 mg/ml) para ambas nanopartículas. Os valores obtidos para os parâmetros supracitados são semelhantes aos encontrados na literatura para estes tipos de nanopartículas. A irradiação das nanopartículas com LBP 660 ou 830 nm (4 J/cm2) não induziu mudanças no tamanho médio da partícula, potencial zeta e teor.

Terapias complementares às medicamentosas têm sido propostas para o tratamento da AAG. O LBP foi desenvolvido em meados de 1960, mas apenas recentemente tem sido mais amplamente empregado na dermatologia. A terapia com LBP consiste na exposição de células ou tecidos a baixos níveis de energia luminosa (<500 mW), normalmente na faixa do vermelho (625-740 nm) ou infravermelho próximo (780-1000 nm). Este método é considerado de “baixa potência” e “frio” porque a densidade de energia depositada no tecido é pequena e insuficiente para gerar calor local comparada com outras formas de laser, capazes de cortar, queimar ou coagular um tecido vivo (MANDEL e HAMBLIN, 2012).

O uso do LBP como estratégia fototerápica utiliza fótons (pequenos “pacotes” de energia) para alterar atividades biológicas (AVCI *et al*., 2013). Embora ainda não estejam

bem estabelecidos os mecanismos de interação entre as células ou tecidos e o LBP, a literatura apresenta cromóforos intramitocondriais (complexo IV ou citocromo *c* oxidase, um complexo de proteínas transmembrana) como os principais fotoaceptores, resultando em aumento da produção de adenosina trifosfato (ATP), NADH, modulação de espécies reativas de oxigênio e indução de fatores de transcrição que desencadeiam uma cascata de eventos celulares que levam à síntese de proteínas (CHUNG *et al*., 2012). Algumas das aplicações da terapia com LBP incluem regeneração tecidual (queimaduras, úlceras, processos cicatriciais), redução da inflamação e alívio da dor (MANDEL e HAMBLIN, 2012). Uma das aplicações mais comercialmente aceitas do LBP é a estimulação do crescimento capilar em indivíduos com algum tipo de alopecia. Apesar de existirem dezenas de artigos publicados sobre o tema, apenas um apresenta um estudo clínico duplo-cego, controlado, multicêntrico e randomizado com duração de 26 semanas que demonstra a efetividade do LBP em homens com AAG (LEAVITT *et al*., 2009).

O efeito destas nanoestruturas e da associação com o LBP foi avaliado *in vivo* e *in vitro*. De acordo com os dados do ensaio HET-CAM, as nanoformulações não foram irritantes, independentemente da presença de BS. O tratamento das linhagens celulares 3T3 e HaCaT com as nanopartículas (com ou sem BS) não induziu citotoxicidade nem proliferação celular, bem como os tratamentos associando o LBP. A viabilidade celular diminuiu apenas para ambas as linhas celulares quando a solução de BS foi utilizada, demonstrando a capacidade das nanoestruturas de proteger contra a citotoxicidade do BS. No ensaio *in vivo Allium cepa*, o índice mitótico não foi influenciado pelas formulações, independentemente da presença de BS e da sua nanoencapsulação.

Levando estes achados em consideração e visando à avaliação dos efeitos da associação do LBP às nanopartículas contendo BS sobre o crescimento capilar, conduziu-se o experimento *in vivo* em modelo de alopecia induzida pela administração de testosterona em ratos Wistar. Simultaneamente à indução, os animais foram tratados com BS-NC, BS-NC+LBP ou finasterida (solução etanólica 1%, p/v). Para reduzir o número de animais necessários para o experimento, apenas as nanocápsulas desenvolvidas foram avaliadas, devido a sua superior estabilidade. Embora o LBP tenha sido estudado separadamente em dois comprimentos de onda (660 e 830 nm) nos demais experimentos deste artigo, não foram encontradas diferenças estatisticamente significativas nos resultados obtidos. Para o experimento *in vivo*, empregou-se ambos comprimentos de onda, concomitantemente, pois atuam em diferentes níveis do tecido biológico. Comprimentos de onda mais curtos (faixa

vermelha, 660 nm) são usados para tratar condições superficiais e comprimentos de onda mais longos (faixa de infravermelho próximo, 830 nm) são empregados para tratar tecidos mais profundos (CHUNG *et al*., 2012).

Durante o experimento, observou-se uma perda notável de pelos após 12 dias de injeção subcutânea de testosterona, mas nenhuma área de alopecia foi detectada até o final do experimento (21 dias), diferente do que foi obtido por Upadhyay e colaboradores (2012). As fotomicrografias da pele mostraram uma maior quantidade de folículos nos grupos tratados com a combinação de BS-NC e LLL (660 + 830 nm), comparável com o grupo tratado com finasterida e superior ao grupo BS-NC e ao controle, mostrando que a combinação de BS-NC com o LLL é benéfica para o crescimento capilar.

A administração oral de finasterida resulta em vários efeitos adversos sistêmicos, sendo assim a aplicação tópica deste e de outros ativos com potencial para o tratamento da AAG têm sido proposta. O sistema ideal deve fornecer alta retenção na derme e epiderme, enquanto permite algum controle da liberação do fármaco. Nanopartículas ("polimerosomos") à base de poliestireno e poli(ácido acrílico) contendo finasterida e revestidas com quitosana foram desenvolvidas e o perfil de liberação e de permeção do fármaco foram avaliados. Os achados demonstraram que o maior tempo de latência foi obtido para a solução hidroetanólica de finasterida, associada a uma menor permeação, quando comparado ao sistema nanoparticulado. Isto sustenta a hipótese de que sistemas nanoestruturados podem ser necessários para aumentar a penetração e permeação de fármacos (CAON *et al*., 2014).

Dando continuidade aos estudos, na seção "Apêndice A" está apresentado um experimento complementar que objetivou analisar como as nanopartículas desenvolvidas (NC e CLN, contendo ou não BS) interagiriam com a fibra capilar. Para tanto, pequenos fragmentos (5 cm, 30 mg) de uma mecha de cabelo caucasiano, virgem, foram colocados em contato com 300 µL das nanoformulações por 48 h e, posteriormente, sua topografia foi analisada por microscopia eletrônica de varredura e comparada com uma amostra sem qualquer tratamento. A mecha não tratada apresentou superfície homogênea, com cutículas com padrão normal e sem qualquer depósito em sua superfície, como espera-se para um cabelo saudável (KALIYADAN *et al*., 2016). As mechas que tiveram contato com as nanocápsulas (B-NC ou BS-NC) apresentaram aglomerados de partículas arredondadas na sua superfície, de distribuição heterogênea e com localização preferencial junto às terminações cuticulares, independente da presença de BS. Quando as mechas foram tratadas com os CLN

(B-CLN ou BS-CLN), a distribuição das nanopartículas na superfície da fibra foi mais uniforme e com menos agregados.

Embora as nanopartículas desenvolvidas não sejam destinadas a tratar a fibra capilar, este experimento foi conduzido para avaliar como interagiriam a haste do cabelo e as nanoformulações. Pensando em uma situação de uso real dessas nanopartículas, é inevitável o contato do produto com a fibra capilar. O movimento contínuo do cabelo que acontece normalmente em circunstâncias *in vivo* pode atuar como um mecanismo de bombeamento que direciona as nanopartículas para o folículo piloso (LADEMANN *et al*., 2007). Se ocorrer penetração folicular das nanoestruturas, sua capacidade de permanência no folículo piloso é maior do que para as moléculas não particuladas (LADEMMAN *et al*., 2015).

Shamma e Aburahma (2014) desenvolveram CLN contendo espironolactona, um fármaco antiandrogênico de prescrição *off label*, por via oral, para o tratamento da AAG em mulheres. Sua aplicação tópica evitaria efeitos adversos sistêmicos. Os pesquisadores observaram a distribuição dos nanocarreadores marcados com fluorescência na pele e nas estruturas foliculares de camundongos e encontraram uma ampla distribuição da fluorescência ao redor dos folículos pilosos e dos fios de cabelo, provavelmente devido ao acúmulo de CLN nessas áreas. O ducto folicular tem sebo e a afinidade dos CLN com essa área pode ser, em parte, conseqüência disso. Da mesma forma, a fibra capilar saudável é recoberta por moléculas de ácidos graxos, cujo representate majoritário é o ácido 18-metil eicosanóico (TANAMACHI *et al*., 2010), o que poderia justificar, por afinidade, a distribuição mais uniforme dos CLN sobre a fibra capilar em relação às NC desenvolvidas neste trabalho.

Alguns experimentos complementariam os resultados aqui obtidos e permitiriam uma discussão mais aprofundada do tema, como por exemplo, a avaliação do perfil de liberação *in vitro* do BS a partir das nanopartículas desenvolvidas, bem como da sua penetração folicular, associadas ou não à laserterapia. Estes experimentos não foram incluídos nesta tese devido à limitação analítica do BS em baixas concentrações, pela indisponibilidade de equipamentos adequados. A adição de antioxidantes e a desidratação dos CLN poderiam auxiliar na estabilidade dos sistemas. No experimento *in vivo*, utilizando o modelo animal de alopecia induzida pela administração de testosterona, três novos grupos (LBP 660+830 nm, NC-B e BS-solução) foram realizados durante a finalização da redação desta tese e seus resultados ainda não estão nela contidos. A análise destes resultados permitirá incrementar a discussão sobre os benefícios da nanoencapsulação, bem como da associação das nanoestruturas à laserterapia. Levando em consideração as muitas vantagens do sistema nanoparticulado para a

liberação intrafolicular de ativos, espera-se que em um futuro próximo uma formulação nanotecnológica seja uma opção de prescrição para o tratamento de AGA. Além disso, a associação do LBP a sistemas nanoestruturados pode ser melhor explorada no que tange à penetração folicular.

CONCLUSÃO

Neste trabalho foram desenvolvidas duas formulações nanotecnológicas contendo β-sitosterol, uma nanocápsula polimérica e um carreador lipídico nanoestruturado. As mesmas foram caracterizadas quanto a sua distribuição granulométrica, tamanho médio de partículas, polidispersão, pH e teor de β-sitosterol e apresentaram resultados compatíveis com os sistemas nanométricos. Como o objetivo central deste trabalho foi avaliar os efeitos da associação das nanopartículas desenvolvidas com o laser de baixa potência no tratamento da alopecia androgenética, os efeitos da irradiação do laser sobre as nanopartículas foram investigados e os resultados demonstram que não ocorreram alterações nos parâmetros físico-químicos avaliados.

As formulações foram consideradas não irritantes quando avaliadas pela técnica de HET-CAM. Sua citotoxicidade, quando associadas ou não ao laser de baixa potência, foi avaliada por dois métodos, *in vitro* e *in vivo*, e os resultados demonstraram que as formulações, o laser e a combinação de ambos não apresentaram efeitos citotóxicos para as linhagens celulares (fibroblastos e queratinócitos) e a espécie vegetal *A. cepa*. Além disso, a nanoencapsulação do β-sitosterol contornou a citotoxicidade apresentada pelo mesmo em solução frente a duas linhas celulares (fibroblastos murinos e queratinócitos humanos). Por fim, no modelo animal de alopecia induzida por administração de testosterona empregando ratos Wistar, a combinação do laser de baixa potência (660 nm + 830 nm, fluência total de 2 J/cm^2) com nanocápsulas contendo β-sitosterol apresentou eficácia semelhante à finasterida em estimular a atividade dos folículos pilosos, além de ser mais eficaz que as nanocápsulas. Sendo assim, conclui-se que a associação do laser de baixa potência com os sistemas nanoestruturados pode fornecer resultados superiores no tratamento do alopecia androgenética e deve ser melhor investigada.

REFERÊNCIAS

ADIL, A.; GODWIN, M. The effectiveness of treatments for androgenetic alopecia: a systematic review and meta-analysis. **Journal of the American Academy of Dermatology,** v. 77, p. 136-141, 2017. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/28396101>. Acesso em: 07 fev .2019. DOI: 10.1016/j.jaad.2017.02.054.

AYAZ, M. et al. Cytotoxicity and molecular docking studies on phytosterols isolated from Polygonum hydropiper L. *Steroids,* v. 141, p. 30-5, 2019.

AVCI, P. *et al*. Low-level laser (light) therapy (LLLT) for treatment of hair loss**. Lasers in Surgery and Medicine**, v. 46, p. 144-151, 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/23970445>. Acesso em: 07 fev. 2019. DOI: 10.1002/lsm.22170.

CAON, T. *et al.* Chitosan-decorated polystyrene-b-poly(acrylic acid) polymersomes as novel carriers for topical delivery of finasteride. **European Journal of Pharmaceutical Science**, v. 52, p. 165-172, 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/24262075>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.ejps.2013.11.008.

CASERINI, M. *et al*. A novel finasteride 0.25% topical solution for androgenetic alopecia: pharmacokinetics and effects on plasma androgen levels in healthy male volunteers. **International Journal of Clinical Pharmacology and Therapeutics**, v. 52, p. 842-849, 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/25074865.>. Acesso em: 07 fev. 2019. DOI: 10.5414/CP202119.

CHEN, L. *et al*. Blockade of androgen markers using a novel betasitosterol, thioctic acid and carnitine-containing compound in prostate and hair follicle cell-based assays. **Phytotherapy Research**, v. 30, p. 1016-1020, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/26990224>. Acesso em: 07 fev. 2019. DOI: 10.1002/ptr.5611.

CHEN, M.; LIU, X.; FAHR, A. Skin penetration and deposition of carboxyfluorescein and temoporfin from different lipid vesicular systems: In vitro study with finite and infinite dosage application. **International Journal of Pharmaceutics**, v. 408, p. 223-234, 2011. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/21316430>. Acesso em: 07 fev. 2019. DOI: **10.1016/j.ijpharm.2011.02.006.**

CHITTUR, S.; PARR, B.; MARCOVICI, G. inhibition of inflammatory gene expression in keratinocytes using a composition containing carnitine, thioctic acid and *Saw palmetto* extract. **Evidence-based Complementary and Alternative Medicine: eCAM,** v. 2011, p. 985345, 2011. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/19692448>. Acesso em: 07 fev. 2019. DOI: 10.1093/ecam/nep102.

CHUNG, H. *et al.* The nuts and bolts of low-level laser (light) therapy. **Annals of Biomedical Engineering,** v. 40, p. 516-533, 2012. Disponível em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3288797/>. Acesso em: 07 fev. 2019. DOI: 10.1007/s10439-011-0454-7.

CONTRI, R. V. *et al.* Drug Transport across Skin. In: MURO, S. (Ed.) **Drug Delivery Across Physiological Barriers**, 1 ed., Boca Raton, FL: CRC Press, 2016. Cap 5. Disponível em: <https://www.crcpress.com/Drug-Delivery-Across-Physiological-Barriers/Muro/p/book/9789814669405>. Acesso em: 07 fev. 2019.

CONTRI, R. V. *et al*. The use of nanoencapsulation to decrease human skin irritation caused by capsaicinoids. **International Journal of Nanomedicine**, v. 9, p. 951-962, 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/24611011>. Acesso em: 07 fev. 2019. DOI: 10.2147/IJN.S56579.

CRABTREE, J. S. *et al*. A mouse model of androgenetic alopecia. **Endocrinology**, v. 151, p. 2373-2380, 2010. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/20233794>. Acesso em: 07 fev. 2019. DOI: 10.1210/en.2009-1474.

CRANWELL, W.; SINCLAIR R. Male Androgenetic Alopecia. In: De Groot, L. J., Chrousos, G., Dungan, K., Feingold, K. R., Grossman, A., Hershman, J. M., … Vinik, A. (Eds.), **Endotext**, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/books/NBK278957/>. Acesso em: 07 fev. 2019. PMID: 25905192

FERNANDEZ, M. L.; VEGA-LÓPEZ, S. Efficacy and Safety of Sitosterol in the Management of Blood Cholesterol Levels. **Cardiovascular Drug Reviews**, v. 23, p. 57-70, 2005. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/15867948>. Acesso em: 07 fev. 2019. PMID: 15867948.

GOMES, M. J *et al*. Lipid nanoparticles for topical and transdermal application for alopecia treatment: development, physicochemical characterization, and in vitro release and penetration studies. **International Journal of Nanomedicine**, v. 9, p. 1231-1242, 2014. Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24634584>. Acesso em: 07 fev. 2019. DOI: *1*0.2147/IJN.S45561.

GONUL, M. *et al.* Comparison of quality of life in patients with androgenetic alopecia and alopecia areata. **Anais Brasileiros de Dermatologia**, v. 93, p. 651-658, 2018. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/30156613>. Acesso em: 07 fev. 2019. DOI: 10.1590/abd1806-4841.20186131.

HAJHEYDARI, Z. *et al.* Comparing the therapeutic effects of finasteride gel and tablet in treatment of the androgenetic alopecia. **Indian Journal of Dermatology, Venereology and Leprology**, v. 75, p. 47-51, 2009. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/19172031>. Acesso em: 07 fev. 2019. PMID: 19172031.

HAMEED, A. *et al.* Scope of Nanotechnology in Cosmetics: Dermatology and Skin Care Products. **Journal of Medicinal and Chemical Sciences**, v. 2, p. 9-16, 2019. Disponível em: <http://www.jmchemsci.com/article_68859_eea7053f5914c4e3f29aee91c77fdbc6.pdf>. Acesso em: 08 abr. 2019. DOI: 10.26655/JMCHEMSCI.2019.6.2

HAMISHEHKAR, H. *et al*. Histological assessment of follicular delivery of flutamide by solid lipid nanoparticles: potential tool for the treatment of androgenic alopecia. **Drug**

Development and Industrial Pharmacy, v. 42, p. 846-853, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/26154267>. Acesso em: 07 fev. 2019. DOI: 10.3109/03639045.2015.1062896.

HAN, S-H. *et al.* Quality of Life Assessment in Male Patients with Androgenetic Alopecia: Result of a Prospective, Multicenter Study. **Annals of Dermatology**, v. 24, p. 311-318, 2012. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/22879715>. Acesso em: 07 fev. 2019. DOI: 10.5021/ad.2012.24.3.311.

HEILMANN, S. *et al.* Evidence for a polygenic contribution to androgenetic alopecia. **British Journal of Dermatology**, v. 169, p. 927-930, 2013. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/23701444>. Acesso em: 07 fev. 2019. DOI: 10.1111/bjd.12443.

KALIYADAN, F. *et al*. Scanning electron microscopy study of hair damage secondary to cosmetic treatment of the hair. **International Journal of Trichology**, v. 8, p. 94-98, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4989398/>. Acesso em: 07 fev. 2019. DOI: 10.4103/0974-7753.188035.

KAUFMANN, K. D. Androgens and alopecia. **Molecular and Cellular Endocrinology**, v. 198, p. 89-95, 2002. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/12573818>. Acesso em: 07 fev. 2019. PMID: 12573818.

KELLY, Y.; BLANCO, A.; TOSTI, A. Androgenetic Alopecia: An Update of Treatment Options. **Drugs**, v. 76, p. 1349-1364, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/27554257>. Acesso em: 07 fev. 2019. DOI: 10.1007/s40265-016-0629-5.

KHAN, I.; SAEED, K.; KHAN, I. Nanoparticles: Properties, applications and toxicities. **Arabian Journal of Chemistry**, *in press*, 2017. Disponível em: <https://www.sciencedirect.com/science/article/pii/S1878535217300990>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.arabjc.2017.05.011.

KUMARI, A.; YADAV, S. K.; YADAV, S. C. Biodegradable polymeric nanoparticles based drug delivery systems. **Colloids and Surfaces. B, Biointerfaces**, v. 75, p. 1-18, 2010. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/19782542>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.colsurfb.2009.09.001.

LADEMANN, J. *et al.* Nanoparticles - An efficient carrier for drug delivery into the hair follicles. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 66, p. 159-164, 2007. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/17169540>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.ejpb.2006.10.019.

LADEMANN, J *et al.* Penetration and storage of particles in human skin: Perspectives and safety aspects. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 77, p. 465-468, 2011. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/21056659>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.ejpb.2010.10.015.

LADEMANN, J. *et al.* Hair follicles as a target structure for nanoparticles**. Journal of Innovative Optical Health Sciences**, v. 8, p. 1530004 (8 pages), 2015. Disponível em:

<https://www.worldscientific.com/doi/abs/10.1142/S1793545815300049>. Acesso em: 07 fev. 2019. DOI: 10.1142/S1793545815300049.

LEAVITT, M. *et al.* HairMax LaserComb laser phototherapy device in the treatment of male androgenetic alopecia: A randomized, double-blind, sham device-controlled, multicentre trial. **Clinical Drug Investigation**, v. 29, p. 283-292, 2009. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/19366270>. Acesso em: 07 fev. 2019. DOI: 10.2165/00044011-200929050-00001.

MANDEL, A.; HAMBLIN, M. R. A renaissance in low-level laser (light) therapy – LLLT. **Photonics & Lasers in Medicine**, v. 1, p. 231-234, 2012. Disponível em: <https://www.degruyter.com/view/j/plm.2012.1.issue-4/plm-2012-0044/plm-2012-0044.xml>. Acesso em: 07 fev. 2019. DOI: 10.1515/plm-2012-0044.

MATOS, B. N. *et al*. Chitosan nanoparticles for targeting and sustaining minoxidil sulphate delivery to hair follicles. **International Journal of Biological Macromolecules**, v. 75, p. 225-229, 2015. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/25647618>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.ijbiomac.2015.01.036.

MÜLLER, R. H.; RADTKE, M.; WISSING, S.A. Nanostructured lipid matrices for improved microencapsulation of drugs. **International Journal of Pharmaceutics**, v. 242, p. 121-128, 2002. Disponível em: <https://www.sciencedirect.com/science/article/pii/S0378517302001801>. Acesso em: 07 fev. 2019. DOI: 10.1016/S0378-5173(02)00180-1.

MÜLLER, R. H. *et al.* Nanostructured lipid carriers (NLC) in cosmetic dermal products. **Advanced Drug Delivery Reviews**, v. 59, p. 522-530, 2007. Disponível em: <https://www.sciencedirect.com/science/article/pii/S0169409X07000464>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.addr.2007.04.012.

NOOR, N. M. *et al*. Preparation and characterization of dutasteride-loaded nanostructured lipid carriers coated with stearic acid-chitosan oligomer for topical delivery. **European Journal of Pharmaceutics and Biopharmaceutics,** v. 117, p. 372-384, 2017. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/28412472>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.ejpb.2017.04.012.

PARK, W-S. *et al.* The extract of *Thujae occidentalis* semen inhibited 5a-reductase and androchronogenetic alopecia of B6CBAF1/j hybrid mouse. **Journal of Dermatological Science**, v. 31, p. 91-98, 2003. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/12670719>. Acesso em: 07 fev. 2019. PMID: 12670719.

PRAGER, N. *et al*. A Randomized, Double-Blind, Placebo-Controlled Trial to Determine the Effectiveness of Botanically Derived Inhibitors of 5AR in the Treatment of Androgenetic Alopecia. **Journal of Alternative and Complementary Medicine**, v.8., p. 143-152, 2002. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/12006122>. Acesso em: 07 fev. 2019. DOI: 10.1089/acm.2002.8.143.

RAMOS, P. M.; MIOT, H. A. Female Pattern Hair Loss: a clinical and pathophysiological review. **Anais Brasileiros de Dermatologia**, v. 90, p. 529-543, 2016. Disponível em:

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4560543/>. Acesso em: 07 fev. 2019. DOI: http://dx.doi.org/10.1590/abd1806-4841.20153370.

REJUVIANCE PRODUTCTS CORP. **Super Strength DHT Blocker.** Disponível em: <http://rejuviance.com/Super-Strength-DHT-blocker--5-beta-Sitosterol_p_53.html>. Acesso em: 07 fev. 2019.

RINALDI, S., BUSSA, M.; MASCARO, A. Update on the treatment of androgenetic alopecia. **European Review for Medical and Pharmacological Sciences**, v. 20, p. 54-58, 2016. Disponível em: <https://www.europeanreview.org/article/10128>. Acesso em: 07 fev 2019. PMID: 26813453.

ROSSI, A. *et al*. Comparative effectiveness of finasteride vs Serenoa repens in male androgenetic alopecia: a two-year study. **International Journal of Immunopathology *and* Pharmacology**, v. 25, p. 1167-1173, 2012. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/23298508>. Acesso em: 07 fev. 2019. DOI: 10.1177/039463201202500435.

ROSSI, A. *et al.* Multi-therapies in androgenetic alopecia: review and clinical experiences. **Dermatologic Therapy**, v. 29, p. 424-432, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/27424565>. Acesso em: 07 fev. 2019. DOI: 10.1111/dth.12390.

ROY, R. K.; THAKUR, M.; DIXIT, V. K. Hair growth promoting effect activity of *Eclipta alba* in male albino rats. **Archives of Dermatological Research**, v. 300, p. 357-364, 2008. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/18478241>. Acesso em: 07 fev. 2019. DOI: 10.1007/s00403-008-0860-3.

SAYEED, M. S. B. *et al.* Critical Analysis on Characterization, Systemic Effect, and Therapeutic Potential of Beta-Sitosterol: A Plant-Derived Orphan Phytosterol. **Medicines (Basel),** v. 3, p. E29, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/28930139>. Acesso em: 07 fev. 2019. DOI: 10.3390/medicines3040029.

SIAH, T. W., MUIR-GREEN, L.; SHAPIRO, J. Female pattern hair loss: a retrospective study in a tertiary referral center. **International Journal of Trichology**, v. *8*, p. 57–61, 2016. Disponível em: <https://doi.org/10.4103/0974-7753.188033>. Acesso em: 07 fev. 2019. DOI: doi: 10.4103/0974-7753.188033.

SINCLAIR, R. Androgenetic alopecia. Modeling progression and regrowth. **Experimental Dermatology**, v. 25, p. 424-425, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/27061176>. Acesso em: 07 fev. 2019. DOI: 10.1111/exd.13029.

SHAMMA, R. N.; ABURAHMA, M. H. Follicular delivery of spironolactone via nanostructured lipid carriers for management of alopecia. **International Journal of Nanomedicine**, v. 9, p. 5449-5460, 2014. Disponível em: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4251754/>. Acesso em: 07 fev. 2019. DOI: 10.2147/IJN.S73010.

TANAMACHI, H. *et al.* 18-MEA and hair appearance. **Journal of Cosmetic Science**, v. 61, p. 147-160, 2010. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/20447366>. Acesso em: 07 fev. 2019. PMID: 20447366.

TORCHILIN, V. P. Multifuncional nanocarries. **Advanced Drug Delivery Reviews**, v. 64, p. 302-315, 2012. Disponível em: <https://www.sciencedirect.com/science/article/pii/S0169409X12002918>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.addr.2012.09.031.

TRÜEB, R. M. Molecular mechanisms of androgenic alopecia. **Experimental Gerontology,** v. 37, p. 981-990, 2002. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/12213548>. Acesso em: 07 fev. 2019. PMID: 12213548.

UPADHYAY, K.; GUPTA, N. K.; DIXIT, V. K. Development and characterization of phyto-vesicles of β-sitosterol for the treatment of androgenetic alopecia. **Archives of Dermatological Research**, v. 304, p. 511–519, 2012. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/22160579>. Acesso em: 07 fev. 2019. DOI: 10.1007/s00403-011-1199-8.

WEBER, J. *et al.* Association of borage oil and betamethasone dipropionate in lipid-core nanocapsules: characterization, photostability and in vitro irritation test. **Journal of Nanoscience and Nanotechnology,** v. 16, p. 1354-1362, 2016. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/27433586>. Acesso em: 07 fev. 2019. DOI: 10.1166/jnn.2016.11674.

WEI, G.; BHUSHAN, B.; TORGERSON, P. M. Nanomechanical characterization of human hair using nanoindentation and SEM. **Ultramicroscopy**, v. 105, p. 248-266, 2005. Disponível em: <https://www.sciencedirect.com/science/article/pii/S0304399105001464>. Acesso em: 07 fev. 2019. DOI: 10.1016/j.ultramic.2005.06.033.

YAZDANI-ARAZI, S. N. *et al.* Histological evaluation of follicular delivery of arginine via nanostructured lipid carriers: a novel potential approach for the treatment of alopecia. **Artificial Cells, Nanomedicine and Biotechnology**, v. 45, p. 1379-1387. Disponível em: <https://www.ncbi.nlm.nih.gov/pubmed/27797271>. Acesso em: 07 fev. 2019. DOI: 10.1080/21691401.2016.1241794.

APÊNDICE A – TERMO DE CONSENTIMENTO LIVRE ESCLARECIDO

UNIVERSIDADE FEDERAL DE SANTA MARIA
PROGRAMA DE PÓS GRADUAÇÃO EM NANOTECNOLOGIA FARMACÊUTICA

Termo de Consentimento Livre e Esclarecido (TCLE)

Título do projeto: Desenvolvimento de formulações nanotecnológicas contendo beta-sitosterol e l-carnitina-l-tartarato associadas à laserterapia no tratamento de alopecia androgenética

Pesquisador responsável: Cristiane de Bona da Silva (orientadora) e Tatiele Katzer (doutoranda)

Instituição/Departamento: Universidade Federal de Santa Maria (UFSM), Programa de Pós Graduação em Nanotecnologia Farmacêutica

Endereço: Av. Roraima, 1000, prédio 26, sala 1144 – Telefones: (55) 3220-8452; 3220-9576

I. Justificativa e objetivos da pesquisa:
Os cabelos são de extrema relevância para homens e mulheres, tanto do ponto de vista de saúde quanto estético. Frente ao limitado número de opções terapêuticas para contornar os sinais e sintomas da calvície e os efeitos adversos a elas associados, faz-se necessário o desenvolvimento de novas estratégias terapêuticas.

II. Procedimentos a serem utilizados:
Você está sendo convidado a participar de um estudo que procura avaliar a eficácia de uma nova combinação terapêutica para o tratamento da calvície, associando a nanotecnologia e a laserterapia. Para analisar a interação entre as nanopartículas desenvolvidas e a fibra capilar, será necessária uma mecha de cabelos não processados quimicamente. Por isso, caso você aceite participar deste estudo, sua contribuição será única e exclusivamente a doação de uma mecha do seu cabelo (área de 1 cm^2, no alto do couro cabeludo – região parietal, cortada rente ao couro cabeludo), uma única vez. A coleta deste material levará, no máximo, 10 minutos, será indolor (sem cortes ou lesões à pele) e será feita na sala 1106, do prédio 26 (Centro de Ciências da Saúde), da UFSM, campus Camobi. O risco de sua participação é mínimo, pois apenas terá que aguardar o tempo para a realização do procedimento. O benefício será indireto, através do conhecimento gerado.

Eu, Tatiele Katzer, declaro que li e fui informado, de forma clara e detalhada, livre de qualquer forma de constrangimento e tentativas de convencimento, dos objetivos deste estudo, dos procedimentos a que serei submetido, dos riscos e desconfortos e dos possíveis benefícios, todos acima listados, assim como da garantia de receber resposta a qualquer pergunta ou dúvida acerca do procedimento em qualquer momento desta pesquisa, bem como da liberdade de retirar meu consentimento a qualquer momento e deixar de participar do estudo, sem que isto leve a qualquer penalidade ou que tenha que justificar a

minha decisão. Concordo que a amostra cedida (mecha de cabelo) seja manuseada conforme as necessidades da pesquisa e fotografada, dando total direito ao profissional para publicá-las em artigos, livros, revistas e em vários outros veículos de divulgação, desde que seja mantido sigilo sobre o meu nome e que tal procedimento não venha causar qualquer tipo de dolo à minha pessoa.

O presente documento foi assinado em duas vias de igual teor, onde uma fica sob os cuidados do participante da pesquisa e outra com a coordenadora da pesquisa, professora Cristiane de Bona da Silva.

A orientadora e coordenadora da pesquisa, profa. Cristiane de Bona da Silva, poderá ser contatada pelo telefone 55 991379447 para eventuais dúvidas.

Declaro que li e entendi as informações acima descritas.

Tatiele Katzer
Nome do Participante
RG: 3095037093

Cristiane de Bona da Silva
Pesquisador

Santa Maria, 05/04/17.

APÊNDICE B – INTERAÇÃO ENTRE NANOPARTÍCULAS E A FIBRA CAPILAR

Para analisar como as nanopartículas interagem com a fibra capilar, a morfologia do cabelo foi avaliada antes e após serem postas em contato com as nanoformulações. Uma mecha de cabelos caucasiano, virgem, liso, castanho claro, foi recebida como doação. Foi cortada a 5 cm da raiz, apresentava cerca de 20 cm de comprimento e pesava 2,25 g. Primeiramente, a mecha foi lavada com 1 mL de uma solução a 10% (p/p) de lauril sulfato de sódio, massageada digitalmente durante 1 min e enxaguada com água destilada até que não fosse mais percebida a presença de espuma. Os cabelos foram mantidos à temperatura ambiente para secarem naturalmente e posteriormente a mecha foi armazenada em um saco plástico.

Subamostras de 30 mg deste cabelo foram usadas para realizar o experimento. Os fios de cabelo foram cortados em fragmentos menores (cerca de 5 cm de comprimento) e colocados em contato com 300 µL de nanoformulações (NC-B, NC-BS, CLN-B e CLN-BS, n=1) por 48 h antes da análise por microscopia eletrônica de varredura (MEV) (Jeol, JSM-6060, Tóquio, Japão), realizada no Centro de Microscopia Eletrônica (UFRGS, Brasil). Para analisar a topografia das fibras capilares após a exposição das nanoformulações, os cabelos foram posicionados sobre suportes metálicos, utilizando fita adesiva dupla face de carbono. As amostras foram revestidas com carbono e ouro (revestimento por aspersão BAL-TEC, SCD 050, Macclesfield, Inglaterra), em atmosfera de argônio. As amostras foram observadas a 10 kV para avaliar a interação entre os cabelos e as nanopartículas. Uma fibra capilar sem qualquer tratamento foi analisada para permitir a comparação.

Os cabelos que não receberam o tratamento apresentavam uma superfície homogênea com sobreposição regular de cutículas e sem substâncias particuladas sobre sua estrutura, características esperadas para um cabelo saudável (KALIYADAN *et al*., 2016). Por outro lado, após o contato com as nanocápsulas, é possível verificar partículas esféricas e aglomeradas na superfície do cabelo em uma distribuição heterogênea, com localização preferencial próximo às extremidades da cutícula. Quando tratadas com os CLN distribuíram-se na superfície do cabelo mais uniformemente e com menos agregados. A presença de BS nas formulações não alterou seu perfil de distribuição sobre as fibras capilares.

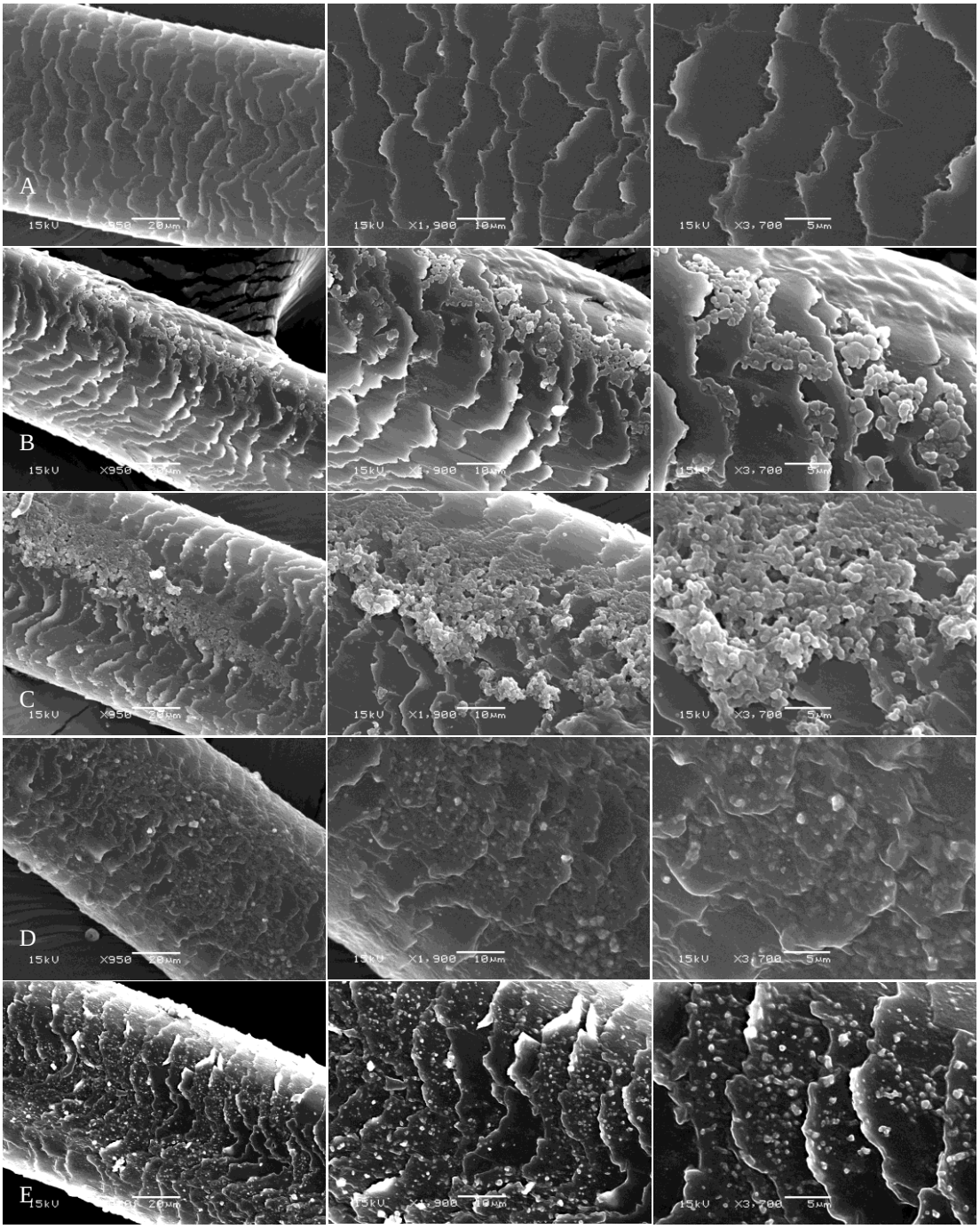

Figura 1: Imagens obtidas por microscopia eletrônica de varredura Scanning electron microscopy images of hair shafts at different magnifications (A) without any treatment, (B) after association with B-NC, (C) BS-NC, (D) B-NLC and (E) BS-NLC.

Dois tipos de interação podem ocorrer entre ingredientes químicos e cabelos: adsorção e absorção. Adsorção é a retenção de ingredientes na superfície do cabelo. A absorção pode ocorrer pela via transcelular (através de células cuticulares, por meio de proteínas de alta e baixa reticulação) e, a via mais comum, por difusão intercelular (entre as células das cutículas, através do complexo de membrana celular e a endocutícula, que apresentam baixo conteúdo de cistina) (WEI; BHUSHAN e TORGERSON, 2005). Para nanopartículas, a difusão

intercelular seria a mais plausível. Além disso, a superfície do cabelo é carregada negativamente, bem como as nanopartículas desenvolvidas. Esta pode ser uma possível explicação para sua distribuição não homogênea, já que cargas iguais se repelem.

Shamma e Aburahma (2014) desenvolveram CLN contendo espironolactona, um fármaco antiandrogênico de prescrição *off label*, por via oral, para o tratamento da AAG em mulheres. Sua aplicação tópica evitaria efeitos adversos sistêmicos. Os pesquisadores observaram a distribuição dos nanocarreadores marcados com fluorescência na pele e nas estruturas foliculares de camundongos e encontraram uma ampla distribuição da fluorescência ao redor dos folículos pilosos e dos fios de cabelo, provavelmente devido ao acúmulo de CLNs nessas áreas. O ducto folicular tem sebo e a afinidade dos CLNs com essa área pode ser, em parte, conseqüência disso. Esta mesma afinidade pode justificar a distribuição mais uniforme dos CLN em relação às NC, pois a fibra capilar saudável é recoberta por moléculas de ácidos graxos, cujo representate majoritário é o ácido 18-metil eicosanóico (TANAMACHI *et al*., 2010).