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CAMILA NUNES LEMOS

Sistemas de liberação para aplicação tópica de peptídeos sob estímulo elétrico para tratamento de feridas

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1.Cicatrização. 2.Iontoforese. 3.Fibroína da seda.

RESUMO

LEMOS, C. N. **Sistemas de liberação para aplicação tópica de peptídeos sob estímulo elétrico para tratamento de feridas**. 2019. 144f. Tese (Doutorado em Ciências). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2019.

O tratamento de feridas crônicas é um grande desafio para a saúde pública na atualidade. Devido à complexidade do processo de cicatrização, envolvendo respostas inflamatórias, de proliferação celular e reconstituição do tecido epitelial, o mercado carece de medicamentos que aliem proteção e modulação desse processo para que as feridas cicatrizem adequadamente. Dentre as diversas estratégias estudadas para modular a cicatrização estão a administração prolongada de peptídeos com ação anti-inflamatória e de proliferação celular, a estimulação elétrica e a aplicação de curativos que protejam a ferida contra infecções e perda de fluidos. O objetivo deste trabalho foi associar, de forma racional, essas estratégias em um único sistema de liberação e verificar sua influência na modulação do processo de cicatrização. Para tanto, filmes de fibroína da seda (FS) carreadores de neurotensina (NT) foram desenvolvidos de forma a sustentar a liberação da NT, proteger a ferida e ainda permitir a aplicação de uma corrente elétrica constante e de baixa intensidade, conhecida como iontoforese. Filmes de FS contendo NT (FS-NT) e glicerina como plastificante foram obtidos por evaporação de solvente. Filmes bilaminados contendo um eletrodo de prata também foram preparados para a aplicação da iontoforese. Os filmes apresentaram-se homogêneos, transparentes, permeáveis ao vapor de água, com baixa capacidade de intumescimento e elevada resistência mecânica. Análises de espectroscopia de infravermelho por transformada de Fourier e calorimetria diferencial sugeriram uma maior organização das fibras de FS do filme em conformação de folhas-β/Seda II. A ocorrência de interações não-covalentes entre NT e FS, capazes de estabilizar a estrutura do filme, foi sugerida por essas análises e confirmada por calorimetria de titulação isotérmica. Assim, o filme de FS-NT sustentou a liberação da NT por até 72 h. Quando aplicada, a iontoforese promoveu uma rápida e alta liberação do fármaco do filme, observada por MALDI *imaging*. Análises por microscopia de força atômica mostraram que na presença do fármaco os filmes apresentaram-se mais rugosos, sugerindo maior aderência ao tecido. Estudos *in vitro* em cultura de macrófagos, fibroblastos e queratinócitos mostraram que tanto os filmes quanto a iontoforese não apresentaram efeitos citotóxicos. Ainda em cultura de macrófagos, foi possível observar que os filmes de FS-NT reduziram significativamente a produção de interleucina 6 e fator de necrose tumoral alfa, o que não foi observado para a solução de NT. A aplicação da iontoforese modulou a secreção dessas citocinas em função do sistema de liberação. A iontoforese anódica, mas não a catódica, foi capaz de inibir o crescimento de bactérias Gram positivas e fungo, mas não teve influência no crescimento de bactérias Gram negativas. *In vivo*, em feridas crônicas induzidas em ratos diabéticos, os filmes de FS protegeram as feridas e suportaram o crescimento de células. Os filmes de FS-NT sustentaram a liberação da NT, modulando a expressão de mediadores pró-inflamatórios, a ativação de macrófagos, de miofibroblastos e de queratinócitos. Associados a iontoforese, os filmes reduziram significativamente a expressão de citocinas pró-inflamatórias, modularam a ativação de neutrófilos e macrófagos, estimularam a angiogênese e a proliferação de fibroblastos. No entanto, quando aplicada na etapa final do processo de cicatrização, principalmente associada a NT, a iontoforese parece ter estimulado excessivamente a proliferação de queratinócitos, sugerindo que seja aplicada apenas na primeira semana do tratamento. A aplicação do filme de FS-NT durante todo o período de cicatrização parece ser importante para a manutenção da modulação desencadeada pela aplicação inicial da iontoforese. Desta forma, os filmes de FS-NT elétrico-estimulados desenvolvidos apresentam-se como uma estratégia promissora para o tratamento tópico de feridas crônicas.

Palavras-chave: Cicatrização. Iontoforese. Fibroína da seda.

ABSTRACT

LEMOS, C. N. **Delivery systems for peptides topical application under electrical stimulation for wounds treatment.** 2019. 144f. Thesis (Doctor of Science). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2019.

The treatment of chronic wounds is still a major public health challenge. Due to the complexity of the healing process, which involves inflammatory responses, cell proliferation and epithelial tissue reconstitution, the market still lacks dressings that combine wound protection and modulation of these processes for proper wounds healing. Strategies that have been studied to modulate wound healing includes the sustained administration of peptides that possess anti-inflammatory and cell proliferating properties, electrical stimulation and application of dressings that protect the wound against infection and fluid loss. The aim of this work was to associate, rationally, these strategies into a single delivery system and verify its influence on the modulation of the wound healing process. To achieve this purpose, neurotensin (NT)-loaded silk fibroin (FS) films were developed to support the release of NT, protect the wound and allow the application of a constant and low intensity of electric current known as iontophoresis. FS films containing NT (FS-NT) and glycerine as plasticizer were developed by film casting, while bi-laminated films containing a silver electrode were also prepared for iontophoresis application. The films were homogeneous, transparent, water vapor permeable, and has low swelling capacity and high mechanical resistance. Fourier transform infrared spectroscopy and differential scanning calorimetry analysis suggested a greater organization of the FS film's fibers into β-sheet/Silk II conformation. The occurrence of non-covalent interactions between NT and FS capable of stabilizing the film structure was suggested by these analyzes and confirmed by isothermal titration calorimetry. FS-NT film sustained the release of NT for up to 72 h, iontophoresis application promoted a fast and high release of NT from the film as observed by MALDI imaging. Atomic force microscopy showed that in the presence of NT, films were rougher, suggesting greater adherence to film structure. *In vitro* studies in macrophages, fibroblasts and keratinocytes showed no cytotoxic effects of the films and iontophoresis. However, FS-NT films significantly reduced interleukin-6 and α-TNF production in macrophages as compared to NT solution. Iontophoresis application modulated the secretion of these cytokines as a function of the delivery system. Anodic, but not cathodic iontophoresis inhibited the growth of Gram positive bacteria and fungus, but had no influence on the growth of Gram negative bacteria. *In vivo*, in diabetic rat-induced chronic wounds, FS films protected the wounds and supported cell growth. FS-NT films supported NT release by modulating the expression of proinflammatory mediators, macrophages, myofibroblasts and keratinocytes activation. When FS-NT was associated with iontophoresis, the films significantly reduced proinflammatory cytokine expression, modulated neutrophil and macrophage activation, stimulated angiogenesis and fibroblast proliferation. However, when applied in the final stage of the healing process, especially in association with NT, iontophoresis appears to have excessively stimulated keratinocyte proliferation suggesting that its application may be more beneficial in the early stage rather than the later stages of wound healing. The application of FS-NT film throughout the healing period seems to be important for maintaining the modulation triggered by the initial application of iontophoresis. Thus, the developed electric-stimulated FS-NT film is a promising strategy for the topical treatment of chronic wounds.

Keywords: Healing. Iontophoresis. Silk fibroin.

1. INTRODUÇÃO

Feridas crônicas, de difícil cicatrização, incluindo úlceras de pressão, venosas e aquelas decorrentes da Diabetes *mellitus* (DM) são um problema de saúde mundial (JAMES et al., 2008; YANG et al., 2016). Elas ocorrem devido a falhas na evolução de uma ou várias etapas do processo de cicatrização fisiológico e, quando finalmente fecham resultam em cicatrizes esteticamente desagradáveis e até em invalidez de partes do corpo, com impactos na economia, na saúde e na qualidade de vida do paciente.

As feridas crônicas ocorrem corriqueiramente em indivíduos com idade avançada, obesos, imunossuprimidos e diabéticos. O processo de cicatrização nessas situações apresenta velocidade similar de progressão e de reparo (STEED, 2003), além de ambiente excessivamente inflamado, com predomínio de neutrófilos que preservam essa inflamação (HU; LAN, 2016). Para reverter esse processo e permitir a cicatrização dessas feridas há a necessidade de modular o processo de cicatrização com estratégias inteligentes. Desta forma, várias abordagens terapêuticas têm sido estudadas para um tratamento mais rápido e efetivo das feridas crônicas.

A terapia tópica é a abordagem mais comum e menos invasiva. Ela possui algumas premissas básicas, como estimular o processo de cicatrização, que é complexo e dinâmico, promover a melhoria da qualidade de vida do paciente, limitar infecções, proteger a ferida da perda de fluidos e proteínas e dissipar o estresse mecânico (BOATENG et al., 2008).

Filmes são sistemas de liberação de fármacos considerados bons candidatos para desempenhar essas funções, mas a grande maioria dos curativos tradicionais presentes no mercado foram desenvolvidos apenas para cobrir a ferida, sem exercer influência significativa no processo de cicatrização. Por isso, o tratamento tópico envolve tradicionalmente, antes da aplicação do curativo, a administração, na ferida limpa, de uma formulação semissólida que contém substâncias destinadas a estimular o processo de cicatrização ou com ação antimicrobiana. Sobre essa formulação, que precisa ser aplicada várias vezes ao dia, é colocado um revestimento (curativo), o qual deve ser impermeável a bactérias e removível sem provocar trauma, que tem a função de manter alta a umidade na interface ferida/curativo, de permitir a troca gasosa e de

fornecer isolamento térmico (KAMOUN; KENAWY; CHEN, 2017; ZARRINTAJ et al., 2017).

O processo de cicatrização fisiológico envolve a ação simultânea de mediadores, células sanguíneas e parenquimais e matriz extracelular. Devido a sua complexidade, as substâncias usadas nas formulações destinadas a acelerar o processo de cicatrização devem idealmente ser capazes de modular esse processo e atuar nas suas 4 fases: homeostática, inflamatória, proliferativa e de remodelamento (MOURA et al., 2013a). Dificilmente, no entanto, uma única substância é capaz de atuar em todas essas etapas. Portanto, mais de uma substância ou estratégias associadas precisam ser empregadas para propiciar uma cicatrização adequada da ferida crônica.

Dentre as várias substâncias que têm mostrado resultados interessantes nessa modulação merecem destaque os neuropeptídeos, dentre eles a neurotensina (NT).

A NT é um neuropeptídeo que participa do controle de várias atividades biológicas, tanto do sistema nervoso central quanto do periférico (KLECZKOWSKA; LIPKOWSKI, 2013). Especificamente na pele, a NT estimula o crescimento celular (SCARPA; CARRAWAY; COCHRANE, 2005) e aumenta a permeabilidade vascular (PEREIRA et al., 2014), funções essas importantes na fase de proliferação do processo de cicatrização. Mas a NT também pode atuar na fase inflamatória, inibindo a cascata de sinalização da via fator nuclear kappa β (NF-kβ), envolvida em diversas respostas imunológicas, e reduzindo a expressão da interleucina 6 (IL-6), interleucina 10 (IL-10) e fator de necrose tumoral α (TNF-α) (PEREIRA et al., 2014). A NT tem mostrado assim, ser capaz de diminuir o estado pró-inflamatório e de atrair fibroblastos, além de aumentar a expressão do fator de crescimento epidérmico (EGF) (PEREIRA et al., 2014). O EGF desempenha papel importante na regeneração e proliferação das células da pele, aumentando a proliferação celular e o acúmulo de colágeno tipo 1α (BALBINO; PEREIRA, LEONARDO MADEIRA; CURI, 2005).

Sendo assim, de acordo com as evidências, a NT influencia no processo de cicatrização de feridas crônicas, visto que pode reduzir a quimiotaxia de células inflamatórias e o próprio estado pró-inflamatório, possibilitando a transição para as fases de proliferação e de remodelamento (PEREIRA et al., 2014) e estimulando a produção de células e mediadores importantes para essas fases (RAMOT; PAUS, 2014).

Para além do uso de substâncias químicas ou biológicas, a estimulação elétrica é outra estratégia que pode contribuir na modulação do processo de cicatrização em feridas crônicas (ISSEROFF; DAHLE, 2012; CASSETTARI et al., 2014; FARBER et al., 2014; KLOTH, 2014; POLAK; FRANEK; TARADAJ, 2014). Já foi demonstrado, por exemplo, que a aplicação de uma corrente elétrica, seja ela pulsátil ou contínua, com densidade de 10-100 μA/cm^2 promove a migração de fibrobrastos e queratinócitos (ZHAO, 2009; KLOTH, 2014), podendo, portanto, modular a fase inflamatória e afetar a fase proliferativa do processo de cicatrização.

Tanto a aplicação da NT quanto a da corrente elétrica requerem, no entanto, sistemas de liberação que permitam sua administração tópica adequada. Por ser um peptídeo, a NT tem meia-vida curta, susceptível a ação das peptidases presentes no ambiente inflamado da ferida e propícia a perder rapidamente sua atividade (SWEITZER, SARAH M.; FANN, STEPHEN A.; BORG, THOMAS K.; BAYNES, JOHN W.; YOST, 2006). Sistemas de liberação capazes de sustentar a liberação da NT são, dessa forma, convenientes para protegê-la da degradação e propiciar sua entrega por períodos prolongados, em várias etapas da cicatrização. A aplicação do estímulo elétrico também requer uma plataforma adequada, que permita a passagem e distribuição da corrente elétrica nas etapas em que possa ser necessária.

A hipótese desse trabalho baseia-se, portanto, na premissa de que o desenvolvimento de um filme que permita a aplicação da corrente elétrica e a liberação sustentada da NT possa servir como um curativo promissor para o tratamento de feridas crônicas.

Para tanto, o filme deve ter características que sirvam ao tratamento e a proteção da ferida. O material que o compõe, dessa forma, requer propriedades que permitam que esse propósito seja alcançado. Dentre os materiais capazes de formar filme e com características promissoras para servir de plataforma curativa está a fibroína da seda (FS).

A FS é uma proteína fibrosa presente no casulo do bicho da seda (*Bomby mori*) (ALTMAN, GREGORY H.; DIAZ, FRANK; JAKUBA, CAROLINE; CALABRO, TARA; HORAN, REBECA L.; CHEN, JINGSONG; LU, HELEN; RICHMOND, JOHN; KAPLAN, 2003). É um biopolímero natural que apresenta baixa reação inflamatória, permeabilidade ao oxigênio e vapor d'água, biocompatibilidade e biodegradabilidade, além de resistência mecânica (WENK; MERKLE; MEINEL, 2011). Estudos revelam ainda que a FS interage com algumas células presentes na pele, como fibroblastos

(SERVOLI et al., 2005), queratinócitos (GUPTA et al., 2007) e células endoteliais (FUCHS et al., 2006). Sendo assim, ela vem sendo utilizada como *scaffold* para a reposição de diversos tecidos (MELKE et al., 2016; ZHOU et al., 2017, 2019; BANDYOPADHYAY; MANDAL, 2019). Também serve como base para o preparo de diversas formas farmacêuticas, como filmes, géis, espumas, nano e/ ou micropartículas (WENK; MERKLE; MEINEL, 2011; KUNDU et al., 2013). Desta forma, pretende-se nesse trabalho desenvolver um curativo a base de FS que sirva como sistema de liberação sustentada da NT, plataforma para aplicação de um estímulo elétrico e *scaffold* para a regeneração da pele ferida.

7. CONCLUSÃO

A metodologia para obtenção de dispersão aquosa de FS a partir de casulos de *Bombyx mori* foi padronizada e permitiu a formação de filmes transparentes e homogêneos, com predominância de folhas-β, baixa hidratação, permeabilidade ao vapor de água e propriedades mecânicas adequadas para administração tópica e proteção de feridas. Os filmes de FS foram ainda capazes de incorporar a NT e de sustentar sua liberação, além de servirem como uma plataforma para aplicação de iontoforese. Essa, por sua vez, apresentou efeito bacteriostático contra microrganismos Gram positivos e modulou a expressão de citocinas pró-inflamatórias quando administrada associada ao filme de FS-NT *in vitro*. Aplicados em feridas crônicas induzidas em ratos, confirmou-se a capacidade dos filmes de FS-NT elétrico-estimulados de diminuírem o processo inflamatório e permitirem a proliferação modulada de fibroblastos e queratinócitos. A investigação do efeito dos tratamentos em dias chaves permitiu compreender a influência do filme de FS, da NT e da iontoforese em todas as etapas do processo de cicatrização. Desta forma, pode-se afirmar que a iontoforese deve ser associada aos filmes na primeira semana de tratamento, mas é prudente não a utilizar nas etapas finais para evitar a proliferação excessiva de queratinócitos e, por consequência, a formação de uma cicatriz patológica. Os filmes de FS-NT deram suporte ao crescimento de células, além da modulação do processo de cicatrização. Estimulados pela iontoforese nas etapas iniciais desse processo, são promissores curativos para o tratamento de feridas crônicas.

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