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FORMULAÇÕES NANOESTRUTURADAS CONTENDO A ASSOCIAÇÃO COENZIMA Q10 E VITAMINA E ACETATO: DESENVOLVIMENTO E AVALIAÇÃO DE ATIVIDADES BIOLÓGICAS

Santa Maria, RS, Brasil
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Dissertação apresentada ao Curso de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração em Desenvolvimento e Avaliação de Produtos Farmacêuticos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Ciências Farmacêuticas.**

Orientadora: Profa. Dra. Letícia Cruz

Co-orientadora: Profa. Dra. Sara Marchesan de Oliveira

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2016

Dedico esta dissertação à minha família,
especialmente à minha mãe e irmã,
por todo o apoio e amor incondicional
a mim dedicados.

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RESUMO

FORMULAÇÕES NANOESTRUTURADAS CONTENDO A ASSOCIAÇÃO COENZIMA Q10 E VITAMINA E ACETATO: DESENVOLVIMENTO E AVALIAÇÃO DE ATIVIDADES BIOLÓGICAS

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A coenzima Q10 é uma molécula sintetizada endogenamente que apresenta atividade antioxidante e participação no processo de respiração celular e síntese de ATP. Além disso, possui atividade neuroprotetora, anti-inflamatória e efeito hepatoprotetor, dentre outros. No entanto, constitui-se de uma molécula de alto peso molecular, baixa solubilidade em água e, consequentemente, baixa biodisponibilidade quando administrada por via oral, além de ser fotolábil, o que dificulta sua utilização terapêutica. Neste sentido, nanocarreadores podem constituir uma interessante alternativa na veiculação desta substância ativa. No primeiro momento, este trabalho objetivou o desenvolvimento de nanocápsulas de poli-ε-caprolactona contendo a associação de dois antioxidantes, coenzima Q10 e vitamina E acetato, e a avaliação da fotodegradação da coenzima Q10 associada às nanoestruturas e do seu efeito antitumoral *in vitro*. Estas suspensões de nanocápsulas apresentaram características físico-químicas satisfatórias para este tipo de sistema de liberação de fármacos, altas taxas de eficiência de encapsulamento e de teor de fármaco. Ainda, verificou-se a influência da concentração de vitamina E sobre a degradação da coenzima Q10 por exposição à radiação ultravioleta do tipo C, a qual foi menor quanto maior a concentração de vitamina E. Ainda, a coenzima Q10 e vitamina E nanoencapsuladas reduziram a viabilidade celular das células tumorais de melanoma (linhagem A375) e glioma humano (linhagem C6). Na segunda abordagem deste trabalho, a partir das suspensões de nanocápsulas, foram preparadas formulações semissólidas pela adição de goma gelana, um polissacarídeo de origem natural. Estas formulações foram, então, utilizadas para a investigação do efeito antiedematogênico, anti-inflamatório e antioxidante em um modelo de edema de orelha induzida por radiação ultravioleta do tipo B (UVB) em camundongos. Todas as formulações testadas, com exceção do veículo (goma gelana e água), levaram à redução do edema de orelha e da atividade das enzimas mieloperoxidase (MPO; indicador da infiltração de neutrófilos) e N-acetil-glucosaminidase (NAGase; marcador da infiltração de macrófagos). Além disso, as formulações semissólidas contendo coenzima Q10 nanoencapsulada levaram ao decréscimo dos níveis de marcadores de estresse oxidativo (níveis de tióis não proteicos e peroxidação lipídica). Considerando os resultados obtidos, as formulações de coenzima Q10 e vitamina E acetato desenvolvidas são uma alternativa interessante para o controle de células tumorais assim como para lesões inflamatórias de pele induzidas por radiação UVB.

Palavras-chaves: nanocápsulas, coenzima Q10, vitamina E acetato, citotoxicidade *in vitro*, atividades biológicas.

ABSTRACT

NANOSTRUCTURED FORMULATIONS CONTAINING THE ASSOCIATION BETWEEN COENZYME Q10 AND VITAMIN E ACETATE: DEVELOPMENT AND EVALUATION OF THE BIOLOGICAL ACTIVITIES

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Coenzyme Q10 is an endogenous synthesized molecule that presents antioxidant activity and acts on cellular respiration process and ATP synthesis. Besides, it presents neuroprotective effect, anti-inflammatory and hepatoprotective effects, among others. However, this molecule has high molecular weight, poor water solubility and, consequently, low bioavailability when administered by the oral route. Besides, coenzyme Q10 is photolabile, that difficults its therapeutic use. In this sense, nanocarriers constitute an interesting alternative to the delivery of this active substance. In the first step, this study aimed the development of poly-ε-caprolactone nanocapsules containing the association of two antioxidant compounds, coenzyme Q10 and vitamin E acetate, and the evaluation of the photodegradation of Q10 associated to nanostructures and their *in vitro* antitumoral effect. This nanocapsule suspensions presented suitable physicochemical characteristics for this type of drug delivery system, high encapsulation efficiency rates and drug content. It was also verified the influence of vitamin E concentration on coenzyme Q10 degradation by ultraviolet C radiation exposure, which was smaller with the increase of vitamin E concentration. Moreover, nanoencapsulated coenzyme Q10 and vitamin E reduced cell viability of tumoral cells melanoma (A375 line) and glioma (C6 line). In the second part of this work, semisolid formulations were prepared from nanocapsule suspensions by the addition of gellan gum, a natural polysaccharide. These formulations were employed in the investigation of the antiedematogenic, anti-inflammatory and antioxidant effects in a model of ear edema induced by ultraviolet radiation type B (UVB) in mice. All formulations tested, excepting the vehicle (gellan gum and water), led to a reduction of the ear edema in mice and in the activities of the enzymes myeloperoxidase (MPO; marker of neutrophil infiltration in the tissue) and β-N-acetylglucosaminidase (NAGase; marker of macrophage infiltration). Besides, semisolid formulations containing nanoencapsulated coenzyme Q10 led to a decrease on oxidative stress biomarkers levels (non-proteic thiols and lipid peroxidation). Considering obtained results, developed formulations of coenzyme Q10 and vitamin E acetate are an interesting alternative to the control of tumoral cells growing, as well as to inflammatory skin wounds induced by UVB radiation.

Keywords: nanocapsules, coenzyme Q10, vitamin E acetate, *in vitro* citotoxicity, biological activities.

LISTA DE ABREVIATURAS E SIGLAS

ATP	adenosina trifosfato
CAT	catalase
CFA	adjuvante completo de Freund (do inglês *Complete Freund's Adjuvant*)
CLN	carreador lipídico nanoestruturado
COX-2	cicloxigenase-2
DMEM	meio Eagle modificado por Dulbecco
DMSO	dimetilsulfóxido
DNA	ácido desoxiribonucléico
DTNB	ácido 3,3'-ditiobis[6-nitrobenzóico]
EDTA	ácido etilenodiaminotetracético
EE	eficiência de encapsulamento
EPR	efeito de permeabilidade e retenção aumentada
ERO's/ROS	espécies reativas de oxigênio
ERN's	espécies reativas de nitrogênio
ES Q10	solução etanólica de coenzima Q10
FBS/SFB	soro fetal bovino
FH	hidrogel contendo coenzima Q10 e vitamina E não-nanoencapsulados
GPx	glutationa peroxidase
H_2O_2	peróxido de hidrogênio
HPLC/CLAE	cromatografia líquida de alta eficiência
K	índice de consistência
MPO	mieloperoxidase
MTX	metotrexato
n	índice de fluxo
NAGase	N-acetil-β-D-glicosaminidase
NBP1	nanocápsula sem coenzima Q10 contendo 1% de vitamina E
NBP2	nanocápsula sem coenzima Q10 contendo 2% de vitamina E
NBP3	nanocápsula sem coenzima Q10 contendo 3% de vitamina E
NCP1	nanocápsula contendo coenzima Q10 e 1% de vitamina E
NCP2	nanocápsula contendo coenzima Q10 e 2% de vitamina E
NCP3	nanocápsula contendo coenzima Q10 e 3% de vitamina E
NLS	nanopartícula lipídica sólida
NO_2	óxido nítrico
NPSH	tiois não-protéicos
$O_2{\cdot}^-$	ânion superóxido
·OH	radical hidroxila
PBS	tampão fosfato-salino
PCL	poli-ε-caprolactona
PdI/IPd	índice de polidispersão
PGD_2	prostaglandina D_2
PGE_2	prostaglandina E_2
PLA	poli (ácido lático)
PLGA	poli (ácido lático-co-glicólico)
Q10	coenzima Q10 ou 3-dimetoxi-5-metil-6-decaprenil-benzoquinona
RL	radical livre
SDS	dodecil sulfato de sódio
SOD	superóxido dismutase

SRB	sulforrodamina B
TBARS	espécies reativas ao ácido tiobarbitúrico
TCA	ácido tricloroacético
TCM	triglicerídeos de cadeia média
TNFα	fator de necrose tumoral alfa
UVA	ultravioleta A
UVB	ultravioleta B
UVC	ultravioleta C
VitE	vitamina E acetato
XTT	2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-2H-tetrazólio-5-carboxanilida

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

PUBLICAÇÃO 1: Improved photostability and antitumor effect of coenzyme Q10 by its association with vitamin E acetate in polymeric nanocapsules

PUBLICAÇÃO 2: Nanoencapsulation of coenzyme Q10 and vitamin E acetate protects against UVB radiation-induced skin injury in mice

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INTRODUÇÃO

A coenzima Q10, que é também conhecida como ubiquinona ou ubidecarenona, é um antioxidante endógeno de caráter lipofílico com função expressiva na cadeia transportadora de elétrons (CRANE et al., 1957). Esta substância lipossolúvel pode ser obtida a partir da dieta, mas também é sintetizada por algumas células do organismo, principalmente em órgãos como fígado, rins, coração e pâncreas. Ela é encontrada principalmente nas mitocôndrias celulares, desempenhando papel importante na produção de ATP. Seu efeito antioxidante tem sido estudado principalmente no desenvolvimento de produtos cosméticos *"anti-aging"* (MÜLLER et al., 2007) e promotores da hidratação da pele (JUNYAPRASERT et al., 2009; PARDEIKE, J.; SCHWABE, K.; MÜLLER, R. H., 2010), assim como as atividades neuroprotetora, antitumoral, anti-inflamatória, além de outras, tem sido investigadas (FULLER et al., 2006; YOUNG et al., 2007; FOUAD; AL-MULHIM; JRESAT, 2013; MACHADO et al., 2013).

Sua molécula é formada por uma quinona, que está ligada a uma cadeia lateral isoprenóide, a qual contém 10 unidades de cinco átomos de carbono cada (CRANE, 2001). A presença desta longa cadeia lateral torna esta coenzima pouco solúvel em água, e sua elevada massa molecular (863,3 g/mol) leva a uma baixa biodisponibilidade oral, fatores que contribuem para a dificuldade da sua utilização terapêutica (SIEKMANN; WESTESEN, 1995; BHAGAVAN; CHOPRA, 2006). Diante disto, e aliado ao fato de ser um composto de caráter fotoinstável (HOPPE et al., 1999; BULE; SINGHAL; KENNEDY, 2010), a coenzima Q10 constitui um potencial fármaco candidato à nanoencapsulação, estratégia que vem sendo utilizada no sentido de contornar obstáculos relacionados à terapêutica de fármacos.

A nanoencapsulação pode conferir vantagens à utilização terapêutica de fármacos, como a vetorização dos mesmos a sítios de ação específicos no organismo e sua liberação sustentada, aumento da eficácia e a redução da toxicidade (MOHANRAJ; CHEN, 2006; REIS et al., 2006; MORA-HUERTAS et al., 2010; ALMOUAZEN et al., 2012). Dentre os sistemas carreadores de fármacos desenvolvidos em nanoescala, encontram-se as nanocápsulas, nanoesferas, nanoemulsões e lipossomas (ANTON; BENOIT; SAULNIER, 2008; VAUTHIER; BOUCHEMAL, 2009; SANTOS et al., 2013).

As nanopartículas poliméricas, que apresentam diâmetro médio geralmente na faixa de 100 a 500 nm, compreendem as nanocápsulas e as nanoesferas (QUINTANAR-GUERRERO et al., 1998; MORA-HUERTAS et al., 2010). Nanoesferas são sistemas matriciais, nos quais o fármaco pode encontrar-se disperso molecularmente, retido fisicamente ou adsorvido no polímero. Nanocápsulas, diferentemente, são sistemas vesiculares nos quais um invólucro polimérico encontra-se disposto ao redor de um núcleo, geralmente oleoso. O fármaco pode

encontrar-se dissolvido neste núcleo e/ou adsorvido à parede polimérica (MEGENHEIM; BENITA, 1991; COUVREUR et al., 2002).

A associação de substâncias antioxidantes pode constituir uma estratégia vantajosa quando se pretende a fotoestabilização de um fármaco. Além disto, esta associação pode também ser útil no sentido de incrementar diferentes atividades biológicas conferidas pelos componentes. Neste contexto, este trabalho propõe a associação de coenzima Q10 e vitamina E acetato em nanocápsulas, visando a avaliação de seu efeito nestas duas abordagens.

A vitamina E é um antioxidante natural que se apresenta sob a forma de tocoferois e tocotrienois. Esta vitamina é obtida da dieta principalmente pela ingestão de amêndoas, nozes, castanha-do-pará, abacate e alguns óleos vegetais (ZIGONEANU et al., 2008; PEH et al., 2015). Ela apresenta importante atividade antioxidante que, aliada ao fato de se apresentar sob a forma de um óleo, vem sendo explorada pelas indústrias farmacêutica e cosmética (CONSTANTINIDES et al., 2006; PARDEIKE, J.; HOMMOSS, A.; MÜLLER, R. H., 2009; NICULAE et al., 2014). Outras atividades biológicas também já foram descritas para a vitamina E, tais como: atividade antioxidante, antiproliferativa, neuroprotetora, entre outras (SHIREEN et al., 2008; JIANG, 2014; PAWAR et al., 2014; ULATOWSKI; MANOR, 2015). Devido ao exposto, a vitamina E acetato foi selecionada para constituir as nanocápsulas preparadas, uma vez que tratando-se de um óleo, pode compor o núcleo oleoso destas nanoestruturas.

Devido às atividades já relatadas para ambos os compostos ativos, a associação dos mesmos poderia levar a uma formulação com potenciais atividades biológicas. Dentre estas, avaliou-se a proteção da fotodegradação da coenzima Q10 nanoencapsulada pela presença da vitamina E acetato e, *in vitro*, a performance antitumoral das mesmas frente a linhagens de melanoma e glioma. Além disso, foram desenvolvidas formulações semissólidas a partir da incorporação de goma gelana às suspensões de nanocápsulas e investigou-se a atividade das mesmas sobre alterações inflamatórias e oxidativas em um modelo de indução de edema de orelha por radiação UVB em camundongos.

Destaca-se a importância deste trabalho para a pesquisa científica e seu caráter inovador, uma vez que, até o presente momento, não foram encontrados estudos na literatura que reportem a associação de coenzima Q10 e vitamina E acetato em nanopartículas poliméricas, tampouco a incorporação de ambos os componentes em nanopartículas e sua adição em formulação semissólida.

1.1 Objetivo geral

Desenvolver nanocápsulas contendo a associação coenzima Q10 e vitamina E acetato e avaliar a fotoestabilidade e a atividade antitumoral *in vitro* destas formulações, bem como investigar as atividades antiedematogênica e antioxidante em um modelo de inflamação *in vivo* induzida por radiação ultravioleta do tipo B.

1.2 Objetivos específicos

- Preparar nanocápsulas de poli-ε-caprolactona contendo a associação coenzima Q10 e vitamina E acetato pelo método de deposição interfacial do polímero pré-formado;
- Caracterizar as formulações desenvolvidas quanto ao teor de fármaco, eficiência de encapsulamento, pH, diâmetro médio de partícula, índice de polidispersão e potencial zeta;
- Avaliar a fotoestabilidade da coenzima Q10 incorporada às preparações frente à radiação ultravioleta do tipo C (UVC);
- Avaliar a atividade antitumoral *in vitro* das nanocápsulas frente a linhagens de glioma (C6) e melanoma humano (A375);
- Preparar formulações semissólidas a partir da incorporação de goma gelana às suspensões de nanocápsulas;
- Caracterizar as formulações semissólidas quanto a teor de fármaco, pH, espalhabilidade e comportamento reológico;
- Avaliar a atividade antiedematogênica das formulações semissólidas empregando um modelo de edema de orelha induzido por radiação ultravioleta do tipo B (UVB) *in vivo*;
- Investigar a possível infiltração de células inflamatórias no tecido da orelha de camundongos submetidos à radiação UVB e tratados com as formulações desenvolvidas através de métodos enzimáticos e histológico;
- Avaliar o potencial antioxidante das formulações semissólidas preparadas empregando os ensaios de espécies reativas ao ácido tiobarbitúrico (TBARS), hidroperóxidos e tióis não-protéicos (NPSH).

1.1 Nanopartículas poliméricas: preparação e caracterização

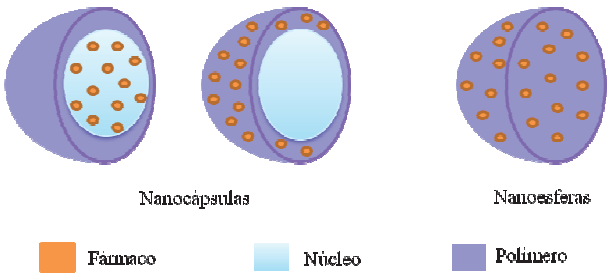
Nos últimos anos, é crescente o interesse pelo estudo de sistemas nanotecnológicos para liberação de fármacos no organismo. Este interesse se deve, em grande parte, às inúmeras potencialidades farmacêuticas associadas às nanopartículas (LETCHFORD; BURT, 2007; BRIGGER; DUBERNET; COUVREUR, 2012). Neste sentido, diversas substâncias antioxidantes tem sido associadas a estes sistemas, tais como a vitamina E e a coenzima Q10, tópicos que serão abordados na sequência desta revisão.

O desenvolvimento de nanocarreadores se dá com o objetivo de alcançar a estabilidade adequada, absorção melhorada e liberação controlada do ativo no organismo, de modo a obter uma melhora no perfil terapêutico (SPEISER, 1991; PANYAM; LABHASETWAR, 2003; NAGAVARMA et al., 2012). Devido à capacidade em controlar e/ou sustentar a cedência de fármacos ao sítio de ação no organismo, estes sistemas podem ser utilizados a fim de aumentar o índice terapêutico de fármacos, aumentando sua eficácia e/ou reduzindo sua toxicidade (REIS et al., 2006; LASALLE; FERREIRA, 2007).

Os sistemas nanoestruturados, caracterizados como dispersões de ordem coloidal, compreendem as nanopartículas poliméricas, as nanoemulsões, os lipossomas e as nanopartículas lipídicas sólidas, além de outros. As nanocápsulas e nanoesferas, denominadas nanopartículas poliméricas, apresentam diâmetro médio geralmente inferior a 500 nm e diferem entre si quanto à composição e organização estrutural. Nanocápsulas são sistemas vesiculares constituídos por um invólucro polimérico disposto ao redor de um núcleo, geralmente de caráter oleoso. Nestas, o fármaco pode estar dissolvido no núcleo e/ou adsorvido à parede do polímero (SCHAFFAZICK et al., 2003; REIS et al., 2006).

Nanoesferas são sistemas matriciais, em que o fármaco pode ficar retido ou adsorvido ao polímero. Assim, não apresentam óleo em sua composição (VAUTHIER; BOUCHEMAL, 2009). Na Figura 1, podem ser observadas as diferenças estruturais entre as nanopartículas poliméricas.

Figura 1 - Representação esquemática das nanocápsulas e nanoesferas.



Vários métodos para a preparação de nanopartículas poliméricas são descritos. Estes métodos podem ser classificados em métodos baseados na polimerização *in situ* de monômeros dispersos, nos quais a formação do polímero acontece no momento da preparação das nanopartículas, ou na precipitação de polímeros pré-formados, em que o polímero já se encontra formado no momento da preparação das nanopartículas (QUINTANAR-GUERRERO et al., 1997; REIS et al., 2006).

A escolha do método de preparação é bastante criteriosa e deve levar em conta as características físico-químicas do fármaco a ser associado, a fim de propiciar uma eficiente associação deste com o nanocarreador. Deve considerar também alguns fatores como o tipo de sistema nanométrico pretendido, o sítio de aplicação da formulação e o tamanho de partícula requerido (RAO; GECKELER, 2011). Independente do método utilizado para a preparação, os produtos obtidos são suspensões coloidais aquosas (SCHAFFAZICK et al., 2003).

No que se refere aos métodos baseados na precipitação de polímeros pré-formados, alcançam maior destaque os métodos de nanoprecipitação e deposição interfacial do polímero pré-formado. Também conhecido como método de deslocamento do solvente, o método de nanoprecipitação foi descrito primeiramente por FESSI e colaboradores (1989). Tal técnica tem como princípio a deposição interfacial do polímero após o deslocamento de um solvente semipolar, miscível com a água, em uma solução lipofílica. Esta técnica é de fácil reprodutibilidade, e tem sido empregada principalmente quando se objetiva a preparação de nanopartículas de poli (ácido lático) (PLA) e poli (ácido lático-co-glicólico) (PLGA) (FESSI et al., 1989; MORA-HUERTAS; FESSI; ELAISSARI, 2011).

O método de deposição interfacial, o qual foi utilizado para preparação das nanopartículas deste estudo, consiste basicamente em verter uma fase orgânica, contendo um solvente miscível com água, o polímero, o óleo, um tensoativo de baixa hidrofilia e o fármaco, em uma fase aquosa contendo um tensoativo de alta hidrofilia (FESSI et al., 1989). A formação das nanoestruturas acontece instantaneamente, enquanto ocorre difusão do solvente pela fase aquosa, e o polímero, insolúvel na fase oleosa, precipita na interface dos componentes imiscíveis. Este método é de grande importância e utilização, pois conduz à obtenção de elevadas eficiências de encapsulamento de fármacos lipofílicos. As nanocápsulas preparadas por este método apresentam diâmetro médio normalmente entre 200 e 500 nm (QUINTANAR-GUERRERO et al., 1998; REIS et al., 2006).

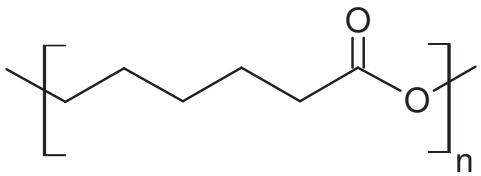
Os constituintes das nanopartículas são, basicamente, um ou dois tensoativos, um polímero (que deve ser biocompatível, podendo ou não ser biodegradável, dependendo da via

de administração pretendida), um óleo (no caso das nanocápsulas) e o fármaco associado (LETCHFORD; BURT, 2007).

Após a preparação dos sistemas nanocarreadores, faz-se necessária a etapa de caracterização físico-química destas suspensões coloidais. Uma vez que se trata de partículas de tamanho muito reduzido, diversas análises de suas propriedades devem ser conduzidas. Os parâmetros mais frequentemente avaliados para as nanopartículas são: diâmetro médio de partícula/gotícula, índice de polidispersão, potencial zeta, pH e eficiência de encapsulamento de fármaco/ativo (SCHAFFAZICK et al., 2003; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Dentre os polímeros mais utilizados na composição de sistemas nanoestruturados figuram os poliésteres de origem sintética, tais como a PCL, o PLA, o PLGA e os derivados acrílicos comumente conhecidos como Eudragit's® (KHOEE; YAGHOOBIAN, 2009). A PCL (Figura 2) é um poliéster alifático semicristalino, insolúvel em água, degradado no organismo humano pela hidrólise das ligações éster (SINHA et al., 2004). Por ser um polímero biodegradável e biocompatível, e de degradação mais lenta quando comparado a outros poliésteres, é muito utilizado em formulações nanoestruturadas, podendo prolongar a liberação de fármacos por longos períodos de tempo (AMIRTHALINGAM; NAYANABHIRAMA; MUTALIK, 2014; XU et al., 2014; KUMAR; VERMA; SINGH, 2015). Além disso, por sua biodegradabilidade e baixa toxicidade, este polímero pode ser administrado pelas vias oral, intravenosa, ocular e tópica, o que o torna muito versátil (SINHA et al., 2004; DAMGÈ; MAINCENT; UBRICH, 2007; LAREDJ-BOUREZG et al., 2015; SILVA et al., 2015).

Figura 2 - Estrutura química da PCL.



Recentemente, formulações nanoestruturadas têm sido direcionadas à aplicação por via tópica, com finalidade cosmética e terapêutica (CHUNG et al., 2010). Devido ao tamanho reduzido e capacidade de apresentar propriedades bioadesivas, o tempo de residência das partículas na pele é prolongado. Além disso, as formulações nanoestruturadas podem

proporcionar aumento da estabilidade química de fármacos e da biodisponibilidade dos mesmos na pele, podendo levar, assim, ao aumento do efeito farmacológico da substância ativa (KOVACEVIC et al., 2011).

1.2 Estresse oxidativo e antioxidantes

O estresse oxidativo é definido como o desequilíbrio entre a produção de espécies reativas de oxigênio/nitrogênio (ERO's e ERN's, respectivamente) e os mecanismos de compensação antioxidantes do organismo humano (PERSSON; POPESCU; CEDAZO-MINGUEZ, 2014). Estas espécies reativas, que compreendem radicais livres (RL) e moléculas que não apresentam elétrons desemparelhados, das quais alguns exemplos são o ânion superóxido ($O_2^{\cdot -}$), o peróxido de hidrogênio (H_2O_2), o radical hidroxila ($\cdot OH$) e o óxido nítrico (NO_2), apresentam elevada reatividade, devido à sua instabilidade, e tempo de meia-vida muito curto. Desta forma, podem reagir com biomoléculas e provocar alterações e dano às células. Dentre os componentes celulares que podem ser afetados, destacam-se as macromoléculas: proteínas, lipídios e ácidos nucleicos (MELLO FILHO; HOFFMAN; MENEGHINI, 1983; LEONARDUZZI; SOTTERO; POLLI, 2010; PISOSCHI; POP, 2015).

O dano a lipídios, o qual é denominado peroxidação lipídica, ocorre por reações que compreendem 3 estágios e leva a formação de produtos como os hidroperóxidos lipídicos e malondialdeído (MDA). Na primeira etapa da reação, a iniciação, é onde ocorre o processo de ataque de um grupo metileno do lipídio pelo radical livre, etapa esta que culmina na formação de um radical peroxil ($ROO^{\cdot}$). Na segunda etapa, a propagação, o radical peroxil reage retirando hidrogênios de cadeias adjacentes e formando o peróxido lipídico (ROOH). Também, nesta etapa, pode ocorrer a reação do radical peroxil com uma ligação dupla na cadeia de ácido graxo, levando à formação de um peróxido cíclico. Por fim, na terceira etapa, denominada terminação, ocorre a formação de produtos estáveis (não-radicais), devido à ação de antioxidantes ou ausência de moléculas reativas (KOHEN; NISKA, 2002).

Devido ao ataque às biomoléculas e, consequentemente, ao dano às estruturas celulares, o estresse oxidativo tem sido associado ao desenvolvimento de patologias. Dentre estas, pode-se destacar: doenças cardíacas, neurodegenerativas (mal de Parkinson, doença de Alzheimer e outras), inflamatórias e neoplasias. Além disso, o processo de envelhecimento cutâneo também está associado ao estresse oxidativo (LÓPEZ-ALARCONA; DENICOLA, 2013; MAULIK et al., 2013).

A geração de ERO's e ERN's ocorre em decorrência de processos aeróbicos, como respiração celular, infecções microbianas que envolvem ativação fagocitária, ou ação de poluentes e toxinas, como o tabagismo, ingestão de bebidas alcoólicas e outros (POLJSAK; SUPUT; MILISAV, 2013; PISOSCHI; POP, 2015). A radiação ultravioleta também pode desencadear estresse oxidativo, o qual pode ser detectado através da peroxidação lipídica pelo aumento da concentração de espécies reativas ao ácido tiobarbitúrico (TBARS) e hidroperóxidos lipídicos e redução dos níveis de tióis não-protéicos (LEE et al., 2000; ROSSATO et al., 2010; TERRA et al., 2012).

Nos organismos aeróbicos, existem sistemas antioxidantes enzimáticos e não-enzimáticos que são responsáveis por manter o equilíbrio entre a produção de espécies pró-oxidantes e antioxidantes (LEONARDUZZI; SOTTERO; POLLI, 2010). As enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) representam os sistemas antioxidantes enzimáticos, enquanto que os sistemas não-enzimáticos são compostos pela glutationa, algumas proteínas (ferritina, transferrina, albumina e outras), substâncias do complexo coenzima Q e ácido úrico (POLJSAK; SUPUT; MILISAV, 2013). Por outro lado, os antioxidantes exógenos, como carotenoides, vitaminas C e E, substâncias pertencentes à classe dos flavonoides, dentre outros, são compostos provenientes da ingestão de alimentos (WILLETT, 2006; PHAM-HUY; PHAM-HUY, 2008).

Os sistemas antioxidantes, endógenos ou exógenos, enzimáticos ou não-enzimáticos, têm a função de inibir ou reduzir os danos causados por espécies reativas através de diversos mecanismos de ação. Dentre os principais mecanismos de ação pelos quais atuam, estão a doação de átomos de hidrogênio ao radical livre, estabilizando-o e interrompendo a reação de oxidação em cadeia, e a remoção do oxigênio presente no meio, átomo que atua na propagação da reação de oxidação (RAJENDRAN et al., 2014).

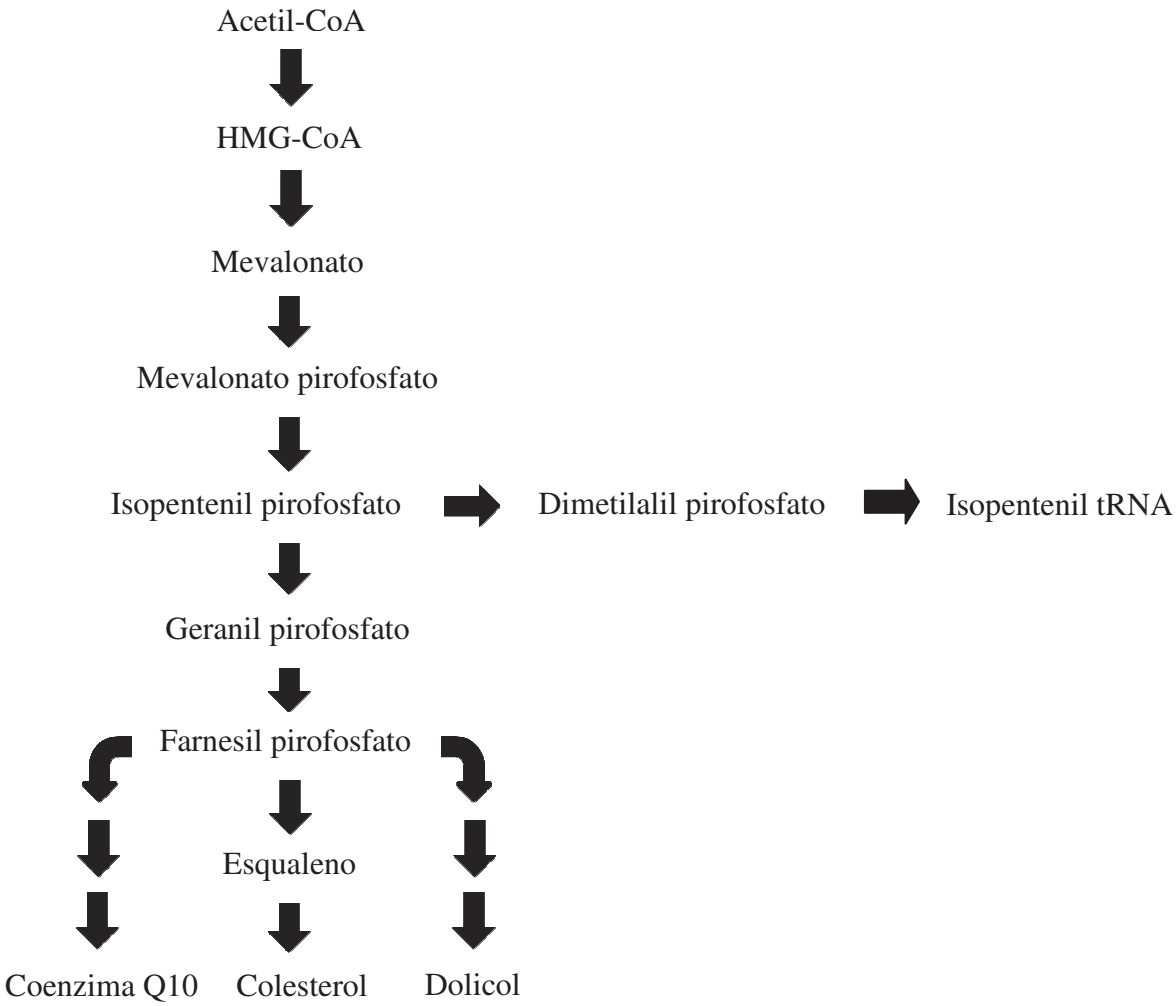
Entretanto, quando há excesso na produção de ERO's e esta não consegue ser compensada, as defesas antioxidantes endógenas se esgotam, e então se faz necessária a ingestão de substâncias antioxidantes no intuito de minimizar o dano oxidativo (WILLETT, 2006; SANTHAKUMAR; BULMER; SINGH, 2014). Neste sentido, acredita-se que terapias por meio de agentes antioxidantes possam ser efetivas em proteger contra danos causados pela radiação ultravioleta e outros processos geradores de ERO's.

1.3 Coenzima Q10

A coenzima Q10 (2,3-dimetoxi-5-metil-6-decaprenil-benzoquinona) (Q10), também denominada ubiquinona ou ubidecarenona, composto ativo de interesse deste estudo, é um importante antioxidante intracelular que também desempenha função na cadeia transportadora de elétrons e produção de ATP. Esta molécula é essencial durante o processo de produção de energia e, por isso, encontra-se presente em maior concentração nos órgãos de maior demanda energética, como fígado, rins, coração e cérebro (ERNSTER; DALLNER, 1995; LÓPEZ-MARTÍN et al., 2007).

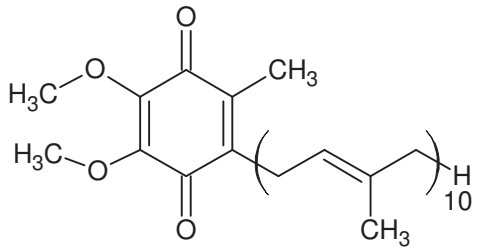
Esta molécula é sintetizada endogenamente a partir de compostos intermediários da síntese do mevalonato, mesma via bioquímica que dá origem ao colesterol (Esquema 1) (ERNSTER; DALLNER, 1995). Estima-se que cerca de 50% da Q10 disponível em nosso organismo seja sintetizada endogenamente, enquanto que os 50% restantes são obtidos a partir da dieta, pelo consumo de carne, ovos, peixes e vegetais, ou também pela ingestão de suplementos alimentares (BHAGAVAN; CHOPRA, 2006; LEE et al., 2012).

Esquema 1 - Rota bioquímica de síntese da Q10 (BUHAESCU; IZZEDINE, 2007).



A Q10 é caracterizada como um pó cristalino, de coloração amarela, com ponto de fusão de 48ºC (BALAKRISHNAN et al., 2009). Sua estrutura química é formada por uma quinona, que está ligada a uma cadeia lateral isoprenóide, contendo 10 unidades de cinco átomos de carbono cada (CRANE, 2001). A presença desta longa cadeia lateral isoprenóide na molécula torna esta coenzima pouco solúvel em água, e sua elevada massa molecular (863,3 g/mol) leva a uma baixa biodisponibilidade oral, dificultando seu uso farmacêutico e cosmético (SIEKMANN; WESTESEN, 1995; BHAGAVAN; CHOPRA, 2006). Além disso, apresenta também instabilidade frente à luz (HOPPE et al., 1999; BULE; SINGHAL; KENNEDY, 2010). A estrutura química da Q10 é mostrada na Figura 3.

Figura 3 - Estrutura química da Q10.



Desde que foi descoberta, em 1957, esta molécula tem sido intensamente estudada. Muitos trabalhos investigam suas aplicações na terapêutica, sugerindo seu emprego no tratamento da doença de Alzheimer (WADSWORTH et al., 2008; SPINDLER; BEAL; HENCHCLIFFE, 2009; MANCZAK et al., 2010), mal de Parkinson (SHULTS et al., 2004; NEZHADI et al., 2011; SIKORSKA et al., 2014), neuropatias e nefropatias associadas a diabetes (HERNÁNDEZ-OJEDA et al., 2012; SOURRIS et al., 2012). Além disso, em termos de aplicabilidade cosmética, sua utilização também promove benefícios no tratamento do envelhecimento cutâneo, hidratação da pele e fotoproteção, além de outros (SCHÖLERMANN et al., 1998; PARDEIKE; SCHWABE; MÜLLER, 2010; YUE et al., 2010).

Apesar de muitos estudos avaliarem a atividade antioxidante da Q10, poucos são os trabalhos que abordam a atividade antioxidante deste ativo em sistemas nanoestruturados. Desta forma, como o objetivo deste trabalho foi a preparação de nanoestruturas, trabalhos envolvendo esse tipo de sistema serão comentados a seguir.

ZHANG e WANG (2009) prepararam lipossomas de Q10 revestidos com trimetil quitosana e realizaram a administração das formulações (5 μL suspensão/20 g de peso

corporal) desenvolvidas em região ocular de coelhos com catarata induzida, objetivando avaliar o efeito anti-catarata com base na sua atividade antioxidante. Este estudo foi o pioneiro em mostrar a eficácia da Q10 como profilática no desenvolvimento de catarata e também no combate à progressão da lesão, a partir da redução da peroxidação lipídica avaliada a partir do conteúdo de MDA no tecido ocular dos animais.

YUE e colaboradores (2010) desenvolveram carreadores lipídicos nanoestruturados (CLN) contendo Q10 e investigaram seu efeito sobre os níveis de peroxidação lipídica e de ERO's após dano celular induzido por radiação UVA. No que diz respeito ao nível de MDA, indicativo de peroxidação lipídica, o mesmo apresentou-se 1,7 vezes maior no grupo emulsão de Q10 do que no grupo de Q10 associada aos carreadores lipídicos nanoestruturados. O mesmo resultado foi encontrado para os níveis de ERO's: apresentaram-se mais elevados no grupo emulsão do que no grupo CLN-Q10.

Em outro estudo, SWARNAKAR e colaboradores (2011) desenvolveram nanopartículas de PLGA contendo Q10 e também estudaram o efeito antioxidante desta coenzima associada ao nanocarreador. Estudos *in vitro* em culturas celulares (macrófagos - linhagem RAW 264.7) indicaram que a Q10 associada às nanopartículas, em concentrações acima de 3 μg/mL, apresentou eficácia em eliminar ERO's cerca de 10 vezes superior à Q10 não-nanoencapsulada.

Recentemente, BRUGÈ e colaboradores (2013) associaram a Q10 oxidada ou reduzida a carreadores lipídicos nanoestruturados e avaliaram seu efeito antioxidante através dos níveis intracelulares de ERO's e funcionalidade mitocondrial. Para isto, empregaram linhagem de fibroblastos humanos como modelo, na qual o estresse oxidativo foi induzido por radiação UVA. As células incubadas com CLN sem Q10 ou com a mesma em sua forma oxidada mostraram maiores níveis de ERO's, em comparação ao controle de células irradiadas e com o CLN contendo Q10 em sua forma reduzida. Igualmente para a funcionalidade mitocondrial, somente o tratamento CLN contendo Q10 em sua forma reduzida apresentou a capacidade de reverter a despolarização mitocondrial induzida por UVA, o que sugere potencial aplicação destes sistemas em formulações cosméticas antienvelhecimento.

Com relação ao efeito antiproliferativo, existem poucos relatos na literatura para a Q10. Esta atividade tem sido atribuída, por muitos autores, às propriedades antioxidante e anti-inflamatória que esta substância apresenta (BAHAR et al., 2010; KIM; PARK, 2010). Em um dos estudos em que foi realizada esta avaliação, SWARNAKAR e colaboradores (2014) investigaram a eficácia antitumoral e segurança da combinação do tratamento com nanopartículas contendo doxorrubicina e nanopartículas contendo Q10 via oral, em modelo de

câncer de mama em ratos, em comparação com o tratamento com doxorrubicina por administração via intravenosa. A administração das nanopartículas de doxorrubicina e de nanopartículas de Q10 levou a efeito 4,5 vezes maior do que a administração de doxorrubicina não-nanoencapsulada por via intravenosa. Os autores correlacionaram este efeito com outros testes que mostraram que a nanoencapsulação levou a maior potencial de apoptose celular e maior dano ao DNA.

LOHAN e colaboradores (2015) desenvolveram carreadores lipídicos nanoestruturados de tamanho muito pequeno (aproximadamente 85 nm) contendo Q10 e avaliaram seu efeito sobre cultura de queratinócitos humanos (HaCaT) pelo método de redução do sal tetrazólio (XTT). Nas concentrações-teste de 10, 25 e 50 μg/mL não houve redução da viabilidade celular, enquanto que para a concentração de 100 μg/mL o declínio de viabilidade celular observado foi de aproximadamente 27%.

Poucos relatos na literatura mostram a atividade anti-inflamatória da Q10, a qual tem sido associada à redução da produção de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNFα) (SCHMELZER et al., 2008). Os estudos presentes evidenciam elevado potencial nesta atividade, potencial que é ainda mais expressivo quando esta molécula é associada a sistemas nanoestruturados. Em um destes estudos, SWARNAKAR e colaboradores (2011), investigaram a atividade da Q10 associada à nanopartículas em modelo de inflamação de pata induzida por carragenina em camundongos. Neste, observaram que as nanopartículas com fármaco levaram à redução de até 89% do edema induzido, enquanto que o fármaco não-associado a nanopartículas mostrou redução de cerca de 60%.

TAWFIK e colaboradores (2015) avaliaram o efeito da Q10 administrada por via intraperitoneal (10 mg/kg) sozinha ou combinada ao metotrexato (MTX) (2 mg/kg) na progressão de artrite em ratos induzida pelo adjuvante completo de Freund (CFA). A administração de Q10 concomitante foi capaz de potencializar o efeito do MTX no tratamento da artrite e, além disso, de reduzir a hepatotoxicidade associada ao tratamento com o mesmo, constituindo uma potencial alternativa terapêutica aos fármacos antirreumáticos convencionais.

1.4 Vitamina E acetato

O óleo, constituinte do núcleo das nanocápsulas, onde o fármaco pode estar dissolvido, deve proporcionar alta solubilização do mesmo, não deve apresentar toxicidade nem ser capaz de solubilizar o polímero. Além disso, o óleo é de fundamental importância em

formulações direcionadas para o uso cosmético, uma vez que apresenta capacidade de conferir emoliência, conduzindo ao aumento da hidratação da pele. Por isso, sua escolha adequada é fundamental para a viabilidade da preparação de nanocápsulas (SCHAFFAZICK et al., 2003; MORA-HUERTAS; FESSI; ELAISSARI, 2010).

Neste sentido, por tratar-se de uma substância oleosa, a vitamina E acetato pode constituir o núcleo das nanocápsulas desenvolvidas. O termo vitamina E refere-se às diferentes formas dos compostos tocoferois e tocotrienois, que estão naturalmente presentes em diversos alimentos de origem vegetal. O tocoferol é um nutriente essencial ao organismo humano, que pode ser obtido da dieta a partir da ingestão de nozes, castanha, amêndoas e derivados de cereais (ITO et al., 2006; ULATOWSKI; MANOR, 2015). No organismo, os principais reservatórios deste nutriente são o tecido adiposo e as glândulas supra-renais (HERRERA; BARBAS, 2001).

Os tocoferois e tocotrienois são compostos derivados do 6-cromanol, dos quais cada um apresenta quatro formas, que são designadas por α, β, γ, e δ, de acordo com o número e posição dos grupos metílicos ligados ao anel aromático. Dentre estes, o α-tocoferol constitui a forma mais abundante e mais ativo biologicamente *in vivo*, sendo considerada a forma genuína da vitamina E. Todos estes compostos apresentam uma hidroxila altamente eletrofílica ligada ao anel aromático, à qual se deve a capacidade de extinguir espécies radicalares, o que torna a vitamina E uma substância com potente atividade antioxidante (LIEN et al., 1999; HERRERA; BARBAS, 2001; CRAFT, 2015).

O α-tocoferol acetato, ou vitamina E acetato, é um éster obtido pela reação entre o ácido acético e o tocoferol, e constitui a principal forma da vitamina E comercializada para uso cosmético. Este é caracterizado como um líquido viscoso oleoso, insolúvel em água e instável na presença de álcali, luz e oxigênio (HERRERA; BARBAS, 2001). Na Figura 4, encontram-se as estruturas químicas do tocoferol e tocoferol acetato.

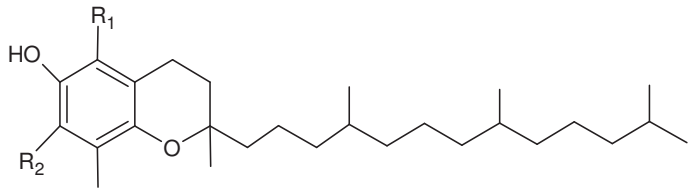
Diversas atividades biológicas já foram descritas para a vitamina E, tais como atividade antioxidante, antiproliferativa, neuroprotetora, entre outras (SHIREEN et al., 2008; JIANG, 2014; PAWAR et al., 2014; ULATOWSKI; MANOR, 2015). Todas as formas de vitamina E apresentam potente atividade antioxidante, a qual se dá através da eliminação de radicais de peroxidação lipídica pela doação do hidrogênio fenólico do anel cromanol ao radical (JIANG et al., 2000).

Além da capacidade de doar o átomo de hidrogênio, as formas de vitamina E não-substituídas na posição 5, como é o caso do γ-tocoferol, conseguem reter eletrófilos, dentre estes as espécies reativas de nitrogênio (por exemplo, NO_2 e peroxinitrito), que tem sua

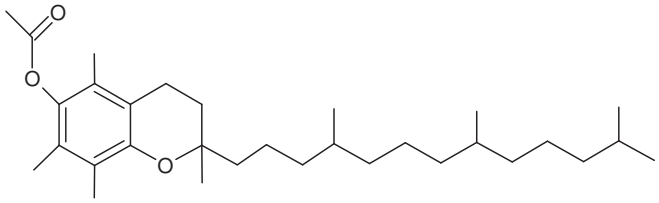
produção aumentada durante processos inflamatórios. Diferentemente, esta atividade não é apresentada pelas formas de vitamina E com um grupamento metil associado à posição 5, como o α-tocoferol (JIANG et al., 2000).

Ainda no que diz respeito à sua atividade anti-inflamatória, a vitamina E, principalmente nas suas formas α-tocoferol e γ-tocoferol, atua em processos inflamatórios pela inibição da formação de prostaglandinas dos tipos D_2 e E_2 (PGD_2 e PGE_2, respectivamente) mediada pela cicloxigenase 2 (COX-2), atividade que já foi demonstrada experimentalmente por JIANG e colaboradores (2000; 2008).

Figura 4 - Estruturas químicas do tocoferol e tocoferol acetato, respectivamente.



	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H



Devido a suas importantes propriedades em sistemas biológicos, os efeitos benéficos da vitamina E vem sendo explorados pelas indústrias farmacêutica e cosmética (CONSTANTINIDES; HAN; DAVIS, 2006; PARDEIKE; HOMMOSS; MÜLLER, 2009; NICULAE et al., 2014). Além disso, tem sido objeto de estudo de inúmeras pesquisas científicas (PARDEIKE; HOMMOSS; MÜLLER, 2009; HATANAKA et al., 2010; CARVALHO et al., 2013). No que diz respeito aos estudos que reportam a associação de vitamina E a nanopartículas, cabe ressaltar os trabalhos a seguir.

BYUN e colaboradores (2011) desenvolveram nanopartículas de PCL contendo α-tocoferol pelo método de emulsificação-evaporação do solvente e ultrasonicação e avaliaram a influência da concentração de PCL (3 ou 5 g/100 mL), do tipo de solvente na fase orgânica (diclorometano ou diclorometano/acetonitrila) e o tempo de ultrassonicação (1, 2 ou 3 minutos) nas características das partículas produzidas. Observaram redução na eficiência de encapsulamento do α-tocoferol com o aumento do tempo de ultrassonicação de 1 para 3

minutos (de 87,73% para 57,45%) e o mesmo foi encontrado para os valores de tamanho de partícula. A formulação contendo concentração de PCL de 5 g/100 mL empregando diclorometano como solvente e tempo de ultrassonicação de 3 minutos apresentou as melhores características quando comparada a outras formulações.

Em outro estudo, NORONHA e colaboradores (2013) prepararam e otimizaram nanocápsulas de PCL contendo α-tocoferol preparadas pelo método de nanoprecipitação. A melhor quantidade de ativo para encapsulação encontrada neste estudo foi de 200 mg, resultando em eficiência de encapsulamento próxima de 100%, diâmetro médio de partícula em torno de 190 nm e índice de polidispersão de 0,11.

Recentemente, NICULAE e colaboradores (2014) desenvolveram formulações de nanopartículas contendo α-tocoferol direcionadas à fotoproteção. Neste contexto, avaliaram a influência do composto antioxidante na fotoestabilidade de um filtro solar UVA incorporado a nanopartículas lipídicas sólidas frente à radiação UVC. As nanopartículas produzidas foram avaliadas quanto ao fator de proteção solar e eritema. Verificaram que a presença do α-tocoferol levou a uma diminuição do eritema quando coencapsulado com o filtro solar, comparado à encapsulação somente do filtro solar. Ainda, o α-tocoferol provou ser eficaz na fotoestabilização do filtro UVA instável encapsulado.

No último ano, ALQAHTANI e colaboradores (2015) formularam e caracterizaram nanopartículas de PLGA e PLGA-quitosana contendo α-tocoferol e γ-tocotrienol, avaliando suas atividades antioxidante, antiproliferativa e a captação celular *in vitro* destes sistemas. As nanopartículas, quando comparadas a formulações micelares de tocoferol e tocotrienol, foram capazes de aumentar a captação dos mesmos pelas células Caco-2, sem provocar toxicidade, sendo que esta absorção foi 3,5 vezes maior para as partículas de PLGA-quitosana. Ainda, os sistemas demonstraram maior capacidade para inibir a oxidação do colesterol e citotoxicidade elevada frente a linhagens de câncer de mama (MCF-7 e MDA-MB-231) ($IC_{50} < 20$ μM).

1.5 Aspectos gerais da pele e formulações semissólidas à base de nanopartículas

A pele, revestimento externo do corpo, desempenha funções vitais para o organismo, mantendo a homeostase, protegendo contra a invasão de microorganismos e oferecendo proteção frente a danos provocados pelo calor, agentes químicos e toxinas. Mais especificamente, também protege frente à perda de água e danos provocados por radiação, executa a regulação da temperatura corporal, além de outras funções fundamentais (FIRESTEIN, 2004; ROBERTS; WALTERS, 2008).

Este órgão apresenta elevada área superficial (aproximadamente 2 m^2) e representa cerca de 16% do peso total do corpo. Quanto à sua estrutura, é composta por três camadas: epiderme, derme e hipoderme. A camada epidérmica é composta majoritariamente por queratinócitos, mas também estão presentes melanócitos, células de Langerhans e células de Merkel, enquanto que na derme encontram-se fibroblastos e fibras de colágeno e elastina, vasos sanguíneos e terminações nervosas. Por fim, a camada mais inferior da pele, hipoderme, é constituída principalmente por adipócitos (VENUS; WATERMAN; MCNAB, 2010; KHAVKIN; ELLIS, 2011).

A administração de fármacos no tecido cutâneo pode possibilitar um efeito local do mesmo ou sua absorção sistêmica. Ainda, apresenta outras vantagens, tais como a menor incidência de efeitos adversos sistêmicos e aplicação específica no local acometido pela patologia, além de ser uma via de administração não-invasiva (TING; VEST; SONTHEIMER, 2004).

Neste sentido, nas últimas décadas, o desenvolvimento de novos sistemas de liberação de fármacos, como as nanopartículas, tem constituído uma abordagem promissora no aumento da penetração e permeação de fármacos na pele e o controle de sua liberação (MÜLLER; RADTKE; WISSING, 2002; ALVAREZ-ROMÁN et al., 2004). Diferentes tipos de nanopartículas tem sido formuladas como carreadores de fármacos para aplicação cutânea, visando tanto aplicações cosméticas, como terapêuticas: nanopartículas lipídicas sólidas (NLS), carreadores lipídicos nanoestruturados (CLN), nanocápsulas, lipossomas e outros (JUNYAPRASERT et al., 2009).

No entanto, estes sistemas nanoestruturados são obtidos como suspensões líquidas, o que dificulta sua aplicação e permanência no local de aplicação (SCHAFFAZICK et al., 2003). Neste sentido, uma estratégia que tem sido empregada recentemente é a incorporação destes nanosistemas em formulações semissólidas, tais como os hidrogéis (LIPPACHER; MÜLLER; MÄDER, 2001). Esta nova abordagem vem ganhando destaque uma vez que as nanopartículas podem conferir inúmeras outras vantagens ao tratamento tópico, tais como a possibilidade de acúmulo nos folículos pilosos, atuando como sistemas reservatórios, além da capacidade de atingir estruturas funcionais em camadas mais profundas da pele (LADEMANN et al., 2007).

Em vista disso, JUNYAPRASERT e colaboradores (2009) compararam nanoemulsões de triglicerídeos de cadeia média (TCM) e carreadores lipídicos nanoestruturados de TCM/palmitato de cetila contendo coenzima Q10 incorporados em hidrogéis de goma xantana quanto à permeação *in vitro* em epiderme humana. Em seu estudo, os autores mostraram que a

oclusão promovida pelas formulações foi o maior promotor de penetração de coenzima Q10 na pele, que também foi afetada, porém em menor intensidade, pela quantidade de óleo presente nas NLC.

TERROSO e colaboradores (2009) desenvolveram formulações cosméticas semissólidas à base de Carbopol® 940 contendo nanocápsulas de coenzima Q10 ou o pó obtido a partir de secagem destas suspensões. Os autores obtiveram formulações com características físico-químicas e propriedades reológicas adequadas, demonstrando assim a viabilidade de preparação de hidrogéis a partir destes produtos intermediários contendo coenzima Q10.

No ano de 2010, MARCHIORI e colaboradores prepararam e caracterizaram hidrogéis de Carbopol Ultrez® 10 NF contendo dexametasona associada a nanocápsulas poliméricas no sentido de aumentar a eficácia deste fármaco no tratamento tópico. Para isso, as suspensões de nanocápsulas foram avaliadas quanto à sua atividade antiproliferativa empregando o modelo *Allium cepa*, as quais demonstraram um menor índice mitótico celular para o grupo contendo as nanoestruturas comparado ao fármaco livre (4,62 % e 8,60%, respectivamente). Os semissólidos preparados apresentaram pH ácido e foram capazes de proporcionar liberação controlada do fármaco encapsulado.

Além dos carbômeros, outra classe de substâncias que tem despertado interesse no desenvolvimento de formulações semissólidas a partir de sistemas nanoestruturados são as gomas naturais. Esta classe de compostos apresenta vantagens na sua utilização, como biocompatibilidade e baixo custo (OSMALEK; FROELICH; TASAREK, 2014). Dentre estas, destaca-se a goma gelana, um polissacarídeo produzido pela bactéria *Sphingomonas* (*Pseudomonas*) *elodea* que possui capacidade de formar um gel elástico (RINAUDO; MILAS, 2000; BAJAJ et al., 2007; PRAJAPATI et al., 2013).

Sua estrutura constitui-se de um esqueleto aniônico linear, composto de unidades repetidas de α-L-ramnose, β-D-glucose e β-D-glucoronato, na proporção molar de 1:2:1 (MILAS; SHI; RINAUDO, 1990). Sua forma de ocorrência natural apresenta dois substituintes acila (L-glicerila e acetila), os quais são removidos por hidrólise alcalina dando origem à gelana desacetilada, denominada "low-acetyl" ou "low-acyl". Ambas as formas, natural e desacetilada, apresentam a propriedade de formação de hidrogéis na presença de cátions mono-, di- ou trivalentes (KANG et al., 1982; OSMALEK; FROELICH; TASAREK, 2014).

TAYEL e colaboradores (2013) empregaram a goma gelana no desenvolvimento de géis a partir de nanoemulsões contendo cloridrato de terbinafina, objetivando propor uma

alternativa para administração ocular deste fármaco. Para tal, realizaram a incorporação das nanoemulsões em dispersões de goma gelana (0,2%) e caracterizaram-nas em termos de transparência, comportamento reológico, mucoadesividade, liberação de fármaco e análise histológica para avaliação de irritação. Os hidrogéis obtidos tiveram coloração transparente, fluxo do tipo pseudoplástico, mucoadesividade e liberação controlada de fármaco.

Recentemente, D´ARRIGO e colaboradores (2014) desenvolveram um nanohidrogel de goma gelana para liberação simultânea dos fármacos prednisolona e paclitaxel e sua utilização nas terapias antitumoral e anti-inflamatória. Neste nanohidrogel, a prednisolona encontrava-se quimicamente ligada aos grupos ácidos carboxílicos da goma gelana, e o paclitaxel estava retido fisicamente. O nanohidrogel estudado pelos autores promoveu melhor efeito citotóxico *in vitro* em diferentes linhagens de células tumorais devido ao sinergismo promovido pela associação de um fármaco antitumoral e de um anti-inflamatório.

PUBLICAÇÃO 1: Improved photostability and antitumor effect of coenzyme Q10 by its association with vitamin E acetate in polymeric nanocapsules

Artigo a ser submetido em revista indexada

Improved photostability and antitumor effect of coenzyme Q10 by its association with vitamin E acetate in polymeric nanocapsules

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Abstract

The present study showed the development of nanocapsules containing the association of coenzyme Q10 and vitamin E acetate, purposing this formulation as an alternative to the treatment of malignant glioma and melanoma cells. In order to investigate if nanocapsules are able to protect the coenzyme Q10 from degradation under UVC radiation, a photostability study was carried out. For this, three concentrations of vitamin E acetate were evaluated (1, 2 or 3%). Nanocapsules presented suitable physicochemical characteristics and were able to protect the coenzyme Q10 from photodegradation. Besides, this protection was influenced by higher vitamin E acetate concentrations, attributing to this oil an important role on coenzyme Q10 photostabilization. Regarding to anticancer activity, tested nanocapsules presented the capability to reduce cell viability of glioma and melanoma cell lines. In this sense, these formulations represent interesting platforms to the delivery of coenzyme Q10 and vitamin E acetate to the treatment of these types of cancers.

Keywords

Coenzyme Q10, vitamin E, antioxidants, nanocapsules, cell culture, *in vitro* cytotoxicity, antitumoral activity.

1 Introduction

Coenzyme Q10, also called ubiquinone or ubidecarenone (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone) (Q10), is a lipophilic molecule that presents important antioxidant activity, by scavenging free radicals and inhibiting lipid peroxidation (ABERG et al., 1998; ERNSTER; DALLNER, 1995; BHAGAVAN et al., 2007). Q10 molecule is synthesized in the human body through mevalonate pathway and is composed by a benzoquinone ring and by an isoprenoid side chain, the last comprising 10 isoprene units (BHAGAVAN et al., 2007).

Concerning to its biological activities, Q10 acts as a cofactor in the mitochondrial electron transport chain, being essential for the ATP synthesis (ERNSTER; DALLNER, 1995; BHAGAVAN et al., 2007; GUEVEN; WOOLLEY; SMITH, 2015). This molecule also presents other important activities as neuroprotective, cardioprotective, antiinflammatory and antitumor effects, among others (JOO, 2005; MANCZAK et al., 2010; LOHAN et al., 2015).

Another substance with expressive biological activities is vitamin E (VitE). This vitamin is composed by tocopherols and tocotrienols and also presents antioxidant activity. This function of VitE is due to its capability to stabilize reactive species (JIANG et al., 2000). Beyond this effect, it also presents anticancer function (PRASAD et al., 2003; DONG; GU, 2009).

Melanoma is the most dangerous type of skin cancer, affecting all age groups and representing the main cause of death from skin disease (WHO, 2010). Around the world, the incidence of this type of cancer is continuing to increase in the last years (ERDEI; TORRES, 2010). The treatment of this cancer varies depending of the stage of the disease (NIEZGODA; NIEZGODA; CZAJKOWSKI, 2015).

Gliomas are tumors of neuroepithelial tissue which occur on glial cells line and constitute the most common malignant tumor of the central nervous system, representing about 77% of cases. Although the occurrence of this type of tumor is rare, they present rapid proliferation and represent one of the most fatal malignancies (BEHIN et al., 2003; SCHWARTZBAUM et al., 2006**).**

Despite the advances on medicine in the treatment of cancers, it is still difficult, since most of all malignant tumors present resistance to therapeutic strategies. In this sense, new approaches on therapeutics of these tumors are required. In the last few years, nanocarriers have emerged as alternative platforms for cancer therapy (PEER et al., 2007).

Polymeric nanocapsules are vesicular systems formed by a polymeric wall surrounding a liquid drug-containing core, generally oily (MISHRA; PATEL; TIWARI,

2010). They present relevant advantages that allow these systems to constitute an alternative to drug delivery on cancer therapy: controlled drug delivery, possibility of funcionalization of nanoparticles surface and tumor-specific deposition of nanoparticles, due to the enhanced permeability and retention (EPR) effect (GREF et al., 2000; GU et al., 2007; WANG et al., 2012). Taking into account the potentialities of Q10 and VitE in respect to antitumor effect and the advantages of nanoparticles, it is relevant to study the association of these molecules on nanocapsules in order to purpose an alternative for cancer treatment. In addition to its potentialities, VitE is used in this work to form the liquid core of nanocapsules since it is an oily substance.

Considering all of this, the aim of this study was to prepare and characterize nanocapsules containing the association Q10 and VitE, as well as to evaluate their anticancer activity against glioma and melanoma cells and their ability to protect Q10 from photodegradation.

2.1 Materials

Q10 and vitamin E acetate were obtained from Mapric (São Paulo, Brazil). Poly-ε-caprolactone (PCL), polysorbate 80 (Tween® 80) and sorbitan monooleate (Span® 80) were provided by Delaware (Porto Alegre, Brazil). Acetone was purchased from Proquimios (Rio de Janeiro, Brazil). Anhydrous ethanol and methanol (both HPLC grade) were obtained from Tedia (São Paulo, Brazil). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, 0.25% trypsin/EDTA solution, Fungizone and fetal bovine serum (FBS) were obtained from Gibco (Gibco BRL, Carlsbad, CA, USA). Dimethylsulphoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and solvents were of analytical grade and used as received.

2.2 Methods

2.2.1 Dissolution/swelling experiments of polymer films

With the aim to evaluate the compatibility between the polymer and the oil used to prepare the polymeric nanocapsules, the assay of dissolution/swelling of PCL films was performed. For this, PCL films weighing about 20 mg were prepared employing hydraulic press (5 tons during 5 min). In the sequence, these films (n=3) were completely immersed in VitE in flasks maintained at room temperature. During 60 days, the weight variation of the films was evaluated by removing them of the contact with the oil and drying with an absorbing paper. An analytical balance was used to determine weight variation.

2.2.2 Preparation of nanocapsule suspensions

Nanocapsules were prepared following the interfacial deposition of preformed polymer method, according to described by Fessi and co-workers (1989). For this, an organic phase constituted by PCL (0.100 g), Span® 80 (0.077 g), VitE (0.100, 0.200 or 0.300 g - 1, 2 or 3%, respectively), coenzyme Q10 (0.010 g) and acetone (27 mL) was kept under moderate magnetic stirring at 40°C during 60 min. After the complete dissolution of the components, this phase was added into an aqueous phase containing Tween®80 (0.077 g) and the magnetic stirring was sustained for 10 min. After this, the organic solvent and part of the water were eliminated by evaporation under reduced pressure in order to obtain a final volume of 10 mL, corresponding to 1.0 mg/mL of Q10 in the suspensions. With comparison purposes, formulations without the active compound were also prepared. All the formulations were prepared in triplicate. The formulations were abbreviated as following: NCP1 (Q10-loaded nanocapsules containing 1% of VitE), NCP2 (Q10-loaded nanocapsules containing 2% of VitE), NCP3 (Q10-loaded nanocapsules containing 3% of VitE), NBP1 (nanocapsules without Q10 containing 1% of VitE), NBP2 (nanocapsules without Q10 containing 2% of VitE) and NBP3 (nanocapsules without Q10 containing 3% of VitE).

2.2.3 Apparatus and chromatographic conditions

The HPLC system used consisted of a Shimadzu LC-10A system (Kyoto, Japan) equipped with a LC-20AT pump, an UV-VIS SPD-M20A detector, a CBM-20A system controller and a Rheodyne valve sample manual injector with 20 µL loop. Chromatographic conditions were as described by Mattiazzi and co-workers (2014), with some modifications. The column used for the chromatographic separation was a Gemini C_{18} Phenomenex column (150 mm x 4.60 mm, 5 µm, 110 Å), fitted with a guard column (SecurityGuard C_{18} 4 x 3.0 mm), both maintained at room temperature. The mobile phase was a mixture of anhydrous ethanol and methanol (90:10 v/v) that was filtered through a 0.45 µm regenerated cellulose membrane and degassed by sonication before its use. The flow rate of mobile phase was 1 mL/min and the analyses were performed at isocratic conditions. The injection volume was 20 µL. The detection was made at 275 nm. Shimadzu LC Solution was the software program used to integrate peak areas.

2.2.4 Physicochemical characterization of nanocapsule suspensions

2.2.4.1 pH

The pH values of nanocapsule suspensions were measured by direct immersion of the electrode of a calibrated potentiometer (Model pH 21, Hanna Instruments, Brazil) in the nanosuspensions. The measures were made at room temperature (25 ± 2°C) and conducted in triplicate.

2.2.4.2 Particle size analysis, polydispersity index (PdI) and zeta potential

Particle sizes and polydispersity indexes (n = 3) were evaluated using ZetaSizer Nano Series Malvern Instruments (UK), by photon correlation spectroscopy method, after diluting samples in ultrapure water (1:500). Zeta potentials (n = 3) were determined by microelectrophoresis using the same equipment, after diluting samples in 10 mM NaCl (1:500).

2.2.4.3 Coenzyme Q10 content and encapsulation efficiency

Total Q10 content in nanocapsule suspensions (n=3) was evaluated by diluting an aliquot of the sample in anhydrous ethanol (10 mL), followed by sonication during 15 min to extract the drug. Before injecting the samples into the chromatographic system, they were filtered through a 0.45 μm membrane.

For the encapsulation efficiency assay, an aliquot of the suspensions was placed into Amicon®Ultra centrifugal filter devices (10,000 MW; Millipore) and free drug was separated from the nanostructures employing ultrafiltration/centrifugation technique (2200 xg during 10 min). Free Q10 content was determined in the ultrafiltrate by HPLC analysis. The encapsulation efficiency (%) was calculated as the difference among total and free concentrations of coenzyme Q10 determined in the nanocapsule suspensions and in the ultrafiltrate, respectively, employing the following equation:

$$\mathrm{EE} = \frac{\text{Total content} - \text{Free content}}{\text{Total content}} \text{ x } 100$$

2.2.5. Photostability evaluation

Aiming to evaluate the effect of photodegradation by UV light, an ethanolic solution of Q10 (1.0 mg/mL) (ES Q10) and an aliquot of the samples (NCP1, NCP2 and NCP3) were placed in plastic cuvettes and exposed to UVC radiation (Philips lamp - UVC long life, 30 w)

during 4 h in a mirrored chamber (dimensions: 1 m x 0.25 m x 0.25 m). The samples were placed at a fixed distance of 20 cm from the light source. A dark control (plastic cuvette containing the Q10 ethanolic solution protected from radiation with aluminum paper) was also evaluated, in order to discard degradation by temperature or the influence of other experimental conditions. The remaining Q10 was quantified by HPLC. The experiment was conducted in triplicate.

2.2.6 Cell culture and viability assay

C6 and A375 cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in low-glucose DMEM containing 0.1% Fungizone and 100 U/L penicillin/streptomycin and supplemented with 5 or 10% FBS at controlled temperature of 37ºC and 5% CO_2 humidified atmosphere. The cells (5 x 10^4) were plated in 96-well plates and 24 h after plating, treatments were performed. Treatment groups were: control (DMEM), vehicle (DMSO), non-encapsulated Q10 (free Q10), non-encapsulated vitamin E (free vitamin E), nanocapsule NCP2 and NBP2. Free molecule (Q10) was first dissolved in sterile DMSO at a concentration of 100 mM (stock concentration) and after all formulations were diluted in DMEM with 5% or 10% FBS to give final concentration of 100 μM in the well.

Cell viability assay was conducted employing sulforhodamine B dye method (SRB) (OUAÏSSI et al., 2008). After 48 h treatment, cells were washed and 50% trichloroacetic acid was added in the fridge for cells fixation during 45 min. After this period, the acid was removed and plates were washed with distilled water for 5 times to complete removal of the reagent. After, the well was covered with sulforhodamine B solution (0.4% acetic acid), followed by 30 min incubation to stain proteins in solution. Subsequently, cells were washed with 1% acetic acid for five times to remove uncomplexed dye with proteins. Finally, the dye was solubilized with Tris HCl solution pH 10.5 at 10 mM. The plate was stirred in orbital shaker and the absorbance was verified employing spectrophotometer at 530 nm.

2.2.7 Statistical analyses

Formulations were prepared and analyzed in triplicate. The results were expressed as mean ± SD (standard deviation). The software GraphPad Prism version 5 (GraphPad, USA) was used. For *t* test and analyses of variance (ANOVA) one way followed by post-hoc Tukey's test or two way and Bonferroni's post test. Values of $p<0.05$ were considered to be statistically significant.

3 Results

3.1 Dissolution/swelling experiments of polymer films

The compatibility between PCL and VitE to prepare the nanocapsule suspensions was evaluated by monitoring the weight change in the polymer films during 60 days of immersion in the oil (Figure 1). Compared to the initial weight of the films, no significant difference was observed, indicating that there was no swelling/dissolution of the polymer films during the time they were in contact with the oil ($p>0.05$).

3.2 Physicochemical characterization of nanocapsule suspensions

Table 1 shows the physicochemical characteristics of coenzyme Q10-loaded nanocapsules and blank nanocapsules, prepared with different concentrations of VitE (1, 2 or 3%). All formulations presented mean diameter in nanometric range, between 184 and 241 nm, and the values of polydispersity index were less than 0.17. Zeta potentials were close to -11 mV and pH values were in the neutral range. Regarding to Q10-loaded nanocapsules, the total drug content was close to theoretical values (0.97 - 1.00 mg/mL) and encapsulation efficiency was high (above 99%).

The results showed that the increase in VitE concentration in nanocapsule formulations resulted in a tendency of increasing mean diameter and Q10 content of the nanocapsules, but this increase was significant only for NCP1 compared to NCP3 ($p<0.05$). On the other hand, this parameter did not influence pH, polydispersity index and zeta potential of the nanocapsules suspensions ($p>0.05$).

Considering the macroscopic aspect, all formulations presented white/bluish opalescent aspect (Tyndall effect), without visible precipitation.

3.3 Photostability evaluation

Considering that coenzyme Q10 is referred as a photolabile substance (ZHANG; WANG, 2009), the ability of nanocapsules to prevent its degradation under UVC light was evaluated (Figure 2). After 4 h of exposure, ES Q10 showed a decrease more pronounced, resulting in 24.00% of Q10 content. In contrast, it was proved that these nanosystems are capable to protect the drug from degradation, since Q10 remaining contents were 59.00%, 71.44% and 74.39% for NCP1, NCP2 and NCP3 respectively. For dark control, the Q10 concentration was close to 100.00%, which indicates that there was no influence of the temperature or other experimental factors on drug degradation.

The ability of the nanocapsules to delay coenzyme Q10 photodegradation was evident. NCP1 conferred a protection of Q10 degradation that was 2.5 times higher than ES Q10; for NCP2 and NCP3 the protection was 3 and 3.1 times higher than ES Q10. This effect was statistically significant for all nanocapsules from 30 min of experiment compared to ES Q10 ($p<0.05$). It was also noted that VitE concentration in nanocapsules had an important effect in Q10 photoprotection. The higher the VitE concentration, the greater Q10 remaining content. The reduction of drug content in NCP1 was significant higher in 180 and 240 min compared to NCP2 and NCP3 ($p<0.05$). In addition, compared to the initial drug content, photodegradation in ES Q10 began at 30 min of assay, while in NCP1 and NCP2 the photodegradation started at 60 min and, in NCP3, photodegradation began at 120 min of experiment ($p<0.05$).

3.4 Cell viability assay

In order to evaluate the *in vitro* antitumor effect, nanocapsule suspensions NCP2 and NBP2 were tested against glioma (C6) and melanoma (A375) cell lines by the SRB method. As illustrated in Figures 3 and 4, at 100 μM both formulations presented the ability to reduce the viability of tumor cells ($p<0.05$). In contrast, free Q10 and VitE at the same concentration were not able to induce cell viability reduction ($p>0.05$).

4 Discussion

In the preformulation step, it is extremely important to evaluate the compatibility between the oily component and the polymer employed to prepare nanocapsule suspensions, in order to know if the core-shell structure will be formed (SCHAFFAZICK et al., 2003; MORA-HUERTAS; FESSI; ELAISSARI, 2010). In this sense, the swelling/dissolution test is generally employed (SANTOS et al., 2013; SANTOS et al., 2014; CHASSOT et al., 2015). It should be emphasized that if the oil and the polymer interact with each other, the formation of micelles could occur rather than the shell-core structure of nanocapsules (GUTERRES et al., 2000). During 60 days of immersion in VitE, PCL films showed no interaction with this oil, presenting no variations in their weights, neither gain or loss weight. In this sense, these constituents are considered suitable to develop nanosystems as nanocapsules (COUVREUR et al., 2002).

Considering that the development of nanosystems requires a detailed physicochemical characterization, in this study different parameters were evaluated (Table 1). Employing PCL and vit E to prepare the nanocapsules, it was possible obtain particles with average diameter

in agreement with other reported nanosystems obtained by the same preparation method and polymer (BERNARDI et al., 2009; TERROSO et al., 2009; BULCÃO et al., 2014; CHASSOT et al., 2015; CORADINI et al., 2015). Also, in this study it was proved that the increase of VitE concentration promote an increase in mean diameter of nanocapsules. KHAYATA and co-workers (2012) also observed different mean diameters of nanocapsules prepared with 50, 100 and 150 mg of VitE, which was justified by the high viscosity of VitE. PdI values were below 0.17, indicating that the systems presents suitable homogeneity in terms of particle size distribution (SCHAFFAZICK et al., 2003; MORA-HUERTAS; FESSI; ELAISSARI, 2010; RIGO et al., 2015).

Another analysis commonly used to characterize the nanosystems is the measure of zeta potential, which reflects the surface potential of the particles (SCHAFFAZICK et al., 2003; MOHANRAJ; CHEN, 2006; CHO et al., 2013). This parameter can be useful to predict stability of nanocapsule suspensions and it is mainly affected by the polymer nature. In their study, TERROSO and co-workers (2009) also developed coenzyme Q10-loaded nanocapsules employing PCL and obtained negative values of zeta potential, close to -10 mV. Furthermore, as reported by SCHAFFAZICK and co-workers (2003), this value is appropriate to nanocapsules prepared with PCL and polysorbate 80.

Regarding pH measurement, the values obtained for this nanocapsule suspensions were near to the neutral range (6.4 to 7.2). According to MORA-HUERTAS and co-workers (2010), nanocapsules prepared by interfacial deposition of preformed polymer method present pH values between 3.0 and 7.5. These values are in accordance to those normally found on literature for other PCL nanocapsules (CATTANI et al., 2010; CORADINI et al., 2014; CHASSOT et al., 2015).

Coenzyme Q10 content in nanocapsules was close to the theoretical value for all formulations (between 97 and 100 %), indicating that there was no loss of this drug during the preparation process. Encapsulation efficiency, as expected, was about 100 % and can be attributed to the high lipophilicity of Q10 and its affinity with VitE.

In relation to the photostability study (Figure 2), it can be clearly observed the influence of the nanosystems on Q10 content. The photoprotection conferred to Q10 can be attributed to the polymer wall presence in nanocapsules, which could acts as a barrier reflecting and/or scattering the UV radiation (OURIQUE et al., 2008; ALMEIDA et al., 2010; SANTOS et al., 2014). Besides the polymer wall, the effect of VitE in controlling Q10 photodegradation was also observed. The increase of VitE content in nanocapsules core avoided a decrease in drug remaining concentration at the end of the UV exposure. The

efficiency of this oil in Q10 photostabilization can be related to its composition riched in tocopherols and tocotrienols, substances that present high antioxidant activity and prevent oxidation reaction propagation (LIEN et al., 1999; HERRERA; BARBAS, 2001; CRAFT, 2015). SANTOS and co-workers (2014) showed an increased protection of clotrimazole photodegradation by coconut oil in nanocapsules, which was attributed to the antioxidant properties of this oil.

It is already known that nanoparticles present physical and structural properties that attract much attention in the search for new strategies for cancer treatment (ZU; LIAO, 2015). Furthermore, molecules that present antioxidant activity can be considered promising alternatives to the cancer therapy (HAMID et al., 2010; JAIN et al., 2013). By combining both approaches, the nanocapsules developed in this work were evaluated in two different cancer cell lines: a brain cancer model (glioma C6 cell line) and a skin cancer model (human melanoma A375 cell line). It is worth noting that this two approaches, the effects of coenzyme Q10 on skin and brain, are the most studied applications for formulations developed containing this compound (WADSWORTH et al., 2008; SPINDLER; BEAL; HENCHCLIFFE, 2009; LEE; TSAI, 2010; SANTOS et al., 2010; SCHWARZ et al., 2013; LOHAN et al., 2015).

SRB test, which evaluates cell viability based on the measure of protein content (SKEHAN et al., 1990), showed that in the concentration of 100 μM, both nanocapsules (Q10-loaded and unloaded) were capable to induce death of tumor cells ($p<0.05$), while free compounds had no effect on cell viability ($p>0.05$). Furthermore, in unloaded nanocapsules, this cytotoxic effect may be due to VitE, the compound present in nanocapsules core which also presents an important antioxidant activity and reported antiproliferative effects in several cell lines (KLINE; YU; SANDERS, 2004; STONE et al., 2004; ALQAHTANI et al., 2015).

5 Conclusion

It was possible to prepare Q10-loaded nanocapsules containing three different concentrations of vitamin E, and also the respective unloaded nanocapsules. This nanocapsules presented suitable physicochemical characteristics (average diameter, PdI, zeta potential, pH and drug content) and encapsulation efficiency of Q10 close to 100%. The presence of Vitamin E in nanosystems showed an important role in the reduction of coenzyme Q10 photodegradation. Additionally, nanocapsules prepared showed important antitumor activity, reducing viability of C6 and A375 cell lines.

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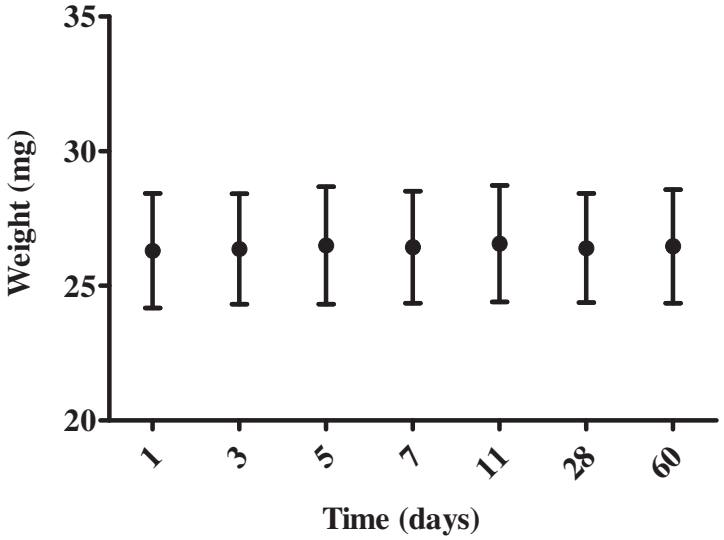

Figure 1 - Weight of PCL films during 60 days of immersion in VitE.

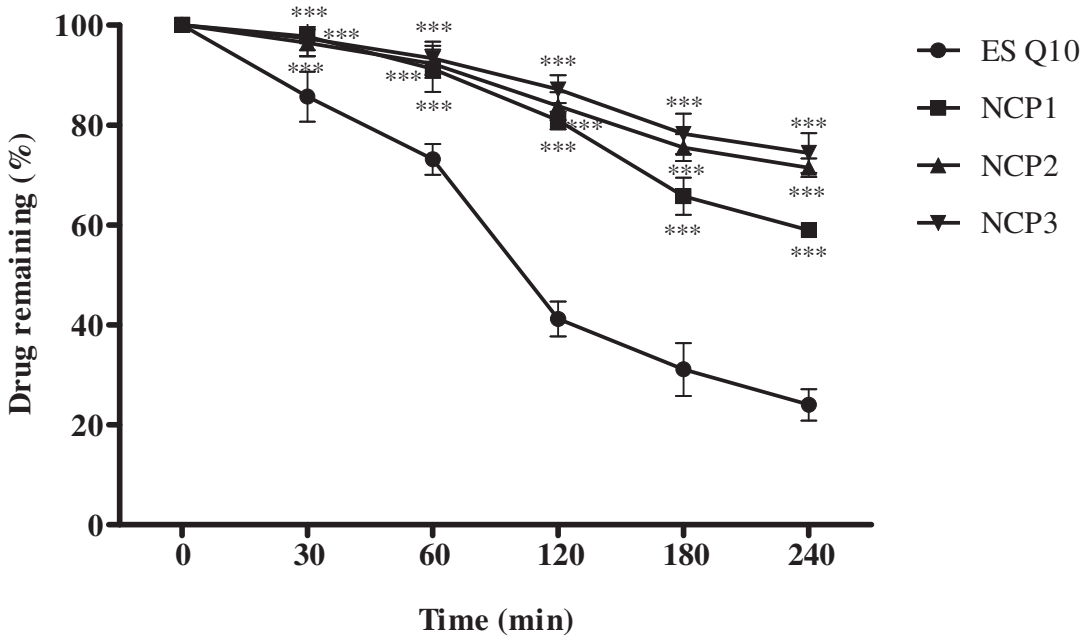

Figure 2 - Photodegradation profiles of Q10 versus exposure time. The asterisks denote the significant levels when compared to Q10 ethanolic solution (ES Q10) (two-way ANOVA) (*) $p<0.05$. NCP1 means: Q10-loaded nanocapsules containing 1% of VitE; NCP2 means: Q10-loaded nanocapsules containing 2% of VitE; NCP3 means: Q10-loaded nanocapsules containing 3% of VitE.

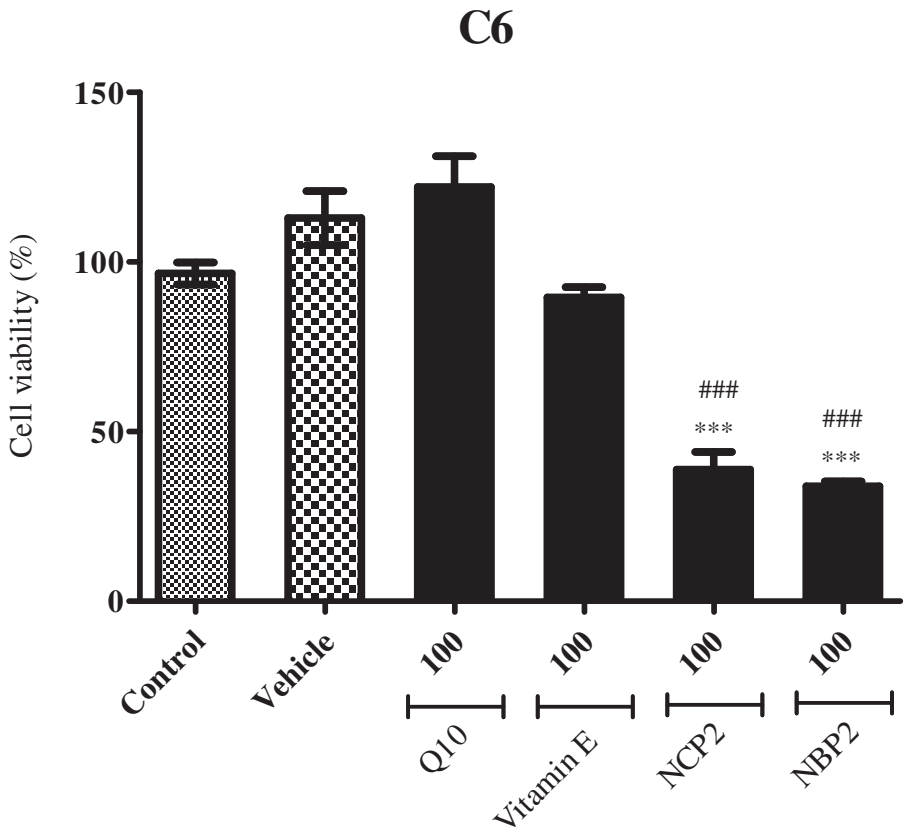

Figure 3 - *In vitro* cytotoxicity assay of free coenzyme Q10 (Q10), VitE and Q10-loaded and blank nanocapsules in C6 glioma cell line. Asterisks (*) denote significant difference from control group. Sharp (#) in NCP2 denotes significant difference from free Q10 and in NBP2 denotes significant difference from VitE ($p<0.05$).

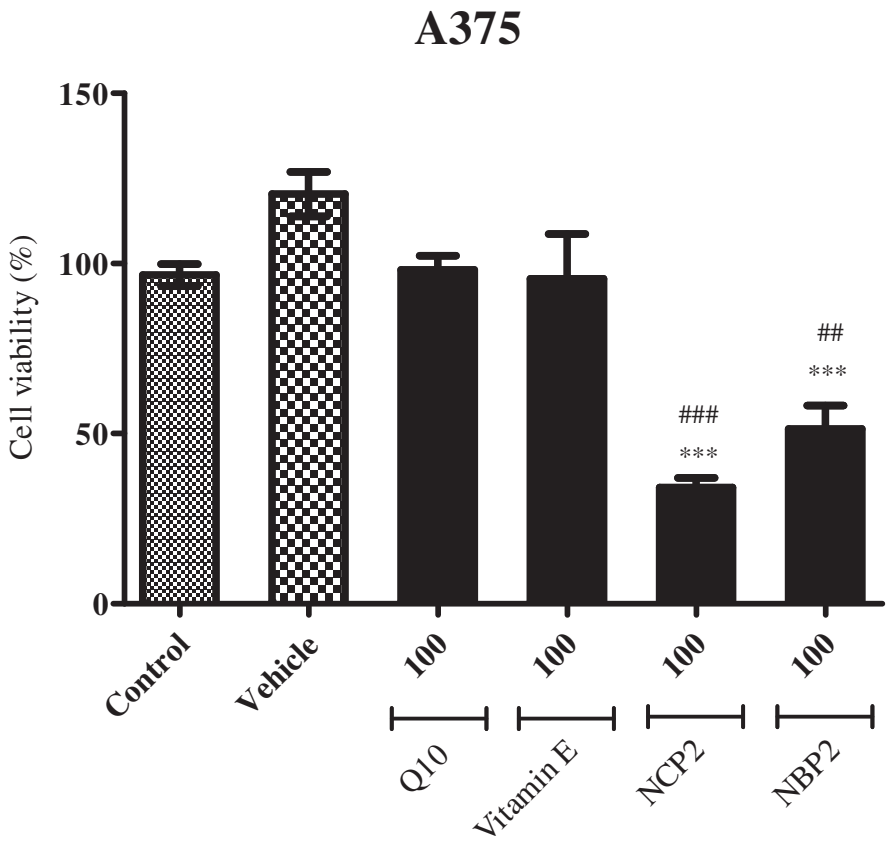

Figure 4 - *In vitro* cytotoxicity assay of free coenzyme Q10 (Q10), VitE and Q10-loaded and blank nanocapsules in A375 human melanoma cell line. Asterisks (*) denote significant difference from control group. Sharp (#) in NCP2 denotes significant difference from free Q10 and in NBP2 denotes significant difference from VitE ($p<0.05$).

Table 1 - Physicochemical characterization of coenzyme Q10-loaded nanocapsules and blank nanocapsules.

Formulation	pH	Mean diameter (nm)	PdI	Zeta Potential (mV)	Coenzyme Q10 content (mg/mL)
NCP1	7.2 ± 0.6	195 ± 06	0.14 ± 0.01	- 10.4 ± 1.3	0.97 ± 0.01
NCP2	6.9 ± 0.3	226 ± 19	0.16 ± 0.03	- 12.0 ± 0.8	0.98 ± 0.02
NCP3	6.5 ± 0.1	241 ± 20	0.14 ± 0.02	- 11.4 ± 0.8	1.00 ± 0.01
NBP1	6.4 ± 0.2	184 ± 10	0.13 ± 0.02	- 10.3 ± 1.0	-
NBP2	6.6 ± 0.6	202 ± 12	0.15 ± 0.03	- 11.4 ± 1.6	-
NBP3	6.6 ± 0.2	220 ± 11	0.17 ± 0.03	- 11.6 ± 3.2	-

PUBLICAÇÃO 2: Nanoencapsulation of coenzyme Q10 and vitamin E acetate protects against UVB radiation-induced skin injury in mice

Artigo a ser submetido em revista indexada

Nanoencapsulation of coenzyme Q10 and vitamin E acetate protects against UVB radiation-induced skin injury in mice

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Abstract

This study evaluated the feasibility of the development of semisolid formulations based on nanocapsule suspensions containing the association of coenzyme Q10 and vitamin E acetate from the addition of gellan gum (2%) to the suspensions. Moreover, we studied their application as an alternative to the treatment of inflammation induced by ultraviolet B (UVB) radiation. For this, an animal model of injury induced by UVB-radiation was employed. All semisolids presented pH close to 5.5, drug content above 95% and mean diameter on the nanometric range, after redispersion in water. Besides, the semisolids presented non-Newtonian flow with pseudoplastic behavior and suitable spreadability factor values. The results showed also that the semisolid containing coenzyme Q10-loaded nanocapsules with the higher concentration of vitamin E acetate reduced in 73 ± 8% the UVB radiation-induced ear edema. Moreover, all formulations tested were able to reduce inflammatory parameters evaluated through MPO activity and histological procedure on injured tissue and the encapsulated hydrogel formulation containing coenzyme Q10 reduced oxidative parameters assessment through of the non protein thiols levels and of the lipid peroxidation.

Keywords

Coenzyme Q10, vitamin E, nanocapsules, ear edema, UV radiation.

1 Introduction

The skin is often exposed to several harmful sources, among these ultraviolet (UV) radiations. UV radiation consists of wavelengths shorter than visible light and is classified as UVA, UVB and UVC. Among these, UVB radiation is the main responsible by skin cytotoxic events, inducing a number of pathologic changes in skin such as erythema, edema, hyperplasia, sunburn, immune suppression and skin cancer. It leads to changes and often to destruction of skin structures, which can result in changes in their normal function (VALKO et al., 2007; BHATIA et al., 2011; ALSHAAL et al., 2011).

One of the major alterations that UV radiation lead to deleterious outcomes on the skin is the production of the highly unstable products, called reactive oxygen species (ROS), that contribute to oxidize and cellular damage (BRIEGER et al., 2012; KVIETYS et al., 2012). Sunscreens are recommended for protection against UV light-induced skin damage. They contain organic or inorganic filters, which absorb, reflect or scatter UV light. However, often these sunscreens can cause adverse effects such as allergic reactions and contact dermatitis which limit their use (NOHYNEK et al., 2010; RIGO et al., 2015).

In this sense, the search for new sunscreen alternatives is growing especially sunscreens associated with antioxidant molecules which can be an useful strategy in prevention and repair UV-mediated cutaneous damage, through benefic effects such as anti-inflammatory, antioxidant and DNA repair (MUKHERJEE et al., 2011; RAMOS et al., 2013).

Coenzyme Q10, also known as ubiquinone or ubidecarenone (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone) (Q10) is a lipophilic antioxidant essential in human body (MOLYNEUX et al., 2007). It plays a key role on ATP synthesis, being fundamental on mitochondrial electron transport chain. This compound can be obtained in diet from sources as red meat, fish and poultry or from supplementation (DOS SANTOS et al., 2009). Numerous studies have shown the potentialities of Q10 in health. Among these, its function in Parkinson's and Alzheimer's diseases, neuropathy and nephropathy associated to diabetes and others have been investigated (MANCZAK et al., 2010; SOURRIS et al., 2012; SIKORSKA et al., 2014). Moreover, concerning to cosmetic applications, there are studies showing benefits on anti-aging therapy, skin hydration and photo-protection (PARDEIKE; SCHWABE; MÜLLER, 2010; YUE et al., 2010; BRUGÈ et al., 2013).

In addition to Q10, other antioxidant substance which has biological activity is Vitamin E (VitE) which is composed by tocopherols and tocotrienols. This vitamin can be obtained from diet by the ingestion of vegetable oils, nuts, almonds (PEH et al., 2015). Its antioxidant function is due to its capability to stabilize reactive species (JIANG et al., 2000)

and provides benefits in anticancer therapy, ischemic heart failure, protection against atherosclerosis, among inflammatory disorders and others (PRASAD et al., 2003; DONG; GU, 2009; IDRIS et al., 2014; JIANG, 2014). Besides, its cosmetic use was described on association with UV filters, based on its antioxidant properties that play an essential role on skin repair (JACOB; BAKER; FARRIS, 2012; MERCURIO et al., 2015).

In the last decades, nanoparticles have emerged as promising alternatives on drug delivery systems. Nanocapsules are vesicular systems composed by a central core, generally an oily core, surrounded by a polymeric wall and have been extensively studied mainly due to their ability to control drug release and to improve drug stability (MIYAZAKI; ISLAM, 2007; FRANK et al., 2015). Their promising applications have been demonstrated for the treatment of several diseases and also their administration by different routes (SANTOS et al., 2014; CHASSOT et al., 2015; KLIPPSTEIN et al., 2015).

In this context, in this study, we verified the effect of nanocapsules containing the association of Q10 and VitE acetate incorporated in a semisolid formulation on ear edema induced by UVB irradiation in mice, comparing to the semisolid formulations containing non-encapsulated compounds.

2 Materials and methods

2.1 Materials

Coenzyme Q10 and vitamin E acetate were obtained from Mapric (São Paulo, Brazil). Poly-ε-caprolactone (PCL), polysorbate 80 (Tween® 80) and sorbitan monooleate (Span® 80) were provided Delaware (Porto Alegre, Brazil). Gellan gum was kindly donated by CP Kelco (Georgia, USA). Acetone was purchased from Proquimios (Rio de Janeiro, Brazil). Anhydrous ethanol (HPLC grade) were obtained from Tedia (São Paulo, Brazil). Dimethylsulphoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents and solvents were of analytical grade and used as received.

2.2 Methods

2.2.1 Preparation of nanocapsule suspensions and semisolid formulations

Nanocapsule suspensions were prepared following the interfacial deposition of preformed polymer method, according to described by Fessi and co-workers (1989). An organic phase constituted by PCL (0.100 g), Span® 80 (0.077 g), VitE (0.1 or 0.3 g - 1 or 3%, respectively), Q10 (0.0100 g) and acetone (27 mL) was kept under moderate magnetic stirring at 40°C during an hour. After the complete dissolution of the components, this phase was

added into an aqueous phase containing Tween®80 (0.077 g) and the magnetic stirring was sustained for 10 min. After this, the organic solvent and part of the water were eliminated by evaporation under reduced pressure in order to obtain a final volume of 10 mL and 1.0 mg/mL of Q10. Formulations without the active compound were also prepared. The formulations were abbreviated as following: NCP1 (Q10-loaded nanocapsules containing 1% of VitE), NCP3 (Q10-loaded nanocapsules containing 3% of VitE), NBP1 (nanocapsules without Q10 containing 1% of VitE and NBP3 (nanocapsules without Q10 containing 3% of VitE).

Semisolid formulations were prepared using mortar and pestle by adding 0.2 g of gellan gum (2%) directly in nanocapsule suspensions (10 mL), in order to obtain about 10 g of semisolids. For semisolid formulations containing free compounds, both Q10 (1mg/g) and VitE (3%) were solubilized in DMSO (1 mL) and added into a dispersion of gellan gum (2%) in water (9 mL). Vehicle was prepared following the same methodology, but only dispersing gellan gum at the same concentration in water (10 mL).

2.2.2 Characterization of semisolid formulations

2.2.2.1 pH determination

pH values were evaluated by immersing a calibrated potentiometer (Model pH 21, Hanna Instruments, Brazil) in a dispersion of the semisolid formulations in ultrapure water (10%, w/v).

2.2.2.2 Evaluation of mean particle size

The evaluation of mean particle size of nanoparticles in the semisolid formulations was carried out using ZetaSizer Nano Series Malvern Instruments (UK), by photon correlation spectroscopy method, by dispersing an aliquot of the samples in ultrapure water (1:500).

2.2.2.3 Coenzyme Q10 content

The analysis of total Q10 content in the prepared semisolids was performed by HPLC employing the methodology described by Mattiazzi and co-workers (2014), with some modifications. For this, an aliquot of the semisolid was solubilized in ethanol and submitted to sonication for 10 min. Then, the sample was centrifuged at 3000 rpm (664 xg) during 10 min. Subsequently, the samples were filtered through a 0.45 μm membrane and injected into HPLC system.

2.2.2.4 Determination of spreadability

Spreadability of semisolid formulations was evaluated according to the parallel plate method described by Borghetti and Knorst (2006) and Rigo and co-workers (2012). For this, an aliquot of the sample was put in a central hole of a mold glass plate, that was in the surface of a scanner (HP Officejet, model 4500 Desktop). Then, the mold glass was carefully removed and upon the sample were placed glass plates with known weights. Each plate was placed with an interval of 1 min of the subsequently plate, in a total of 10 plates. One image was captured at each interval of 1 min, employing the desktop scanner and the software ImageJ (Version 1.49q, National Institutes of Health, USA) was used to calculate the spread areas of the captured images. The spreadability profiles were obtained by plotting spreading area versus cumulative weight of the plates. Finally, the spreadability factor (Sf) was also calculated for all formulations. This factor represents the ability of the formulation in expand on a smooth horizontal surface when a gram of weight is added on it, under the conditions of the test. This equation (Eq. (1)) was employed to calculate the spreadability factor:

$$\mathrm{Sf} = \frac{\mathrm{A}}{\mathrm{W}}$$

in which Sf is the spreadability factor ($mm^2\ g^{-1}$), (A) is the maximum spread area (mm^2) after addition of the total number of plates, and (W) is the total weight added (g).

2.2.2.5 Rheological behavior

Rheological analyses were conducted at 25 ± 1ºC employing viscometer (RVDV-I-PRIME model, Brookfield, USA) with a RV06 spindle. For this, about 50 g of the formulations was used and submitted to a range of speed between 5-100 rpm. The data obtained were analyzed to the best fit using Bingham, Casson, Ostwald and Herschel-Bulkley models (Eq. 2 - 5) (KIM et al., 2003), employing graphical model, in order to determine rheological behavior.

(2) $$\tau = \tau_0 + \eta\,\gamma$$

(3) $$\tau^{0.5} = \tau_0^{0.5} + \eta^{0.5}\,\gamma^{0.5}$$

(4) $$\tau = \mathrm{K}\,\gamma^{n}$$

(5) $$\tau = \tau_0 + \mathrm{K}\,\gamma^{n},$$

where τ_0 is the yield stress, η is the viscosity, n is the index of flow, K is the index of consistency, τ is the shear stress and γ is the shear rate (KIM et al., 2003).

2.2.3 Animals

Male Swiss mice (25-30 g; n total = 114) were used in all experiments. Animals were kept under controlled temperature (22 ± 2°C) on a 12 h light-dark cycle and with standard laboratory chow and water *ad libitum*. The animals were habituated to the experimental room at least 1 h before the experiments. All of the experiments were carried out between 8:00 a.m. and 5:00 p.m. The experiments were performed in accordance with current ethical guidelines for the care of laboratory animals (ZIMMERMANN, 1983) and all procedures were approved by our Institutional Ethics Committee (Process number 4921060715/2015). Animals were randomly assigned in different treatment groups and the all experiments were performed blindly. The number of animals and intensity of stimuli was the minimum necessary to demonstrate the consistent effects of treatments.

2.2.4 UVB irradiation model

The UVB source of irradiation consisted of a Philips TL40W/12 RS lamp (Medical-Eindhoven, Holland) mounted 20 cm above the table on which the mice were placed, and which emitted a continuous light spectrum between 270 and 400 nm with a peak emission at 313 nm. UVB output (80% of the total UV irradiation) was measured using a model IL-1700 Research Radiometer (International Light, USA; calibrated by IL service staff) with a radiometer sensor for UV (SED005) and UVB (SED240). The UVB irradiation rate was 0.27 mW/cm^2 and the dose used was 0.5 J/cm^2. The mice were firstly anesthetized (90 mg/kg of ketamine plus 3 mg/kg of xylazine) with a single intraperitoneal injection and then exposed to UVB irradiation. Only right ear of each animal was exposed to UVB irradiation (CASAGRANDE et al., 2006; VICENTINI et al., 2010).

2.2.5 Formulation administration and experimental design

Swiss mice were randomly divided into nine groups with six animals in each following groups: Naïve (non-irradiated); untreated irradiated; treated with vehicle (gel base), treated with hydrogel containing non-encapsulated Q10 and VitE (FH), treated with hydrogel containing encapsulated Q10 and VitE 1% (NCP1), treated with hydrogel containing encapsulated VitE 1% without Q10 (NBP1), treated with hydrogel containing encapsulated Q10 and VitE 3% (NCP3) and treated with hydrogel containing encapsulated VitE 3%

without Q10 (NBP3). As positive control to treat the burn was used silver sulfadiazine (3%). Mice were topically treated on the ear surface with different semisolid formulations (15 mg/ear) immediately after to UVB irradiation (GODWIN et al., 2006). Non-irradiated and untreated irradiated groups were included in the experiments.

2.2.6 Ear edema measurement

Skin photodamage was induced by UVB irradiation and the inflammatory process was assessed through ear edema formation. Edema was measured by the increase in ear thickness after the inflammatory stimuli. Ear thickness was evaluated before and 24 h after UVB irradiation using a digital micrometer (Digimess) in animals anesthetized with isofluorane (SILVA et al., 2011). The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges. The thickness was expressed in μm. To minimize variation, a single investigator performed the measurements throughout each experiment.

2.2.7 Assessment of leukocyte infiltration

2.2.7.1. Myeloperoxidase (MPO) and N-acetyl-β-Dglucosaminidase (NAGase) activities

The UVB irradiation-induced leukocytes migration in the skin was assessed using the MPO and NAGase activities assay as previously described (OLIVEIRA et al., 2014; RIGO et al., 2015). The activity of MPO and NAGase enzymes is used as biochemical marker of the polymorphonuclear leukocyte influx (mostly neutrophil and macrophages, respectively) to the injured tissue. Twenty-four hours after UVB irradiation, the mice were euthanized and the ears were removed to determine MPO and NAGase activities. Tissue samples were homogenized in acetate buffer (80 mM, pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide and kept at 4ºC. Immediately before assay, the tissue homogenate was centrifuged at 11,000 xg for 20 min and the supernatant was collected for assay.

For evaluation of MPO enzyme activity, sample was incubated with acetate buffer and 3,3',5,5'-tetramethylbenzidine solution (18.4 mM) for 3 min at 37 ºC. The reaction was stopped on ice by addiction of acetic acid. The color formed was analyzed by a spectrophotometer at 630 nm. Otherwise, in order to determine NAGase enzyme activity, the sample was mixed with sodium citrate buffer (50 mM, pH 4.5) and p-nitrofenil-2-acetamide-β-D-glucopyranoside (NAG 2.25 nM) and incubated for 60 min at 37 ºC. After incubation time, the reaction was stopped on ice by addition of glycine buffer (0.2 μM, pH 10.4). The color formed was analyzed by a spectrophotometer at 405 nm. Both reactions were read on a

Fisher BiotchMicrokinetics BT 2000 microplate reader. The results were expressed as optical densities (OD)/mL of the sample.

2.2.7.2 Histology

Separate groups of mice were used to evaluate histological changes in ear tissue 24 h after UVB-irradiation or UVB-irradiation plus hydrogel formulations. Mice were euthanized and the right ear were removed and fixed in an alfac solution (16:2:1 mixture of ethanol 80%, formaldehyde 40% and acetic acid). Each sample embedded in paraffin was sectioned at 5 μm and stained with hematoxylin-eosin. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response with a 20x and 40x objectives (OLIVEIRA et al., 2014; PIANA et al., 2016). To minimize a source of bias, the investigators did not know the group that they were analyzing.

2.2.8 Oxidative stress evaluation

In order to verify the effect of different hydrogel formulations on oxidative damage induced by UVB irradiation we determined the non protein thiol levels and lipid peroxidation. After 24 h of UVB irradiation, the mice were euthanized and the ears were removed and homogenized with Tris/HCl buffer (50 mM, pH 7.4) and centrifuged at 665 xg for 10 min. The supernatants were used to continue the analysis. To determine non protein thiol (NPSH) levels, the protein supernatant was precipitated with TCA 10% and centrifuged at 5000 g for 10 min. The deproteinized supernatant was incubated with Tris/HCl (200 mM, pH 8.9) and 5-5'dithiobis- (Z-nitro-benzoicacid) (DTNB – 2.5 mM) at room temperature for 5 min (ROSSATO et al., 2010). The color of the solution resulting from the reaction was measured at 405 nm with a Fisher BiotchMicrokinetics BT 2000 microplate reader.

The lipid peroxidation was determined through of the measurement of lipid hydroperoxide and thiobarbituric acid reactive substances (TBARS) assay. The concentration of lipid hidroperoxide was estimated by the FOX assay. For this purpose, the tissue homogenate supernatant was incubated with FOX reagent (sulfuric acid 250 mM, xylenol orange 0.8 μM, ferrous sulfate and ammonium sulfate 3 μM and butyl hydroxytoluene 0.04 mM) at 37 ºC for 30 min. After, the absorbance of the solution was read at 560 nm (MANNA et al., 2008). The lipid hydroperoxide content (μM) was calculated using the molar extinction coefficient of 4.6 x 10^4M/cm.

The extent of lipid peroxidation in terms of malondialdehyde (MDA) formation was measured by TBARS assay. The supernatant was incubated with TBA 0.8%, acetic acid

buffer and sodium dodecyl sulfate (SDS) 8.1% at 90 ºC for 90 min. The solution color resulting from the reaction was measured at 535 nm with a Fisher BiotchMicrokinetics BT 2000 microplate reader. Both the lipid peroxidation by TBARS and non protein thiol contents were corrected for mL of sample (SILVA et al., 2014).

2.2.9 Statistical analysis

The results are presented as mean + SEM with exception of the ID_{50} values (dose required to reduce the responses of the treated groups by 50% relative to the control group), which are reported as geometric means plus their respective 95% confidence limits. The maximum inhibitory effect (E_{max}) was calculated based on the response of the control groups. The statistical significance between the groups was assessed by one-way analysis of variance (ANOVA) followed by a post hoc Newman-Keuls test. All tests were carried out using GraphPad 5.0 Software (San Diego, CA, USA).

3 Results

3.1 Characterization of semisolid formulations

3.1.1 pH determination

The characteristics of semisolid formulations are presented in Table 1. pH values were in the acid range, close to 5.5 for all formulations, which is compatible with skin pH and suitable for cutaneous administration (SCHMID-WENDTNER; KORTING, 2006; PROW et al., 2011). Semisolids containing NCP1, NBP1 and NBP3 nanocapsule suspensions presented higher pH values ($p<0.05$) than vehicle (gellan gum + water).

3.1.2 Evaluation of mean particle size

The measures of mean diameter of the semisolids after redispersion in water are showed in Table 1. Nanostructured formulations and vehicle (dispersion of gellan gum in water) presented mean diameter on the nanometric range. For the nanostructured ones, values of mean diameter were significantly higher to the nanoformulations containing coenzyme Q10 (NCP1 and NCP3) ($p<0.05$). All formulations showed significant difference when compared to vehicle mean diameter ($p<0.05$).

3.1.3 Coenzyme Q10 content

Concerning Q10 content, the semisolid formulations containing NCP1 and NCP3 nanocapsules and free compounds revealed Q10 content close to 100%. The presence of

nanostructures or vitamin E concentration on the samples did not influenced this parameter ($p>0.05$).

3.1.4 Determination of spreadability

In relation to spreadability evaluation (Figure 1), the values of spreadability factors were 4.61 ± 0.83 and 4.63 ± 0.86 mm^2/g for NCP1 and NCP3 semisolid formulations, respectively (Table 1). For NBP1 and NBP3 they were 3.79 ± 0.35 and 3.63 ± 0.67 mm^2/g, respectively. In this case, concentration of VitE or drug presence on nanocapsules presented no influence on spreadability factor ($p>0.05$).

3.1.5 Rheological behavior

Regarding the rheological properties of semisolids, all samples evaluated showed non-Newtonian flow with pseudoplastic behavior. The rheograms, that were analyzed using different models, fit better to Herschel-Bulkey model (Figure 2 and Table 2). Flow index (n) and consistency index (K) were also established (Table 3).

3.2 *In vivo* experiments

3.2.1 Assessment of ear edema and leukocyte infiltration-UVB irradiation-induced

An UVB irradiation-induced ear edema model was used to assess the influence of Q10 and VitE nanoencapsulation on inflammatory parameters induced by UVB irradiation. The UVB irradiation on the ear in mice induced a marked increase in ear thickness with an E_{max} of 108 ± 5 μm when evaluated 24 h after UVB irradiation. Upon topical treatment with hydrogel formulations the UVB irradiation-induced ear edema and inflammatory cell infiltration were reduced effectively. All hydrogel formulations tested, except gel base (vehicle), caused a decrease of ear edema of mice with inhibitions of 33 ± 7%, 29 ± 3%, 66 ± 5%, 38 ± 9% and 73 ± 8% for FH, NBP1, NCP1, NBP3 and NCP3, respectively. Silver sulfadiazine, used as a positive control, was able to reduce UVB irradiation-induced ear edema in 70 ± 5% (Figure 3A).

MPO and NAGase play an important role in the innate immune system and they are considered biochemical markers of the inflammatory cells infiltration in the injured tissue (mostly neutrophil and macrophage, respectively). In order to evaluate the effect of the hydrogel formulations on inflammatory cells infiltration, the MPO and NAGase activities were assessed 24 h after UVB irradiation. The irradiation with a UVB source promoted an increase of MPO and NAGase activities in the untreated and vehicle-treated irradiated groups

when compared with naïve group. All hydrogel formulations tested, except gel base (vehicle), were able to decrease the MPO activity induced by UVB irradiation with a maximum inhibition of 44 ± 6%, 27 ± 4%, 42 ± 5%, 28 ± 8% and 64 ± 5% for FH, NBP1, NCP1, NBP3 and NCP3, respectively. On the other hand, only encapsulated hydrogel formulations containing VitE at the concentration of 3% (NBP3 and NCP3) reduced the NAGase activity with a maximum inhibition of 28 ± 7% for NBP3 and 25 ± 4% for NCP3. The positive control, silver sulfadiazine, decreased MPO and NAGase activities in 60 ± 3% and 40 ± 5%, respectively (Figures 3B and 3C).

Since MPO and NAGase enzyme activities indicated the inflammatory cells infiltration in untreated and vehicle-treated animals, we carried out histological analysis to confirm the inflammatory cell infiltration. We observed that UVB irradiation promoted intense inflammatory cell infiltration (92 ± 6 inflammatory cell per field) when compared with the naïve (9 ± 2 inflammatory cell per field) group. The topical treatment with encapsulated hydrogel formulations decreased the cell infiltration (21 ± 4 and 26 ± 1 inflammatory cell per field to NCP1 and NCP3, respectively) when compared with untreated UVB irradiation group (Figures 4A and 4B).

3.2.2 Oxidative stress evaluation

It has been reported that UV radiation generates ROS and their production is involved in UV-induced skin inflammatory and photodamage process. To investigate the possible antioxidant action of the coenzyme Q10 nanoencapsulated in hydrogel formulations, we evaluated the capacity of the treatments to reduce oxidative stress parameters through non protein thiols levels and lipid peroxidation. UVB irradiation promoted an increase in all oxidative stress parameters assessed. The nanoencapsulated hydrogel formulations containing coenzyme Q10 increased non protein thiols levels (100% to NCP1, NBP3 and NCP3) and decreased lipid peroxidation through lipid hydroperoxide content (100% to NCP1 and NCP3) and through TBARS assay (28 ± 4% and 100% to NCP1 and NCP3, respectively) (Figures 5A, 5B and 5C).

4 Discussion

Gellan gum has advantageous physicochemical properties that led to multiple applications on different industries: food, personal care, pharmacy and others. In pharmaceutical area, among the advantages of its use, can be emphasized: non-toxicity, biodegradability, rapid gelation in cations presence and mucoadhesive potential,

characteristics that allow its use as component of oral, ophthalmic, nasal and other formulations. Due to this, it was chosen to the development of these semisolid formulations (OSMALEK; FROELICH; TASAREK, 2014).

Some analyses were made aiming to prove the suitable characteristics of these formulations. pH values were around 5.5, which is compatible with skin pH range (SCHMID-WENDTNER; KORTING, 2006; PROW et al., 2011). This result is in agreement the study of TERROSO and co-workers, which also developed nanohydrogels of Carbopol® 940 containing coenzyme Q10 and PCL. After redispersion in water, the nanoformulations showed mean diameter values on the nanometric range, confirming the existence of the nanostructures on the semisolids. Regarding Q10 content, all values were close to 100%, indicating that no loss or minimal loss occurred during the incorporation of Q10-loaded nanocapsules in the semisolids.

In the formulation of semisolids, spreadability and rheological analysis are usually carried out. The efficacy of a topical therapy depends on its ease of spread on the substrate (GARG et al., 2002). Values of spreadability of the developed semisolids were between 3.63 ± 0.35 and 4.63 ± 0.86 mm^2/g. The higher this value, the greater the ease of spread and less force is required for application (GARG et al., 2002).

With respect to rheological properties, this evaluation can help to predict the stability of the semisolids and to control the influence of the components on the flow behavior of the formulations. All samples presented pseudoplastic non-Newtonian flow behavior, which is interesting for topical application. In this type of flow behavior, when an external force is applied, the formulation become less viscous, that favors its topical application (CHHABRA; RICHARDSON, 2008). This semisolids developed present suitable physicochemical and flow characteristics required to a formulation aimed to a topical administration.

Exposure to UV radiation, particularly UVB radiation (280-315 nm) causes skin photodamage, resulting in sunburn, immunosuppression and cancer. The skin damage promotes an inflammatory process which is characterized by the edema formation and inflammatory cell infiltration to the injured tissue (BISHOP et al., 2007; GUSTORFF et al., 2013). Moreover, UVB irradiation-induced inflammation can be mediated in affected tissue through impairment of the oxidant/antioxidant balance, which generates an increase in the cellular levels of ROS leading to damage of cellular proteins and lipids and deoxyribonucleic acid (DNA) oxidation (VALKO et al., 2007; FONSECA et al., 2010).

Sunscreens are recommended to prevent UV irradiation-induced skin injury. However, the use of the synthetic molecules in sunscreens can generate an allergic skin reaction which

limits its use long-term. This way, there is growing the search for formulations with natural compounds as new sources of protective agents (MUKHERJEE et al., 2011; YIN et al., 2013). Here, we demonstrated that hydrogel formulations, except gel base (vehicle), reduced the ear edema UVB irradiation-induced, suggesting that these formulations can inhibit some skin inflammatory responses caused by UVB exposure. The inflammatory events occur once the exposure to UVB light leads to development of skin edema, which can be considered a marker of the skin inflammation (BHATIA et al., 2011; RIGO et al., 2015). The encapsulated formulations containing coenzyme Q10 and VitE (NCP1 and NCP3) were more effective in reducing ear edema than non-encapsulated formulations containing coenzyme Q10 and VitE (FH) (Figure 3A). Besides, the coenzyme Q10 nanoencapsulation (NCP1 and NCP3) enhanced the antiedematogenic effect when compared with encapsulated formulations without coenzyme Q10 (NBP1 and NBP3), suggesting that not only the encapsulation process or vitamin E but also the presence of coenzyme Q10 contribute for the antiedematogenic property of NCP1 and NCP3. These results can be associated with the ability of nanocapsules, due to their small size, to penetrate and be situated in different layers of the skin (PROW et al., 2011). Additionally, these nanostructures can be retained in hair follicles which act as reservoirs of nanoparticles after topical administration (LADEMANN et al., 2007).

UVB irradiation promotes edema and cells inflammatory recruitment to the injured tissue (NOTARA et al., 2015). Neutrophil and macrophage tissue infiltration after UVB irradiation can be evaluated by MPO and NAGase enzymes activities. All hydrogel formulations, except gel base (vehicle), decreased neutrophil infiltration induced by UVB irradiation and NCP1, NBP1 and NBP3 were as effective as FH in reducing cell infiltration (Figure 3B). On the other hand, NCP3 presented better effect than FH, NBP1, NCP1 and NBP3 on neutrophil infiltration. Probably the presence of the vitamin E 3% in NCP3 contributed for this effect. Besides, NCP3 presented similar effect to silver sulfadiazine. Otherwise, only nanoencapsulated hydrogel formulation with vitamin E 3% was able to reduce NAGase activity since this formulation presents higher content of vitamin E (Figure 3C). These results demonstrate greater anti-inflammatory efficacy of encapsulated hydrogel formulation than non-encapsulated hydrogel formulations.

Furthermore, ROS production UVB irradiation-induced also contributes to development of inflammatory process, such as neutrophil and macrophage recruitment (HATTORI et al., 2010; DHANASEKAR et al., 2015). The encapsulated hydrogel formulations containing coenzyme Q10 were significantly more effective to reduce oxidative stress parameters than the encapsulated hydrogel formulations NBP1 and NBP3 (without

coenzyme Q10). Moreover the results indicate that the antioxidant capacity of the encapsulated hydrogel formulation is due to the coenzyme Q10 presence, but also by nanoencapsulation contribution.

The *in vivo* results suggest the potential of the purposed nanohydrogels containing the association coenzyme Q10 and vitamin E acetate in reduce effects induced by UVB irradiation on skin (inflammation and oxidative damage), showing an interesting formulation with anti-aging and anti-inflammatory properties.

5 Conclusion

In this study, we developed for the first time semisolid formulations based on nanoparticulate systems by the simple addition of gellan gum in nanocapsule suspensions. Furthermore, we have investigated anti-edematogenic, antiinflammatory and antioxidant activities of the semisolid formulations of coenzyme Q10 and VitE associated to nanocarriers and we have demonstrated their promising effects in animal model of UVB irradiation-induced inflammation.

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Table 1 - pH values, drug content, mean diameter and spreadability factors of semisolid formulations.

Formulation	pH	Coenzyme Q10 content (mg/g)	Mean diameter (nm)	Spreadability factor ($mm^2\ g^{-1}$)
Vehicle	5.2 ± 0.06	-	283 ± 16	-
Free	5.3 ± 0.08	0.95 ± 0.07	-	-
NCP1	5.7 ± 0.10	1.06 ± 0.03	331 ± 15	4.61 ± 0.83
NCP3	5.4 ± 0.08	1.05 ± 0.06	346 ± 23	4.63 ± 0.86
NBP1	5.5 ± 0.13	-	219 ± 20	3.79 ± 0.35
NBP3	5.5 ± 0.13	-	145 ± 13	3.63 ± 0.67

Table 2 - Regression coefficient (r) for various flow models in shear rate–shear stress curve obtained for semisolids.

Formulation	Bingham	Casson	Ostwald	Herschel-Bulkley
NBP1	0.920 ± 0.034	0.926 ± 0.029	0.955 ± 0.052	0.990 ± 0.001
NBP3	0.899 ± 0.009	0.940 ± 0.012	0.984 ± 0.009	0.989 ± 0.006
NCP1	0.901 ± 0.019	0.947 ± 0.013	0.989 ± 0.004	0.994 ± 0.005
NCP3	0.885 ± 0.015	0.935 ± 0.014	0.983 ± 0.004	0.990 ± 0.001
Free	0.909 ± 0.007	0.951 ± 0.007	0.989 ± 0.003	0.993 ± 0.001
Vehicle	0.895 ± 0.022	0.939 ± 0.015	0.985 ± 0.005	0.994 ± 0.003

Table 3 - Flow index (n) and consistency index (K) of semisolid formulations, according to Herschel-Bulkley flow model.

Formulation	n	K
NBP1	0.106 ± 0.031	4370 ± 745
NBP3	0.114 ± 0.006	4481 ± 345
NCP1	0.122 ± 0.010	4124 ± 401
NCP3	0.115 ± 0.003	4664 ± 499
Free	0.133 ± 0.009	4371 ± 391
Vehicle	0.137 ± 0.009	3856 ± 273

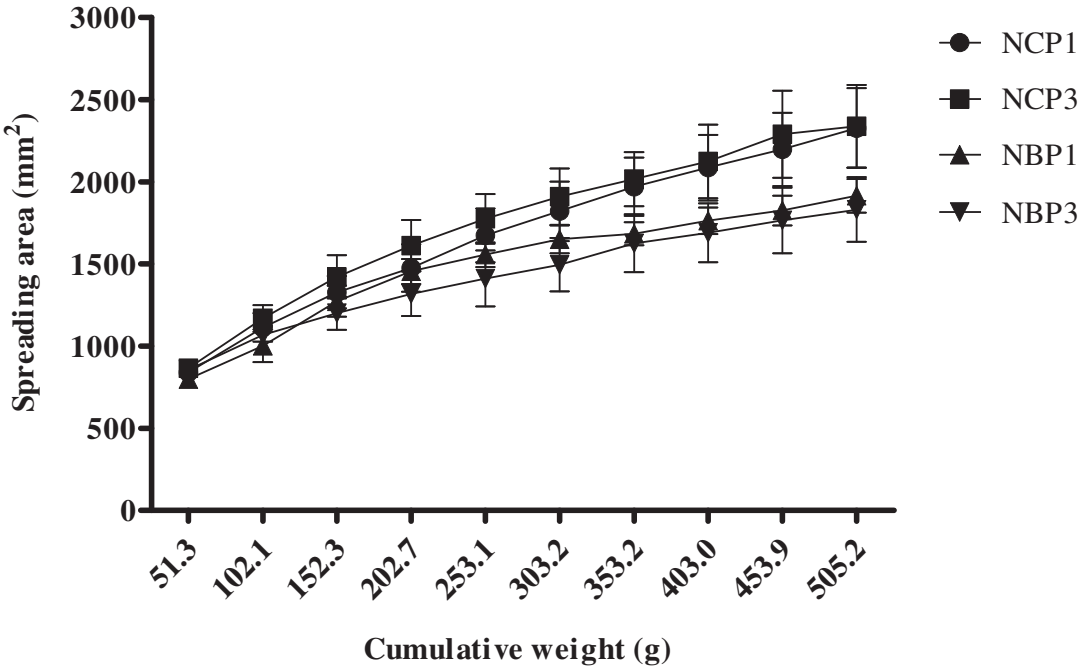

Figure 1 - Spreadability (mm^2) of semisolid formulations of gellan gum with nanoencapsulated systems.

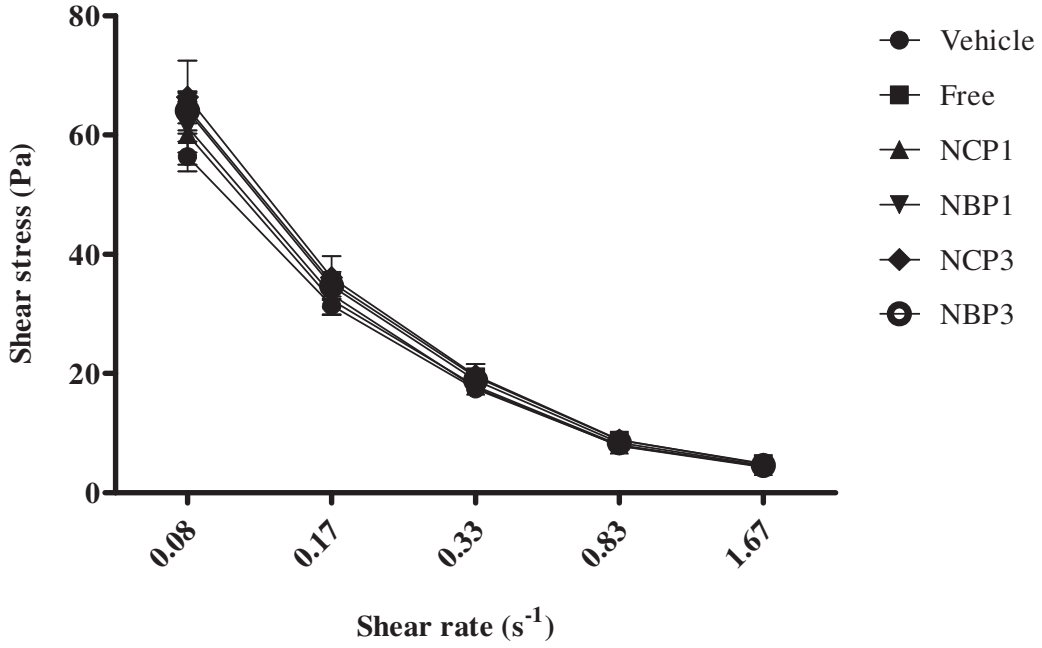

Figure 2 - Rheological behavior of the semisolid formulations of gellan gum with coenzyme Q10 and VitE non-encapsulated (free) or nanoencapsulated systems (NBP1, NBP3, NCP1, NCP3).

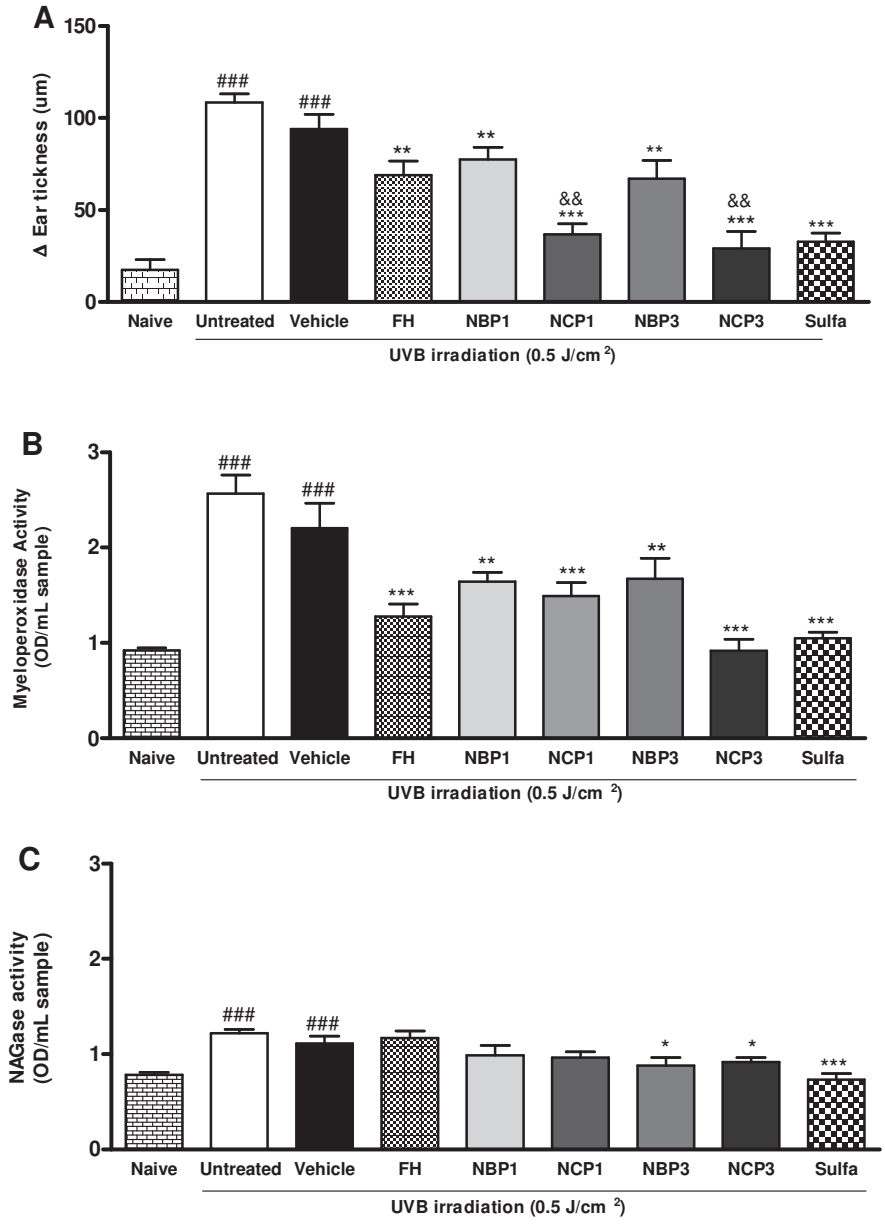

Figure 3 - Anti-inflammatory effects of hydrogel formulations on UVB irradiation-induced skin injury in mice. Ear edema (A) and Myeloperoxidase (B) and NAGase (C) activities in mice submitted to UVB irradiation (0.5 J/cm^2). All formulations (15 mg/ear) were applied immediately after UVB irradiation. Ear edema and cell infiltration were measured 24 h after irradiation. Each bar represent the mean + SEM (n=6-7); $^{###}$P<0.001 when compared with the naïve group. *P<0.05, **P<0.01 and ***P<0.001 when compared with the untreated group. $^{&&}$P<0.01 shows significant different between encapsulated formulations containing coenzyme Q10 (NCP1 and NCP3) with encapsulated formulations without coenzyme Q10 (NBP1 and NBP3). One-way ANOVA followed by post hoc Newman-Keuls test.

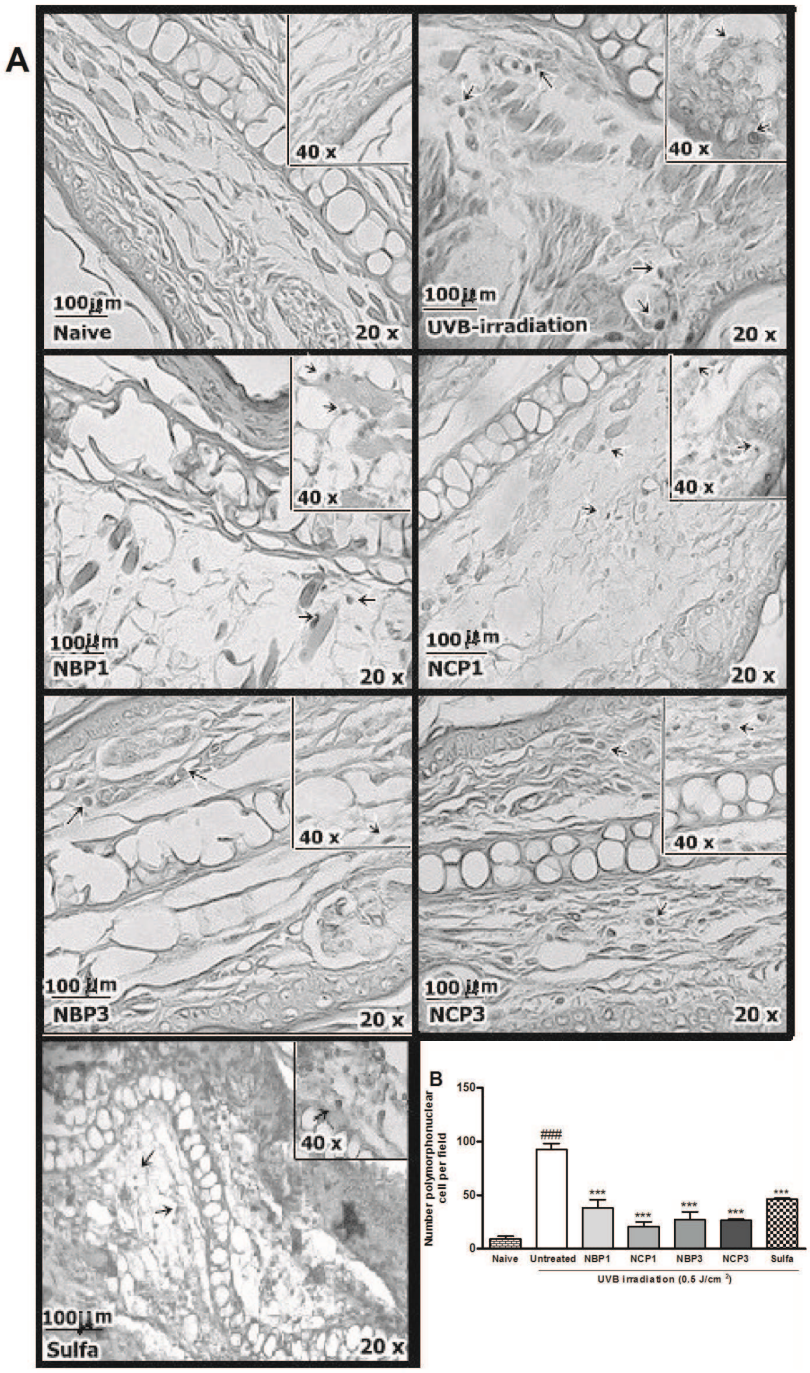

Figure 4 - Anti-inflammatory (cell infiltration) effects of hydrogel formulations on UVB irradiation-induced skin injury in mice. Ear representative light microphotographic (arrows indicate polymorphonuclear cells) and quantification of polymorphonuclear cells per field (B) of the ear tissue of mice 24 h after UVB irradiation or UVB-irradiation plus hydrogel formulations. Each bar represent the mean+ SEM (n=6-7); $^{###}$P<0.001 when compared with the naïve group. ***P<0.001 when compared with the untreated group. One-way ANOVA followed by post hoc Newman-Keuls test.

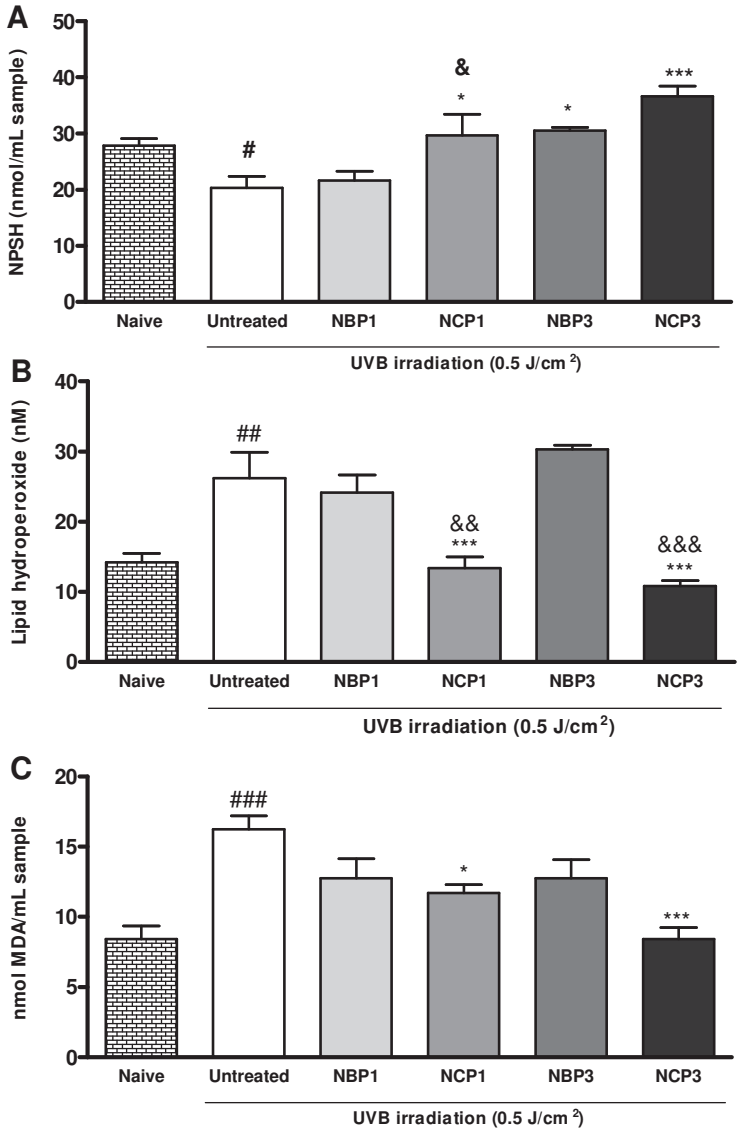

Figure 5 - Effect of hydrogel formulations on stress oxidative parameters induced by UVB irradiation in mice. Non protein thiols (NPSH) levels (A) and lipid peroxidation (B) and (C) in mice submitted to UVB irradiation (0.5 J/cm^2). The oxidative stress parameters were evaluated 24 h after UVB irradiation. Each bar represent the mean+ S.E.M (n=6-7); $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ when compared with the naïve group. *P<0.05 and ***P<0.001 when compared with the untreated group. $^{\&}P<0.05$, $^{\&\&}P<0.01$ and $^{\&\&\&}P<0.001$ show significant different between encapsulated formulations containing coenzyme Q10 (NCP1 and NCP3) with their encapsulated formulations without coenzyme Q10 (NBP1 and NBP3). One-way ANOVA followed by post hoc Newman-Keuls test.

DISCUSSÃO GERAL

A nanotecnologia, nas últimas décadas, tem sido proposta como uma importante alternativa às formulações convencionais na aplicação de fármacos pelas diferentes vias de administração. Isto se deve às inúmeras vantagens conferidas pelas nanopartículas, tais como a vetorização de fármacos ao sítio específico de ação e sua liberação controlada no organismo, podendo resultar na redução da dose administrada e sua frequência, assim como na redução de efeitos adversos. Por isso, diversas classes de fármacos já têm sido incorporadas a diferentes tipos de nanoestruturas, visando benefícios na terapêutica de inúmeras doenças e condições. No que diz respeito às características do fármaco incorporado, os nanocarreadores podem ainda conferir aumento da solubilidade e proteção frente à degradação, sendo muitas vezes utilizados no sentido de reduzir alguns tipos de instabilidade destas moléculas.

A coenzima Q10 é uma substância ativa que apresenta inúmeras limitações quando se trata do desenvolvimento de formulações farmacêuticas: elevada massa molecular, que implica em baixa solubilidade e baixa biodisponibilidade oral, e instabilidade frente à luz. No entanto, apresenta inúmeras potencialidades na terapêutica, as quais ainda são, de certa forma, comprometidas por estas limitações. Neste sentido, buscou-se o desenvolvimento de uma formulação alternativa, com o propósito de ampliar o leque de aplicações terapêuticas desta molécula antioxidante.

Como primeira abordagem deste trabalho, foram desenvolvidas suspensões de nanocápsulas contendo coenzima Q10 na concentração de 1 mg/mL, empregando como polímero a PCL. Paralelamente, tentou-se desenvolver a mesma formulação com a utilização do polímero Eudragit® RS100, porém o mesmo apresentou interação com a vitamina E acetato no teste de inchamento/dissolução de filme polimérico sendo, portanto, esta formulação excluída do estudo. Associou-se a vitamina E acetato em três concentrações (1%, 2% e 3%) às nanocápsulas de PCL, uma vez que, tratando-se de uma matéria-prima de caráter oleoso, poderia formar o núcleo das nanocápsulas.

Estas formulações foram primeiramente utilizadas para investigar a capacidade do sistema desenvolvido em proteger a coenzima Q10 frente à degradação por radiação, empregando, para isto, câmara espelhada com lâmpada UVC acoplada. Para a determinação do teor remanescente de coenzima Q10 nas suspensões nanoestruturadas, utilizou-se metodologia analítica previamente validada por MATTIAZZI e colaboradores (2014) para a determinação por cromatografia líquida de alta eficiência (CLAE). A metodologia analítica foi validada para os seguintes parâmetros: linearidade, especificidade, precisão, exatidão e robustez.

No estudo de fotoestabilidade, observou-se que, quanto maior a concentração de vitamina E acetato na formulação, maior o teor remanescente de coenzima Q10 após 4 horas de exposição à luz UVC, demonstrando assim a importância da presença deste óleo como componente das nanocápsulas. Em um estudo paralelo conduzido em nosso grupo de pesquisa, o qual avaliou a fotoestabilidade da coenzima Q10 associada a nanocápsulas de óleo de pracaxi, empregando as mesmas condições experimentais, mostrou-se que estas nanoestruturas foram capazes de proporcionar teor remanescente de fármaco entre 51 e 56% (MATTIAZZI, 2014).

Em outro estudo de nosso grupo, o qual avaliou a fotoestabilidade da coenzima Q10 associada a nanocápsulas com núcleo de óleo de linhaça ou de óleo de argan, as mesmas apresentaram teor remanescente de coenzima Q10 entre 41 e 51% ao final das 4 horas de exposição à radiação UVC (STANGARLIN, 2014). Estes dois trabalhos ajudam a reforçar a hipótese de que a vitamina E acetato, devido a sua constituição rica em compostos antioxidantes, teve papel fundamental na fotoestabilização da coenzima Q10.

Como etapa de desenvolvimento de formulação, pretendia-se também conduzir o experimento de liberação *in vitro* da coenzima Q10 a partir das nanocápsulas, utilizando sacos de diálise. No entanto, devido à elevada lipofilia desta molécula, não foi possível estabelecer, fazendo o uso da infraestrutura e materiais que dispomos em nosso laboratório, uma condição experimental que favorecesse a realização do experimento e o estudo do mecanismo de liberação da coenzima Q10 a partir das nanocápsulas.

Posteriormente, selecionou-se a formulação com concentração intermediária de vitamina E acetato (2%), a qual apresentou características físico-químicas adequadas e considerável proteção do fármaco frente à degradação, para os experimentos de citotoxicidade *in vitro*. Para isto, o desempenho destas formulações foi avaliado contra duas linhagens de células tumorais de expressiva ocorrência mundial: melanoma e glioma humano. Mostrou-se o efeito citotóxico nas duas linhagens tumorais na concentração de 100 μM, tanto para as formulações contendo coenzima Q10 e vitamina E acetato encapsulados, quanto para a formulação com apenas vitamina E encapsulada. Diferentes concentrações abaixo de 100 μM foram também testadas, porém as mesmas não levaram à morte celular nas condições experimentais a que foram submetidas.

Para o estudo *in vivo*, a segunda etapa de desenvolvimento deste trabalho, fez-se necessário o desenvolvimento de um semissólido, uma vez que o modelo de inflamação escolhido requer o tratamento dos animais por via tópica. Estes semissólidos foram

produzidos a partir da incorporação de goma gelana (2%) às formulações de nanocápsulas com a maior e menor concentração de vitamina E acetato (1% e 3%).

Estudos preliminares conduzidos em nosso laboratório empregando goma gelana mostraram resultados interessantes no uso deste polímero no desenvolvimento de formulações. Neste sentido, pensou-se em associar o mesmo à proposta inicial deste trabalho. Além da concentração de 2%, também foi testada a concentração de 2,5% de goma gelana para incorporação nas suspensões de nanocápsulas. No entanto, as formulações resultantes apresentaram-se muito consistentes, sendo descartado o emprego desta concentração de goma, pois isto poderia dificultar a aplicação dos semissólidos.

As formulações desenvolvidas foram então caracterizadas quanto a pH, teor de fármaco, diâmetro médio de partícula pós-redispersão em água, espalhabilidade e comportamento reológico.

Escolheu-se o modelo de inflamação induzida por radiação em camundongos, uma vez que já tínhamos conduzido o experimento de fotodegradação da coenzima Q10 e que o foco principal do trabalho seria a administração tópica. Além disso, outros fatores levaram a pensar neste modelo como uma boa proposta: sabe-se da importância da presença de antioxidantes na potencialização do efeito de fotoprotetores e dos efeitos provocados por radiação UV como, além de inflamação, o dano oxidativo ao tecido cutâneo e seu envelhecimento. Neste estudo, avaliaram-se diferentes tipos de atividades biológicas para as formulações desenvolvidas: antiedematogênica, anti-inflamatória e antioxidante.

A atividade antiedematogênica foi avaliada a partir da medida da espessura da orelha irradiada dos camundongos, após 24 horas de tratamento com a formulação. Todas as formulações testadas, exceto o veículo, foram capazes de levar à inibição do edema, sendo que o maior nível de inibição, 73 ± 8%, foi demonstrado para o semissólido contendo a suspensão de nanocápsulas com coenzima Q10 e com a maior concentração de vitamina E acetato. Esta atividade foi superior à inibição provocada pela sulfadiazina de prata, 70 ± 5%, a qual é utilizada como controle positivo do tratamento. Este resultado evidencia não só a atividade antiedematogênica da Q10, mas também o efeito da vitamina E acetato variando com a sua concentração na amostra.

Subsequentemente, a atividade anti-inflamatória foi elucidada a partir da avaliação da atividade de enzimas marcadoras de infiltração celular no tecido injuriado: MPO e NAGase, e da análise histológica. Para a atividade da enzima MPO, todas as formulações, exceto o veículo, levaram à redução da atividade, cuja maior redução foi de 64 ± 5%, para o semissólido contendo nanocápsulas com coenzima Q10 e com a maior concentração de

vitamina E acetato, comparada à redução de 60 ± 3% provocada pelo controle. Para a enzima NAGase, apenas as formulações contendo a maior concentração de vitamina E acetato, contendo ou não coenzima Q10, promoveram redução da atividade, a qual esteve em torno de 25%, comparada à redução de 40 ± 5% da substância controle. Aqui, mais uma vez destaca-se a importância, não só da substância ativa coenzima Q10, mas também do componente oleoso na atividade biológica da formulação.

No intuito de confirmar a infiltração de células inflamatórias no tecido injuriado, também foi realizada uma análise histológica. Corroborando com o resultado da atividade das enzimas, esta análise mostrou a redução do número de células inflamatórias por campo para as duas formulações investigadas: ambas contendo coenzima Q10 e com concentrações de vitamina E acetato de 1% e 3%.

Por fim, investigou-se também a capacidade dos tratamentos em reduzir o estresse oxidativo induzido. As formulações contendo coenzima Q10 e vitamina E acetato nas concentrações de 1% e 3% (NCP1 e NCP3) e a formulação contendo apenas vitamina E acetato na concentração de 3% (NBP3) reduziram o nível de tióis não-protéicos em 100%. NCP1 e NCP3 reduziram o conteúdo de hidroperóxidos lipídicos 49 ± 6% e 59 ± 3%, respectivamente. No que diz respeito ao conteúdo de substâncias reativas ao ácido tiobarbitúrico (TBARS), o mesmo foi reduzido em 28 ± 4% pelo tratamento com NCP1 e em 48 ± 5% pelo tratamento com NCP3.

CONCLUSÕES

Em conclusão, o conjunto dos resultados obtidos demonstra que os sistemas nanoestruturados desenvolvidos com a associação entre coenzima Q10 e vitamina E acetato são alternativas promissoras tanto para o tratamento do glioma e melanoma, quanto para proporcionar proteção contra lesão de pele induzida por radiação ultravioleta. Por fim, como perspectiva para a conclusão final deste trabalho e submissão a periódicos científicos, destaca-se a dosagem de citocinas pró-inflamatórias e anti-inflamatórias, a fim de elucidar seu envolvimento no dano tecidual induzido.

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