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**Iontoforese de nanodispersões visando a imunoterapia
associada à terapia fotodinâmica tópica do melanoma**

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**Iontoforese de nanodispersões visando a imunoterapia associada à
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RESUMO

DALMOLIN, Luciana Facco. **Iontoforese de nanodispersões visando a imunoterapia associada à terapia fotodinâmica tópica do melanoma**. 2019. 137 f. Tese (Doutorado em Ciências) – Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2019.

A associação da terapia fotodinâmica (TFD) e da imunoterapia foi a estratégia investigada neste trabalho para o tratamento tópico do melanoma. Para viabilizar a administração do fotossensibilizante lipofílico usado na TFD, a zinco ftalocianina (ZnF), na mesma formulação que o anticorpo monoclonal anti-fator de crescimento endotelial vascular, o bevacizumabe, foi desenvolvida uma nanoemulsão. A influência da nanoemulsão contendo os fármacos na cito e fototoxicidade de células de melanoma e na inibição da proliferação de células do endotélio foi verificada *in vitro*. A influência da iontoforese da nanoemulsão na permeação cutânea desses fármacos e distribuição nas células tumorais foi investigada *in vitro*, em pele de porco, e *in vivo* em melanoma induzido em camundongo. Neste último, a eficiência da TFD e da imunoterapia no crescimento tumoral e na metástase foi investigada após administração via intratumoral e tópica por iontoforese da nanoemulsão. A nanoemulsão O/A desenvolvida apresentou-se estável frente a aplicação da corrente elétrica, capacidade de solubilizar mais de 40 µg/mL da ZnF e de diminuir a agregação da ZnF em meio biológico. Em células de melanoma B16F10, a ZnF mostrou maior potencial fototóxico quando incorporada na nanoemulsão do que quando em solução, apresentando IC₅₀ de 192±6 ng/mL após irradiação, sendo que em associação ao bevacizumabe foi observada uma redução nesse valor para 56±7 ng/mL. Além disso, a nanoemulsão foi capaz de aumentar em 4 vezes o *uptake* celular da ZnF quando comparado à solução. O potencial anti-angiogênico do bevacizumabe incorporado na nanoemulsão, avaliado em células endoteliais CRL-2922, foi confirmado pela sua capacidade de inibir a migração celular de forma mais efetiva do que o fármaco em solução após 24 h de tratamento. *In vitro*, a nanoemulsão aumentou a penetração da ZnF na pele quando comparada a uma emulsão de composição idêntica. Na presença de apenas 30 min de iontoforese, a nanoemulsão aumentou em quase 16 vezes a penetração da ZnF nas camadas mais profundas da pele quando comparada a iontoforese da emulsão. *In vivo*, este período de aplicação permitiu que a ZnF alcançasse o melanoma induzido sob a pele de camundongos C57BL6. O tratamento desse tumor por via intratumoral e iontoforética com a nanoemulsão de ZnF e bevacizumabe seguido da TFD diminuiu em aproximadamente 8 vezes o crescimento tumoral em relação ao controle. A aplicação tópica com iontoforese se mostrou tão ou mais efetiva do que a administração intratumoral da nanoemulsão, uma vez que a inibição do crescimento tumoral foi observada após um período mais curto de tratamento quando a nanoemulsão foi administrada por iontoforese do que por via intratumoral. Os animais tratados por ambas as vias com a nanoemulsão de ZnF e bevacizumabe seguido da TFD não apresentaram metástases, diferentemente dos demais. A análise histológica dos tumores revelou a presença de células apoptóticas nos grupos tratados com a nanoemulsão contendo os fármacos, indicando que este foi um dos mecanismos responsáveis pelo efeito obtido. Desta forma, a iontoforese da nanoemulsão de ZnF e bevacizumabe seguido da TFD demonstrou ser uma estratégia promissora para o tratamento tópico do melanoma.

Palavras-chave: Nanoemulsão. Melanoma. Terapia Fotodinâmica. Terapia Anti-angiogênica. Iontoforese.

ABSTRACT

DALMOLIN, Luciana Facco. **Iontophoresis-facilitated nano-dispersion delivery of immunotherapy associated with topical photodynamic therapy for treatment of melanoma**. 2019. 137 f. Thesis (Doctor of Science) – Faculty of Pharmaceutical Sciences in Ribeirão Preto, University of São Paulo, Ribeirão Preto, 2019.

The association of photodynamic therapy (PDT) and immunotherapy as a strategy for the topical treatment of melanoma was investigated in this work. An O/A nanoemulsion was developed to facilitate the administration of a photosensitizer, zinc phthalocyanine (ZnF) for PDT and anti-vascular endothelial growth factor monoclonal antibody, bevacizumab, for immunotherapy. The cytotoxicity and phototoxicity of drug-loaded nanoemulsion on melanoma cells and inhibition of endothelial cell proliferation was evaluated *in vitro*. The influence of iontophoresis on the cutaneous permeation of loaded drugs in the nanoemulsion and distribution in tumor cells was investigated *in vitro* in pig skin and *in vivo* in mouse-induced melanoma. In the latter, the efficiency of PDT and immunotherapy on tumor growth and metastasis was investigated following intratumoral and iontophoretic topical administration of nanoemulsion. The O/A nanoemulsion developed was stable under the application of electric current, solubilized more than 40 µg/mL of ZnF and decreased the aggregation of ZnF in biological medium. In B16F10 melanoma cells, ZnF-loaded nanoemulsion showed a higher phototoxic potential than ZnF solution with an IC₅₀ of 192 ± 6 ng/mL after irradiation, while in combination with bevacizumab, a further reduction of IC₅₀ to 56 ± 7 ng/mL was observed. In addition, the nanoemulsion increased by 4 folds, the cellular uptake of ZnF when compared to ZnF solution. The anti-angiogenic potential of nanoemulsion incorporated bevacizumab, evaluated in CRL-2922 endothelial cells, was confirmed by its ability to inhibit cell migration more effectively than the drug in solution after 24 h of treatment. *In vitro*, nanoemulsion increased ZnF skin penetration when compared to an emulsion of identical composition. With the application of only 30 min iontophoresis, nanoemulsion increased by almost 16 folds the penetration of ZnF into the deeper layers of the skin when compared to iontophoresis of the emulsion. *In vivo*, 30 min iontophoresis application allowed ZnF to reach melanoma induced under the skin of C57BL6 mice. Intratumoral and iontophoretic treatment of the induced tumor with the ZnF and bevacizumab loaded nanoemulsion followed by PDT decreased tumor growth by approximately 8 folds in comparison to control. Topical application through iontophoresis was observed to be as or more effective than intratumoral administration of nanoemulsion, as the inhibition of tumor growth was observed within a shorter period of iontophoretic nanoemulsion delivery rather than intratumoral treatment. Animals treated with both ZnF and bevacizumab loaded nanoemulsion followed by PDT showed no metastasis, unlike other treatment groups. Histological analysis of tumors revealed the presence of apoptotic cells in the groups treated with drug-loaded nanoemulsion, indicating apoptosis as one of the mechanisms responsible for the effect observed. Thus, iontophoretic delivery of ZnF and bevacizumab loaded nanoemulsion followed by PDT was shown to be a promising strategy for the topical treatment of melanoma.

Keywords: Nanoemulsion. Melanoma. Photodynamic Therapy. Anti-angiogenic therapy. Iontophoresis.

INTRODUÇÃO

O melanoma cutâneo é o tipo mais agressivo de câncer de pele. Apesar de sua incidência ser considerada baixa em relação aos outros tipos de câncer de pele, seus números triplicaram nas últimas três décadas provocando uma situação alarmante, visto que sua taxa de letalidade é bastante elevada em função da rápida progressão e disseminação da doença para outros órgãos (INCA, 2018).

Os tratamentos convencionais disponíveis incluem a intervenção cirúrgica, a radioterapia e a quimioterapia, as quais podem ser combinadas. Existem muitos problemas associados a estes tipos de tratamento, como os graves efeitos adversos sistêmicos e a resistência aos fármacos (CHIN; MERLINO; DEPINHO, 1998; MONGE-FUENTES; MUEHLMANN; DE AZEVEDO, 2014). Neste contexto, na tentativa de contornar esses problemas, novas alternativas têm sido exploradas, dentre elas a imunoterapia e a terapia fotodinâmica (TFD).

A imunoterapia baseia-se na administração de citocinas (interferons e interleucinas) e/ou anticorpos monoclonais que estimulam o sistema imunológico, modificando assim a resposta biológica. O bevacizumabe é um anticorpo monoclonal que vem sendo avaliado e utilizado na imunoterapia de diversos tipos de câncer em associação com outros quimioterápicos (FUJITA et al., 2007; GERBER; FERRARA, 2005; LEE et al., 2008). Conhecido comercialmente como Avastin, o bevacizumabe foi o primeiro agente anti-angiogênico aprovado pelo *Food and Drug Administration* (FDA) para uso intravenoso no tratamento do câncer colorretal metastático em humanos (FERRARA et al., 2004). No tratamento do melanoma, pelo menos dois estudos de fase II mostraram um aumento na taxa de resposta objetiva e de sobrevida global dos pacientes quando o bevacizumabe foi administrado por via endovenosa em associação com carboplatina e paclitaxel (PEREZ et al., 2009) e com interferon- α (VARKER et al., 2007).

Apesar das evidências da efetividade da associação do bevacizumabe com outras terapias, um grande problema associado a sua administração sistêmica é a ocorrência de efeitos adversos como proteinúria, hipertensão, hemorragia, epistaxe, má cicatrização de feridas e o tromboembolismo venoso e arterial (FERRARA et al., 2004; GERBER; FERRARA, 2005; KIM et al., 2012).

A utilização da via tópica para administração do bevacizumabe vem a ser uma

estratégia interessante para tratar tumores de pele, pois pode proporcionar uma diminuição destes efeitos adversos e uma liberação direta do anticorpo no local pretendido. No entanto, a camada mais externa da pele, o estrato córneo, constitui uma eficiente barreira para a absorção de substâncias tóxicas do ambiente e também para a penetração de fármacos (JEPPS et al., 2013). A penetração cutânea passiva do bevacizumabe, que é uma macromolécula de caráter hidrofílico, até as camadas da pele onde as células tumorais estão localizadas é, portanto, dificultada pelo estrato córneo.

A iontoforese é um método físico que tem sido utilizado com o objetivo de aumentar a penetração de fármacos através do estrato córneo. Ela é uma técnica não-invasiva que utiliza uma corrente elétrica fraca, de até 0,5 mA/cm² (KALIA et al., 2004). Especificamente, no tratamento do câncer de pele, a iontoforese tem sido utilizada para aumentar a penetração de quimioterápicos (HUBER et al., 2015; TAVEIRA et al., 2014) e de agentes fotossensibilizantes (FSs) utilizados na TFD (GELFUSO et al., 2008, 2011; LEMOS et al., 2016; LOPEZ et al., 2001).

A TFD tópica é uma modalidade terapêutica que vem sendo largamente utilizada para várias doenças cutâneas, inclusive no tratamento do melanoma (BEACK et al., 2015; MICHAILOV et al., 1997). Na TFD tópica, o FS deve penetrar na pele e acumular-se nas células tumorais. A pele é então exposta à luz no comprimento de onda adequado de acordo com o espectro de absorção do FS. O FS absorve fótons e os fotoprodutos gerados por este processo (espécies reativas de oxigênio e oxigênio singlete) levam a morte das células tumorais por meio de uma série de reações fotoquímicas (DOLMANS; FUKUMURA; JAIN, 2003). Os principais eventos biológicos provocados pela TFD que são de grande relevância na terapia antitumoral são: morte direta das células tumorais por necrose, apoptose ou autofagia, isquemia tumoral seguida de danos vasculares induzidos pela TFD e ativação do sistema imune contra os antígenos tumorais (AGOSTINIS et al., 2011).

Entretanto, a aplicação da TFD deve utilizar comprimentos de onda da luz em que o tecido biológico seja mais transparente a radiação luminosa, uma vez que componentes do próprio tecido, como a melanina, podem competir com o agente fotossensibilizante pela absorção da luz. Devido a isso, gerações modernas de FS, que absorvem luz em comprimentos de onda entre 600 e 850 nm (considerada a janela terapêutica da TFD), têm sido preferidas para o emprego no tratamento do

melanoma (MACDONALD; DOUGHERTY, 2001). É o caso da zinco ftalocianina (ZnF), principal FS de segunda geração, que exibe um elevado efeito fotodinâmico (SHAO et al., 2013). Entretanto, a ZnF é uma molécula extremamente lipofílica, o que acaba dificultando seu uso clínico e a aplicação da iontoforese (GELFUSO et al., 2011).

Os benefícios da TFD com a administração tópica da ZnF podem ser aliados aos benefícios da ação do bevacizumabe no tratamento do melanoma, uma vez que a TFD deve atuar matando as células tumorais já instaladas no tecido e o bevacizumabe deve atuar impedindo o crescimento do tumor. Para isso, é necessária a utilização de um sistema de liberação capaz de possibilitar a administração dos dois fármacos associados, auxiliar na penetração na pele e ainda permitir a aplicação da iontoforese, necessária para direcionar os fármacos para as células tumorais e permitir que altas concentrações dos mesmos cheguem às camadas da pele onde estas células estão localizadas.

As nanoemulsões óleo-em-água (O/A) têm sido propostas como potenciais sistemas nanodispersos para a veiculação de moléculas lipofílicas e de macromoléculas como anticorpos (HAGIGIT et al., 2012; RAI et al., 2018). Devido as gotículas nanométricas da fase oleosa que as compõem (entre 50 e 500 nm), nanoemulsões são sistemas altamente estáveis e de natureza fluida. Quando aplicada topicamente, a nanoemulsão pode interagir com a barreira do estrato córneo, desorganizando-a e permitindo desta forma o aumento da penetração e partição dos fármacos que ela carrega para as camadas mais profundas da pele (MD et al., 2017; RAI et al., 2018). Ainda, nanoemulsões O/A podem permitir a passagem de corrente elétrica, possibilitando assim a aplicação da iontoforese, melhoram a estabilidade de fármacos e são capazes de veicular altas concentrações de substâncias lipofílicas, como a ZnF (SIBATA; TEDESCO; MARCHETTI, 2004; YU et al., 2018).

Portanto, o presente trabalho visa o desenvolvimento de nanoemulsões contendo bevacizumabe e ZnF para administração por iontoforese visando o tratamento tópico de melanoma cutâneo por imunoterapia e TFD.

CONCLUSÃO

Uma nanoemulsão O/A catiônica com alta concentração de óleo e baixa concentração de tensoativos foi desenvolvida com sucesso por emulsificação ultrassônica, apresentando tamanho médio inferior a 200 nm, baixa polidispersividade e estabilidade frente a corrente elétrica. A nanoemulsão foi capaz de incorporar o bevacizumabe e até 50 $\mu\text{g/mL}$ de ZnF, a qual foi quantificada seletivamente também na pele por um método espectrofluorimétrico validado. Em células de melanoma B16F10, a ZnF demonstrou ter maior potencial fototóxico quando incorporada na nanoemulsão e a associação do bevacizumabe acentuou ainda mais esse efeito, já que o valor de IC50 obtido para a nanoemulsão contendo ZnF e bevacizumabe após irradiação foi pelo menos 3 vezes menor do que o valor de IC50 da nanoemulsão contendo apenas ZnF. Além disso, a veiculação pela nanoemulsão aumentou em 4 vezes a internalização celular da ZnF pelas células tumorais, quando comparado ao fármaco em solução. A incorporação do bevacizumabe na nanoemulsão possibilitou que este fármaco inibisse a migração de células do endotélio mesmo após longo período em contato com as células, evitando sua rápida degradação. A iontoforese da nanoemulsão aumentou a permeação da ZnF na pele em relação ao controle (emulsão contendo os mesmos componentes da nanoemulsão) e permitiu que a ZnF alcançasse o melanoma induzido em camundongos após 30 min de aplicação tópica. A contribuição da iontoforese da nanoemulsão para a permeação cutânea da ZnF e do bevacizumabe resultou numa resposta biológica ao tratamento do melanoma, seguido ou não da TFD, até mesmo mais promissora do que a injeção intratumoral da formulação, uma vez que a inibição do crescimento tumoral se deu mais rapidamente após administração da nanoemulsão por iontoforese do que por via intratumoral. A análise histológica dos tumores tratados indicou que o mecanismo de inibição do crescimento tumoral após a TFD aliada a imunoterapia está relacionado a apoptose das células tumorais. Desta forma, a administração iontoforética da associação de ZnF e de bevacizumabe possibilitada pela nanoemulsão mostrou ser uma alternativa terapêutica promissora para a TFD e imunoterapia do melanoma cutâneo.

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