UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Desenvolvimento tecnológico de nanoemulsões catiônicas contendo isoflavonóides de *Glycine max* (soja) visando ao tratamento do herpes

Débora Fretes Argenta

Porto Alegre, 2015

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Tese apresentada por **Débora Fretes Argenta** para obtenção do TÍTULO DE DOUTOR em Ciências Farmacêuticas

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Porto Alegre, 2015

Tese apresentada ao Programa de Pós Graduação em Ciências Farmacêuticas, em nível de Doutorado, da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul e aprovada em 20.03.2015, pela Banca Examinadora constituída por:

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Desenvolvimento tecnológico de nanoemulsões catiônicas contendo isoflavonóides de Glycine max (soja) visando ao tratamento do herpes / Débora Fretes Argenta. -- 2015.
197 f.

Orientador: Helder Ferreira Teixeira.
Coorientadora: Cláudia Maria Oliveira Simões.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Farmácia, Programa de Pós-Graduação em Ciências Farmacêuticas, Porto Alegre, BR-RS, 2015.

1. Genisteína. 2. Cumestrol. 3. Nanoemulsão. 4. Pele/mucosa. 5. Herpes. I. Teixeira, Helder Ferreira, orient. II. Simões, Cláudia Maria Oliveira, coorient. III. Título.

Agradecimentos a CAPES, órgão financiador da bolsa de estudos para desenvolvimento desta tese, a FAPERGS pelo auxílio financeiro na realização dos experimentos, ao Laboratório de Desenvolvimento Galênico da Universidade Federal do Rio Grande do Sul e ao Laboratório de Virologia Aplicada da Universidade Federal de Santa Catarina, que disponibilizaram todos os equipamentos necessários para a realização dos experimentos.

AGRADECIMENTOS

Ao meu orientador, Prof. Dr. Helder Ferreira Teixeira e a minha coorientadora Profa Dra Cláudia Maria Oliveira Simões, pela oportunidade, ensinamentos, pelos valiosos conselhos, pela confiança e, sobretudo pela amizade.

Aos meus pais Laércio e Dory, pelo amor e apoio incondicional, e por me guiarem até aqui.

Aos meus irmãos Filipe e Vinícius, meus orgulhos, obrigada por estarem na minha vida.

Ao meu marido, Mauricio, que não mediu esforços para me apoiar. Obrigada pela paciência, compreensão e amor.

À toda minha família, aos meus sogros, cunhados e cunhadas.

A todos os funcionários da Faculdade de Farmácia-UFRGS que possibilitaram a realização deste trabalho.

Aos professores do Laboratório de Desenvolvimento Galênico (LDG), Prof. George Ortega, Prof. Pedro R. Petrovick, Profa. Letícia Koester e Profa. Valquíria Linck Bassani.

Aos colegas do Laboratório de Desenvolvimento Galênico, por toda contribuição científica, pela amizade, carinho e momentos de descontração.

Em especial, à Cristiane Mattos e Juliana Bidone, minhas grandes amigas.

Ao Roger, pela amizade e boas conversas

Aos colegas do Laboratório de Virologia Aplicada-UFSC, pelo acolhimento, ensinamentos e pela amizade.

Aos colegas e professores do Laboratório de Farmcognosia e do Laboratório de Farmacotécnica da UFSC por cederem o espaço e disponibilizarem os equipamentos durante a realização deste trabalho.

RESUMO

A atividade antiviral de compostos flavonoídicos tem sido amplamente investigada nos últimos anos. Nesse contexto, a primeira etapa da presente tese teve por objetivo avaliar a atividade anti-herpética *in vitro* dos principais isoflavonóides de *Glycine max* (soja): genisteína, daidzeína, gliciteína e cumestrol. Dentre os isoflavonóides investigados, a genisteína e o cumestrol mostraram uma interessante atividade frente aos vírus HSV-1 (cepa KOS e 29R, sensível e resistente ao aciclovir, respectivamente) e HSV-2 (cepa 333), interferindo em diferentes etapas do ciclo de replicação dos vírus. Devido à reduzida hidrossolubilidade desses isoflavonóides, foi proposta a sua incorporação em nanoemulsões de uso tópico. As formulações foram otimizadas através de um experimento fatorial completo do tipo 2^3. O efeito dos fatores tipo de óleo (óleo de rícino ou miristato de isopropila), co-tensoativo iônico (oleilamina ou ácido olêico) e fosfolipídeo (DSPC ou DOPC) sobre as propriedades das nanoemulsões e a retenção da genisteína na pele de orelha suína foi investigado. Nanoemulsões compostas de miristato de isopropila/ oleilamina/DOPC apresentaram um menor diâmetro de gotícula e uma maior retenção de genisteína na pele de orelha suína enquanto que a combinação miristato de isopropila/oleilamina/DSPC mostrou o menor índice de polidispersão. A viscosidade das formulações selecionadas foi ajustada através do uso de hidroxietilcelulose visando à obtenção de produtos de uso tópico, obtendo-se hidrogéis de comportamento pseudoplástico. Na sequência, um conjunto de resultados obtidos demonstrou que a composição das formulações (especialmente o fosfolipídeo DOPC e o agente espessante hidroxietil celulose) pode influenciar a liberação e a retenção dos isoflavonóides em mucosa esofágica suína. A integridade da mucosa também desempenha um papel no aumento da permeação/retenção do cumestrol, conforme ilustrado nas imagens de microscopia confocal, utilizando vermelho do Nilo como marcador fluorescente. De maneira geral, a incorporação dos isoflavonóides nas nanoemulsões aumenta a atividade anti-herpética desses compostos *in vitro*. O conjunto dos resultados demonstra que as formulações desenvolvidas são potenciais carreadores de uso tópico para genisteína e cumestrol no tratamento das infecções herpéticas.

Palavras-chave: genisteína, cumestrol, nanoemulsão, pele, mucosa, herpes

ABSTRACT

The antiviral activity of flavonoid compounds has been extensively investigated in recent years. In this context, the first step of this study was to evaluate the anti-herpes activity *in vitro* of the main isoflavonoids of *Glycine max* (soybean): genistein, daidzein, glycitein and coumestrol. Among the investigated isoflavonoids, genistein and coumestrol showed interesting activity against HSV-1 (KOS and 29R strains, which are acyclovir-sensitive and acyclovir–resistant strains, respectively) and HSV-2 (333 strain), interfering at different stages of the virus replication cycle. Due to the low hydrosolubility of these isoflavonoids, their incorporation into topical nanoemulsions was proposed in this study. The formulations were optimized through a 2^3 full factorial design. The factors effect of oil type (castor oil or isopropyl myristate), ionic co-surfactant (oleylamine or oleic acid) and phospholipid type (DSPC or DOPC) on physicochemical properties and genistein retention into porcine ear skin was investigated. Nanoemulsions composed of isopropyl myristate/ DOPC/oleylamine showed the smaller particle size and higher genistein retention into skin, whereas the formulation composed of isopropyl myristate/DSPC/oleylamine exhibited the lower polydispersity index. The viscosity of selected formulations was adjusted with hydroxyethyl cellulose to obtain topical products, which showed non-Newtonian behavior. In sequence, a set of results showed that formulations composition (especially the phospholipid DOPC and the thickening agent hydroxyethyl cellulose) could influence the release and retention of isoflavonoids in porcine esophagus mucosa. The integrity of mucosa plays a role on the increase of coumestrol permeation/retention, according to confocal microscopy images, using Red Nile as fluorescent label. In general terms, the incorporation of isoflavonoids into nanoemulsions increases the antiherpes activity of these compounds *in vitro*. The overall results show that the formulations developed in this study are potential topical carriers for genistein and coumestrol in the treatment of herpes infections.

Keywords: genistein, coumestrol, nanoemulsion, skin, mucosa, herpes

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INTRODUÇÃO

Estudos de avaliação da potencialidade terapêutica de extratos, frações e/ou compostos isolados da soja têm sido objeto de diversas pesquisas em diferentes áreas. Dentre os compostos presentes na soja, com elevado potencial terapêutico, encontram-se os isoflavonóides, os quais apresentam distribuição taxonômica restrita no reino vegetal, sendo isolados principalmente da família Fabaceae (Simões *et al*., 2004). Diversos estudos epidemiológicos têm relacionado o consumo de soja com uma menor incidência e mortalidade por doenças crônicas e degenerativas, como afecções cardiovasculares e câncer (Willis e Wians, 2003; Arts e Hollamn, 2005; Sim e Cheng, 2005; González e Duran, 2014). Entre os isoflavonóides mais estudados desse grão destacam-se a genisteína, daidzeína e gliciteína, pertencentes à subclasse isoflavona e o cumestrol pertencente à subclasse dos cumestanos.

Os benefícios descritos para os isoflavonóides podem ser relacionados às atividades estrogênica e antioxidante desses compostos (Cooke, Selvaraj e Yellayi, 2006; Messina, 2014). Por produzirem efeitos análogos aos hormônios estrogênicos, esses isoflavonóides são considerados fitoestrógenos (Cos *et al.*, 2003). Uma vez que receptores de estrogênio são expressos na pele, a aplicação tópica desses compostos pode inibir ou reduzir os efeitos indesejáveis na pele devido ao declínio fisiológico de estrogênio (Masuda *et al*., 2011; Emmerson e Hardman, 2012). Ainda, devido à atividade antioxidante e anti-inflamatória, os isoflavonóides podem inibir ou atenuar o dano oxidativo de processos patológicos envolvidos no desenvolvimento de doenças que afetam a pele (Baxa e Yoshimura, 2003; F'guyer, Afaq e Mukhtar, 2003; Dijsselbloem *et al.*, 2004).

Além dessas propriedades, estudos demonstram que os isoflavonóides apresentam uma potencial atividade antiviral, sendo a genisteína o composto mais investigado. Os estudos demonstram que a genisteína é capaz de reduzir a infectividade de diversos vírus, incluindo os vírus herpes humanos (Andres, Donovan e Kuhlenschmidt, 2009). A resistência dos vírus herpéticos aos fármacos de primeira escolha e a prevalência das infecções em pacientes imunocomprometidos, tornam as infecções herpéticas uma importante questão de saúde pública (Strasfeld e Chou, 2010; Hodge e Field, 2013). Até o momento não existe tratamento definitivo para o herpes simples, uma vez que a farmacoterapia disponível apenas minimiza as crises

e retarda o aparecimento de novas manifestações. Neste contexto, estudos da avaliação anti-herpética de grupos de compostos naturais com potencial atividade antiviral, tais como os isoflavonóides, são de grande interesse.

No entanto, de maneira geral, as atividades biológicas demonstradas para os isoflavonóides da soja são especialmente atribuídas à sua forma livre (aglicona), a qual apresenta reduzida hidrossolubilidade. Essa característica tem impulsionado a investigação de diferentes estratégias tecnológicas (i.e. formação de complexos de inclusão com ciclodextrinas, encapsulamento em lipossomas e a incorporação em nanopartículas) que permitam a sua dispersão em meio aquoso (Nemitz *et al*., 2014), visando o desenvolvimento de produtos de uso tópico.

Recentemente, em nosso grupo de pesquisa, demonstramos a viabilidade de incorporação de agliconas flavonoídicas em nanoemulsões de uso tópico, incluindo compostos da subclasse dos isoflavonóides, como as isoflavonas (genisteína) e cumestanos (cumestrol) (Silva *et al.*, 2009; Argenta *et al.*, 2011). Devido a sua reduzida hidrossolubilidade, esses compostos encontram-se preferencialmente dispersos e/ou adsorvidos na interface das gotículas da fase interna (constituídas de triglicerídeos de cadeia média) de nanoemulsões estabilizadas por lecitina de gema de ovo isolada ou em combinação com tensoativos não-iônicos. Os resultados preliminares indicam um fluxo lento desses compostos a partir das nanoemulsões desenvolvidas através da pele de orelha suína, apontando para uma potencial aplicação destes produtos para uso tópico/local.

Com base nesses resultados, o presente trabalho visa otimizar essas formulações nanoemulsionadas a partir de um planejamento fatorial, com ênfase no desenvolvimento de produtos para o tratamento local (de pele e de mucosas) de infecções herpéticas, bem como avaliar *in vitro* a atividade desses produtos frente a cepas sensíveis e resistentes ao aciclovir. Após a revisão da literatura acerca do tema, a tese apresenta os resultados experimentais em quatro capítulos sequenciais, como segue:

— O primeiro capítulo apresenta os resultados da investigação da atividade anti-herpética *in vitro* dos principais isoflavonóides da soja, bem como o mecanismo de ação dos compostos bioativos.

— O segundo capítulo descreve o desenvolvimento e otimização de sistemas nanoemulsionados contendo genisteína com base nas propriedades físico-químicas das nanoemulsões e retenção cutânea da genisteína, bem como a avaliação da atividade anti-herpética das formulações selecionadas.

— O terceiro capítulo apresenta os resultados obtidos no estudo com nanoemulsões catiônicas espessadas com hidroxietilcelulose sobre a reologia, liberação e permeação/retenção da genisteína em mucosa suína. Esse capítulo também apresenta os resultados da atividade anti-herpética das nanoemulsões contendo genisteína frente a uma cepa resistente ao aciclovir.

— O quarto capítulo descreve as propriedades físico-químicas de nanoemulsões catiônicas contendo cumestrol, a sua permeação/retenção em mucosa suína (íntegra e lesada), bem como a avaliação da atividade anti-herpética *in vitro.*

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OBJETIVOS

Objetivo geral

Preparar, caracterizar as propriedades físico-químicas, avaliar a permeação/ retenção em pele e mucosa suína e atividade anti-herpética de nanoemulsões de uso tópico contendo isoflavonóides da soja (*Glycine max*)

Objetivos específicos

— Avaliar a atividade anti-herpética de isoflavonóides da soja frente aos vírus HSV-1 e HSV-2, bem como investigar o mecanismo de ação antiviral dos compostos bioativos;

— Otimizar a composição de nanoemulsões de uso tópico para a genisteína através de um planejamento fatorial do tipo 2^3, com base nas propriedades físico-químicas e retenção cutânea deste isoflavonóide;

— Preparar e caracterizar propriedades físico-químicas e reológicas de hidrogéis contendo as nanoemulsões otimizadas para os isoflavonóides selecionados genisteína e cumestrol;

— Avaliar a permeação, retenção e distribuição dos isoflavonóides genisteína e/ou cumestrol através de mucosa esofágica suína integra e lesada a partir das formulações;

— Investigar a atividade anti-herpética *in vitro* das nanoemulsões contendo genisteína ou cumestrol frente aos vírus HSV-1 e HSV-2 em cepas sensíveis e resistentes ao aciclovir.

REVISÃO DA LITERATURA

Propriedades biológicas dos isoflavonóides da soja

Diversos estudos têm relacionado o consumo de uma dieta rica em isoflavonóides com uma menor incidência e mortalidade por doenças crônicas e degenerativas, como afecções cardiovasculares e alguns tipos de câncer (Willis and Wians, 2003; Sim e Cheng, 2005; Yang *et al.*, 2012; Tsugane e Sawada, 2014). Dessa forma, o consumo desses compostos parece ter papel protetor contra diversas doenças. Como exemplo, evidências mostram o papel de fatores dietéticos e atividade física no desenvolvimento e progressão do câncer de próstata, sendo que os hábitos alimentares representam os principais elementos que afetam as taxas desse tipo de câncer inter-etnias (Kolonel, Nomura e Cooney, 1999; Willis e Wians, 2003). Os compostos fitoquímicos presentes nos produtos naturais, como a soja, tem o potencial de interagir com um conjunto de vias cancerígenas, incluindo os processos do ciclo celular e apoptose, a manutenção do potencial de membrana mitocondrial e da resposta ao estresse oxidativo (Lindström *et al.*, 2012).

A soja é uma leguminosa rica em isoflavonóides. Entre os isoflavonóides mais estudados desse grão destacam-se a genisteína, daidzeína e gliciteína, pertencentes à subclasse isoflavona e o cumestrol pertencente à subclasse cumestano (Figura 1). Esses compostos se apresentam predominantemente em formas heterosídicas, sendo necessária a hidrólise da ligação heterosídica para liberar a aglicona, composto biologicamente ativo. Após administração oral, esta reação ocorre durante o processo de digestão através das bactérias da flora intestinal (Atkinson, Frankenfeld e Lampe, 2005; Cassidy *et al.*, 2006).

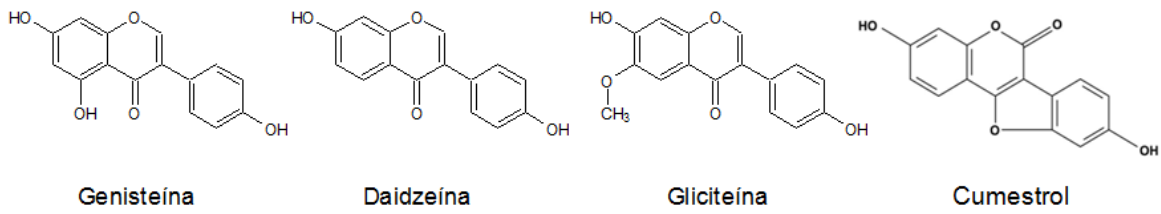

Figura 1. Estruturas químicas dos isoflavonóides encontrados na *Glycine max* (soja).

Os benefícios descritos para os isoflavonóides são relacionados, principalmente, a atividade estrogênica e antioxidante desses compostos (Cooke, Selvaraj e Yellayi, 2006; Messina, 2014).

Atividade estrogênica

Por produzirem efeitos análogos aos hormônios estrogênicos, esses isoflavonóides são descritos como fitoestrogênios (Cos *et al*., 2003). A atividade estrogênica tem sido demonstrada em estudos epidemiológicos e experimentais, que sugerem que o elevado consumo de soja está associado com a redução dos sintomas característicos do período pós-menopausa e doenças relacionadas ao hormônio estrogênio, como câncer de próstata e de mama, e osteoporose (Willis e Wians, 2003; Moraes *et al*., 2009; Wada *et al*., 2013). Os fitoestrógenos são menos potentes que o hormônio estrogênio, portanto, ao competir com esse pela ligação nos receptores, os fitoestrogênios atuam como antiestrógenos, pois impedem que o hormônio exerça seus efeitos. Porém, em ambientes com baixa concentração do hormônio, os fitoestrógenos se ligam aos receptores e apresentam efeitos estrogênicos. Contudo, apesar do mecanismo de atividade dos fitoestrogênios não ser totalmente conhecido, a atividade de agonista ou antagonista parece ser dependente da presença e concentração do hormônio endógeno, bem como da afinidade pelos receptores estrogênicos (ERα e ERβ) e do órgão alvo (Hwang *et al.*, 2006; Thornton, 2013).

A distribuição intracelular dos receptores de estrogênio é tecido dependente. As proteínas do ERα e ERβ têm 97% de homologia, no entanto, no domínio de ligação eles apresentam somente 59% de homologia. Isso indica que os compostos se ligam com diferentes afinidades a esses receptores. Apesar do 17β-estradiol apresentar afinidade semelhante para ambos os receptores, os fitoestrogênios possuem afinidades diferentes em relação ao tipo de receptor estrogênico e são menos potentes que o 17β-estradiol em ambos os subtipos (Thornton, 2013). Kuiper e colaboradores (1998) avaliaram a atividade estrogênica de fitoestrogénos, incluindo genisteína, daidzeína e cumestrol. Os experimentos foram realizados através de ensaios de competição pela ligação às proteínas dos receptores ERα e ERβ e por expressão transiente de gene. Utilizando concentrações de 1 a 10 nM, os autores obtiveram o seguinte ranking de potência estrogênica: zearalenol = cumestrol > genisteina> daidzeina > biochanina A > quercetina para receptores ERα e genisteína > cumestrol > zearalenol > daidzeina > biochanina A > quercetina para

receptores ERβ. Dentre os flavonóides avaliados, os isoflavonóides genisteína e cumestrol apresentaram atividades mais potentes.

Uma vez que receptores de estrogênio estão presentes na pele, sendo o subtipo ERβ amplamente expressado na epiderme, vasos sanguíneos e fibroblastos dérmicos (Hall e Phillips, 2005), o declínio do hormônio estrogênio em mulheres no período pós-menopausa está associado à pele seca, afinamento da epiderme, redução da camada córnea, diminuição do colágeno na derme, formação de rugas e da flacidez. Devido a essas indesejáveis alterações, muitas mulheres buscam a terapia da reposição hormonal, que além de reduzir os efeitos característicos da menopausa, tem gerado efeitos benéficos na pele, como aumento de colágeno e da espessura dérmica. Também se observou uma maior hidratação da pele e o aumento da atividade das glândulas sebáceas (Archer, 2012; Masuda *et al.*, 2011; Uyar *et al.*, 2014)

Atividade antioxidante e anti-inflamatória

Os isoflavonóides são bem conhecidos por sua propriedade antioxidante. Estudos de avaliação da atividade antioxidante dos isoflavonóides da soja têm mostrado que o cumestrol apresenta atividade mais potente que as isoflavonas genisteína e daidzeína (Brozic *et al.*, 2006; Lee *et al.*, 2006). A atividade antioxidante dos isoflavonóides ocorre devido à presença de estruturas de anel conjugados e grupos hidroxilas que têm o potencial para atuar como antioxidantes, sendo que a atividade desses compostos tem sido observada em experimentos *in vitro* com cultura de células ou em sistemas isentos de células, através da eliminação de ânions superóxido, oxigênio singleto, radicais lipoperoxidos e/ou estabilizador de radicais livres envolvidos nos processos oxidativos de hidrogenação ou através da complexação com espécies oxidantes (Robak e Gryglewski, 1988; Shahidi, Janitha e Wanasundara, 1992; Birt, Hendrich e Wang, 2001).

O dano oxidativo desempenha importante papel em processos patológicos e está envolvido diretamente no desenvolvimento de doenças que afetam a pele (F'guyer, Afaq e Mukhatar, 2003). A exposição crônica à radiação ultravioleta (UV) induz reações deletérias na pele que podem causar estresse oxidativo e consequentemente, envelhecimento, imunossupressão, carcinogênese e várias

doenças inflamatórias (Marti-Mestres *et al*., 2007; Quyen, Choi e Kang, 2013). Embora substâncias antioxidantes exógenas possam chegar à pele através da alimentação e suplementação oral, as etapas de biodistribuição limitam a quantidade liberada para a pele, não sendo, muitas vezes, suficiente para eliminar os radicais livres gerados (Xu e Fisher, 2005; Yin *et al*. 2013).

Nesse contexto, pesquisas com pele têm demonstrado que os isoflavonóides presentes na soja são moléculas promissoras na redução de danos cutâneos causados pelos raios UV. Huang e colaboradores (2010) avaliaram o efeito protetor de extratos de soja contendo agliconas (daidzeína, genisteína e gliciteína) ou glicosídeos (acetildaidzeína, acetilgenisteína e acetilgliciteína) frente aos danos induzidos por radiação UVB. Nos ensaios *in vitro*, queratinócitos humanos foram primeiramente tratados com o extrato de soja e após expostos à radiação UVB. Os resultados revelaram uma diminuição da morte das células pré-tratadas com as isoflavonas. Além disso, ensaios *in vivo* foram realizados utilizando camundongos tratados por via tópica com o extrato da soja, antes da exposição aos raios UVB, e os resultados observados pelos autores mostraram menor nível de descamação, menor perda de água transepidérmica (TEWL), eritema e espessura da epiderme na pele dos animais comparados aos animais não tratados.

A inibição da ciclooxigenase, enzima que faz parte da biossíntese das prostaglandinas, sugere que as isoflavonas também atuam na modulação das reações inflamatórias. Outros trabalhos têm sugerido que o mecanismo através da qual a isoflavona genisteína reduz a resposta inflamatória pode ser pela inibição da transcrição do fator nuclear-kB (NF-kB) e a da ativação da secreção de quimiocinas-8, através tanto da diminuição da atividade da caspase-3 como da atividade de proteínas de tirosina quinase (Baxa e Yoshimura, 2003; Dijsselbloem *et al*., 2004).

Atividade antiviral

A atividade antiviral dos isoflavonóides tem sido igualmente investigada, especialmente a atividade da genisteína. A atividade antiviral desses compostos parece ser relacionada com a combinação de vários efeitos, tanto na partícula viral como nas células hospedeiras. Nas células, os compostos podem ter ação na

adsorção e/ou entrada dos vírus, na replicação viral, na produção das proteínas e formação de certos complexos de glicoproteínas do envelope viral. Ainda, os compostos podem afetar a indução de determinados fatores de transcrição e a secreção de citocinas das células hospedeiras (Andres, Donovan e Kuhlenschmidt, 2009). Os estudos com a genisteína mostram que a mesma é capaz de reduzir a infectividade de diversos vírus: vírus encapsulados, não encapsulados, vírus RNA e vírus DNA de fita simples ou fita dupla (Tabela 1).

Tabela 1. Vírus inibidos pela isoflavona genisteína.

Vírus	Referência
Adenovírus	Li *et al*., 2000
Arenavírus	Vela *et al*., 2008; Vela *et al*., 2010
Citomegalovírus	Evers *et al*., 2005
Herpesvírus bovino 1	Akula *et al*., 2002
Herpesvírus humano 1	Yura, Yoshida e Sato, 1993; Lyu, Rhim e Park, 2005
Herpesvírus humano 2	Lyu, Rhim e Park, 2005
Herpesvírus humano 4	Fukuda e Longnecker, 2005
Herpesvírus humano 8	Sharma-walia *et al*., 2004
HIV	Stantchev *et al.*, 2007; Guo *et al.*, 2013; Sauter *et al.*, 2014
Rotavírus	Andres *et al*., 2007

Uma vez que compostos fenólicos, ligam-se às proteínas para formar complexos instáveis, os vírus envelopados, como o HSV, tornam-se vulneráveis à ação desses compostos. Assim, isoflavonóides podem interagir com glicoproteínas de superfície do envelope viral e com as proteínas dos vírus e/ou da superfície das células hospedeiras (Hudson *et al*., 1990; Schnitzler *et al*., 2008). Com relação à genisteína, o mecanismo da atividade anti-herpética tem sido atribuído a sua ação inibitória sobre a fosforilação da tirosina quinase. Yura e colaboradores (1993) investigaram a atividade anti-HSV-1 da genisteína e os resultados obtidos sugerem que essa isoflavona reduz a replicação do HSV-1 através da inibição específica da fosforilação de resíduos de tirosina dos polipeptídios virais.

Segundo Nicola e colaboradores (2005), a entrada dos vírus herpes simples nas células endoteliais ocorre por meio da via endocítica, dependente da ativação da tirosina quinase. Nesse estudo, os autores utilizaram a genisteína como inibidora da tirosina quinase em células de queratinócitos da epiderme humana. A genisteína, ao inibir a atividade dessa enzima, *in vitro*, reduziu a entrada dos vírus HSV nos núcleos dos queratinócitos humanos. Em outro estudo, Lyu e colaboradores (2005) avaliaram a atividade anti-herpética de alguns flavonóides, entre eles a genisteína. Análises dos efeitos citopáticos em linhagem de células Vero, mostraram interessante atividade inibitória dessa molécula contra os vírus HSV-1 e HSV-2. Os resultados mostraram baixa toxicidade da genisteína às células, concentração efetiva (EC50%) de 5 µM, sendo inferior ao encontrado para o controle aciclovir (50 µM) para o HSV-1 e valores de EC50% idênticos ao aciclovir para HSV-2 (50 µM). O valor do índice de seletividade (IS) para o aciclovir foi 10, para ambos os tipos virais, e para a genisteína foi de 50 (HSV-1) ou 5 (HSV-2).

Herpes simples

O herpes simples é uma dermatose infecto-contagiosa crônica e recorrente causada por dois vírus da família *Herpesviridae,* HSV-1 e HSV-2, que mostram similaridades em suas manifestações clínicas, sendo, por isso, difícil distingui-las. No entanto, as diferenças na distribuição anatômica das infecções indicam o agente etiológico da doença, uma vez que o HSV-1 é comumente associado às infecções orofaciais e o HSV-2 às infecções genitais (Dowdle *et al.*, 1967; Lupi, Silva e Pereira Jr, 2000). Porém, nas últimas décadas tem sido observado o aumento da proporção de infecções genitais causadas por HSV-1. Tais mudanças na distribuição e prevalência das infecções herpéticas podem estar relacionadas ao comportamento sexual, especialmente pelo contato oro-genital desprotegido. Desse modo, a identificação do vírus pode ser feita por análise sorológica, uma vez que os vírus apresentam características biológicas e genéticas diferentes (Ellermann-Eriksen, 2005; Goodyear, 2015).

O HSV propaga-se de pessoa a pessoa pelo contato com secreções infectadas e ambos os tipos virais provocam infecção primária em pele/mucosas e latência nos gânglios nervosos sensoriais (Brady e Bernstein, 2004). Após penetrar

na pele/mucosa, o HSV passa a se replicar na membrana parabasal das células epiteliais. A propagação da infecção herpética nos tecidos é devido a capacidade dos vírus de infectarem células adjacentes através das junções celulares e, assim, os vírus não são expostos a substâncias extracelulares como os anticorpos. Na pele e mucosas, a proliferação viral causa lise das células o que acarreta em resposta inflamatória e formação de vesículas eritematosas (Lopes, 2006; Goodyear, 2015).

A infecção primária é, em grande parte, assintomática em indivíduos jovens e saudáveis (Cesario *et al.*, 1969). O ciclo de multiplicação dos vírus herpéticos envolve uma sequência de eventos complexos, sendo que, *in vitro*, a replicação viral ocorre em torno de 18 a 20 horas. Na infecção primária, os vírus herpéticos causam uma infecção latente nos gânglios sensoriais, adjacentes ao local afetado, por toda a vida do hospedeiro. Os vírus são reativados espontaneamente ou por vários fatores como, exposição ao sol, febre, período menstrual, traumatismo e estresse, passando anterogradamente pelo nervo e causando novas infecções em sítios específicos da pele e mucosas (Spear, 2004; Baringer, 2008). As manifestações clínicas variam desde gengivoestomatite, lesões orofaciais, querato-conjuntivites e infecções genitais até doenças graves e muitas vezes fatais como infecção neonatal, encefalites e infecções disseminadas em indivíduos imunocomprometidos (Brady e Bernstein, 2004).

O fármaco de escolha no tratamento do herpes simples é o aciclovir. Esse fármaco atua como inibidor seletivo da replicação dos herpesvírus, sendo administrado como um pró-fármaco para posterior conversão a um derivado fosforilado pela ação da enzima timidina quinase, a qual é produzida pelo próprio vírus. Após, o monofosfato de aciclovir produzido sofre mais duas fosforilações por quinases celulares até a transformação na forma ativa (trifosfato de aciclovir) que inibe, especificamente, a replicação do DNA viral ao competir com o trifosfato desoxiguanosina pela DNA polimerase viral. Dessa forma, o aciclovir impede a incorporação do outro nucleotídeo e inibe a síntese do DNA viral de forma irreversível (Fyfe *et al*., 1978; Elion *et al*., 1999). O aciclovir é utilizado no tratamento do herpes genital e oral através da administração por via tópica, oral ou intravenosa, sendo eficaz em reduzir a duração da lesão e no alívio da dor. Porém, até o momento, não há tratamento definitivo para o herpes simples (Hasler-Nguyen *et al.*,

2009). A via tópica possui a vantagem da administração do fármaco diretamente na lesão, além de evitar o metabolismo de primeira passagem, reduzindo efeitos adversos como distúrbios gastrointestinais e insuficiência renal, quando administrados em grandes doses (Strasfeld e Chou, 2010).

Outros fármacos disponíveis são os análogos de nucleosídeos como penciclovir, ganciclovir, fanciclovir e idofovir que, assim como o aciclovir, atuam na inibição da replicação viral. Já o docosanol tem ação como inibidor da entrada dos vírions nas células (Mamidyala e Firestine, 2006). Dessa forma, outros alvos como proteínas que desempenham funções específicas na replicação dos herpesvírus podem ser explorados para a descoberta de novas moléculas antivirais, sendo que qualquer processo que seja essencial para a replicação do vírus e não para a sobrevivência celular é um alvo potencial para a ação de agentes antivirais (Clercq, 2013; Weller e Kuchta, 2013).

O surgimento da resistência aos fármacos anti-herpéticos é um desafio na terapêutica e está relacionado com as mutações virais. De fato, a existência de cepas resistentes e a prevalência das infecções em pacientes imunocomprometidos tornam as infecções herpéticas uma importante questão de saúde pública (Strasfeld e Chou, 2010; Hodge e Field, 2013). Assim, diversos estudos têm sido realizados em busca de substâncias que apresentem atividade antiviral com reduzida toxicidade às células hospedeiras (Harish *et al.*, 1996; Chiang *et al.*, 2002; Lyu, Rhim e Park, 2005; Rani e Murugesan, 2013).

Os produtos naturais são fontes de substâncias ativas, especialmente para o tratamento de doenças infecciosas. Entre 1981 e 2006, cerca de 44% dos medicamentos antivirais aprovados são derivados de produtos naturais, semi-sintético ou sintéticos baseados em produtos naturais (Newman e Cragg, 2007). Zhong et al (2013) relatam que 222 plantas já foram identificados como potenciais fontes de agentes anti-herpéticos, sendo que a família Asteraceae representa o maior grupo, com 9,0% das plantas pertencentes a esta família. O segundo grupo mais abundante é a família Fabaceae, a qual a soja pertence, que responde por 7,7% do total de plantas com atividade contra os herpesvírus. Essas pesquisas abrem expectativas para o surgimento de novos fármacos anti-herpéticos.

Sistemas nanoemulsionados

Sistemas nanoemulsionados caracterizam-se por apresentar gotículas de fase interna de tamanho nanométrico. Do ponto de vista farmacêutico, esses sistemas podem ser conceituados como dispersões compostas de gotículas oleosas, uniformemente dispersas em uma fase aquosa externa e estabilizadas por tensoativos adequados (Washington, 1996; Trotta, Pattarino e Ignoni, 2002). Apresentam aspecto leitoso e de baixa viscosidade, sendo que nos sistemas do tipo O/A, o núcleo oleoso corresponde de 5 a 20% da formulação. Esses sistemas têm recebido grande atenção devido ao seu potencial uso como carreadores de compostos de reduzida hidrossolubilidade, os quais se encontram dispersos preferencialmente no núcleo oleoso e/ou adsorvidos na interface (Sonneville-Aubrun, Simonnet e L'alloret, 2004; Sakulku *et al.*, 2009; Zahi *et al.*, 2014).

Com relação à composição, óleos de origem natural ou semi-sintética como os triglicerídeos e os álcoois graxos têm sido utilizados nas formulações (Benita, 1998; Yilmaz e Borchert, 2006; Chang e Mcclements, 2014; Abbas *et al.*, 2015). Para conferir estabilidade ao sistema, tensoativos como a lecitina são incluídos na composição das nanoemulsões. Lecitinas são tensoativos de origem natural, formadas por misturas de fosfolipídeos extraídos da gema de ovo ou da soja, cujo componente majoritário é a fosfatidilcolina e apresentam natureza não-iônica e anfótera (Kibbe, 2000). Esse tensoativo apresenta grande afinidade pelas membranas celulares, podendo conduzir em aumento da absorção de fármacos, e geralmente não são irritantes, nem sensibilizantes (Paolino *et al.*, 2002; Hoeller, Sperger e Valenta, 2009; Klang e Valenta, 2011). A associação da lecitina com tensoativos de natureza não-iônica como polissorbatos, poloxâmero e polietilenoglicóis tem sido relatada na literatura (Santander-Ortega *et al.*, 2010; Dora *et al.*, 2012; Ebrahimi e Salmanpour, 2014). Apesar do vasto emprego de misturas de fosfolipídeos em sistemas nanoemulsionados, há poucos estudos mostrando o efeito dos fosfolipídeos isolados nesse tipo formulação. Uma vez que esses compostos não apresentam carga, esses podem ser utilizados em misturas binárias com outros tensoativos de carga para conferir estabilidade ao sistema (Bruxel *et al*., 2011; Fraga *et al*., 2011).

Dentre as principais propriedades dos sistemas nanoemulsionados encontram-se o diâmetro médio das gotículas, normalmente entre 100 e 300 nm (Haskell, 1998) e o baixo índice de polidispersão (IP), sendo que, geralmente valores de IP de até 0,3 indicam uma boa homogeneidade das gotículas na amostra (Alves *et al*., 2007). A técnica mais empregada para analisar esta propriedade é a espectroscopia de correlação de fótons (PCS), também conhecida como espalhamento de luz dinâmico (Benita e Levy, 1993). Outras técnicas complementares, como microscopia eletrônica de transmissão (MET), têm sido empregadas para avaliar a morfologia e o tamanho das gotículas (Ruktanonchai *et al.*, 2009; Klang, Valenta e Matsko, 2013). Os principais fatores que influenciam o tamanho da gotícula são a composição da formulação e o procedimento de preparação. No estudo realizado por Silva et al. (2009) foi avaliada a influencia dos óleos triglicerídeo de cadeia média (TCM) e octildodecanol (ODD) sobre o diâmetro de gotículas de nanoemulsões contendo genisteína. Nesse estudo, foi observado que o tipo de óleo influenciou o tamanho das gotículas, sendo que o óleo de maior viscosidade (ODD) gerou tamanhos de partículas maiores.

Outra propriedade importante é o potencial zeta, o qual é utilizado para avaliar a carga de superfície das partículas, fornecendo informações sobre a estabilidade do sistema, uma vez que o efeito de barreira das forças eletrostáticas pode alterar a velocidade de coalescência das gotículas. O potencial zeta reflete a composição da interface da nanoemulsão, seja em relação aos tensoativos formadores do filme, a presença de fármacos ou outras moléculas associadas à interface (Schaffazick *et al*., 2003). A determinação deste parâmetro é realizada por técnicas eletroforéticas, sendo que valores elevados (>30 mV), em módulo, garantem repulsão entre as gotículas, evitando, a ocorrência de floculação e coalescência (Yilmaz e Borchert, 2005; Teeranachaideekul *et al*., 2007; Ruktanonchai *et al*., 2009). Lecitinas e ácido oléico propiciam uma carga negativa à interface das nanoemulsões, sendo que a carga negativa da lecitina decorre da presença de fosfolipídeos, tais como, ácido fosfatídico e fosfatidilserina (Fang *et al*., 2004; Primo *et al*., 2008). Além de valores de potencial zeta negativos, nanoemulsões podem apresentar elevado potencial zeta positivo, através do uso de tensoativos catiônicos como o brometo de cetil trimetilamônio (CTAB) (Fasolo, Bassani e Teixeira, 2009; Argenta *et al.*, 2011).

Atualmente, diversos métodos de preparação de nanoemulsões têm sido descritos na literatura, incluindo a homogeneização à alta pressão, a microfluidização e a emulsificação espontânea. Essa última técnica apresenta a vantagem de ser simples e rápida, além de possuir baixo custo e permitir o preparo de pequenas quantidades de amostra. Ainda, o uso de baixas temperaturas permite incorporar fármacos termolábeis nas formulações e também evita a degradação dos fosfolipídeos por hidrólise, fenômeno este que pode ocorrer como descrito por Rabinovich-Guilatt et al. (2005). A emulsificação espontânea consiste na adição de uma solução solvente-óleo na fase aquosa, resultando na emulsificação das nano-gotículas da fase oleosa devido a algum tipo de instabilidade interfacial conhecida como efeito Marangoni, originada da rápida difusão do solvente através da interface, com conseqüente diminuição da tensão interfacial (Bouchemal *et al*., 2004).

Nos últimos anos, nanoemulsões têm sido amplamente estudadas para a liberação de fármacos por via tópica. A sua composição e características físico-químicas apresentadas por essas formulações podem modular a permeação transdérmica de fármacos, podendo proporcionar aumento da permeação e do tempo de permanência do fármaco na pele. Com isso, esses sistemas apresentam como vantagens, quando de uso tópico: modulação da barreira do estrato córneo, possível controle da retenção cutânea, aumento da interação do ativo com a pele (devido à grande área superficial e a baixa tensão superficial das gotículas de óleo) e o aumento da estabilidade química do ativo (Alves *et al*., 2007; Fasolo, Bassani e Teixeira, 2009; Vargas *et al.*, 2012; Clares *et al.*, 2014).

Permeação/retenção cutânea de ativos a partir de nanoemulsões

A pele representa uma via atrativa e acessível para a administração de moléculas ativas, uma vez que a liberação tópica pode evitar ou reduzir a toxicidade sistêmica (Guterres, Alves e Pohlmann, 2007). A difusão de moléculas pelo estrato córneo pode seguir dois caminhos: a rota intercelular, entre os corneócitos, e a rota através dos corneócitos. Sendo que, a principal via de permeação cutânea é a intercelular (Figura 2). Assim, a substância permeante difundirá obrigatoriamente pela matriz lipídica, reconhecida como a maior determinante do transporte percutâneo (Potts e Guy, 1992). A via anexial é outra possibilidade de passagem de

fármacos, porém, considerando a área total disponível da pele, os folículos pilosos ocupam de 0,1 a 1%, o que torna essa via insignificante para o transporte de ativos. No entanto, esse ponto de vista tem sido reavaliado devido a vascularização, profunda, invaginação e afinamento do estrato córneo nas vias anexiais. Portanto, os folículos pilosos podem constituir um reservatório para liberação de fármacos à base de nanopartículas, além disso, a penetração pode ser aumentada com a aplicação de massagem no local (Bilia *et al*., 2014). Ulmer e colaboradores (2013) mostraram uma importante aplicação de sistemas nanoemulsionados nos folículos pilosos. Os autores apresentaram a importância da prevenção de infecções causadas pela flora bacteriana da pele em punções cirúrgicas. A antissepsia convencional da pele não alcança os folículos pilosos, local onde há cerca de 25% da flora bacteriana. Assim, os autores investigaram a potencial antissepsia da polihexanida incorporada em sistemas nanoemulsionados, através da monitorizarão do crescimento bacteriano em voluntários. Os resultados mostraram uma antissepsia mais duradoura na pele, após a administração tópica das nanoemulsões do que com a polihexanida em solução, sugerindo o efeito promotor das nanopartículas.

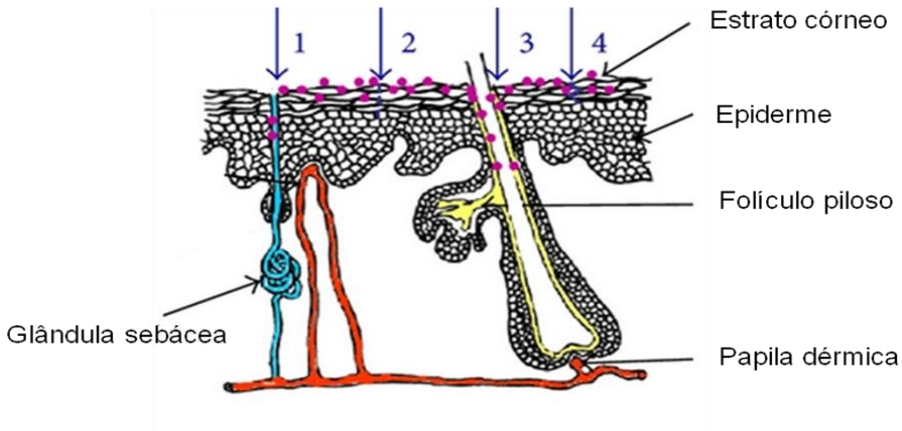

Figure 2. Potenciais rotas para a penetração de nanoemulsões na pele. Glândulas sudoríparas (1); via intracelular (2); folículo piloso (3); via intercelular (4). Adaptado de Bilia et al. (2014).

Fatores como a idade da pele, a integridade do estrato córneo, o fluxo sanguíneo e o metabolismo podem alterar a permeação de moléculas. Além desses,

outros aspectos que afetam a permeação de moléculas são as características físico-químicas das próprias substâncias ativas, como o grau de lipofilia e coeficiente de partição, os veículos e a forma farmacêutica. Com relação às propriedades dos ativos, as características ótimas para a absorção percutânea são, principalmente, baixo peso molecular e solubilidade adequada em meios hidrofílicos e hidrofóbicos (Swart *et al*., 2005; Brain *et al*., 2007). Para avaliar a extensão da absorção de compostos e sua quantificação nas diversas camadas da pele, estudos de difusão em células de Franz têm sido amplamente utilizados (Schwarz *et al*., 2012; Fernández-Campos *et al*., 2013). Atualmente, um dos modelos de pele mais difundidos para os experimentos de permeação *in vitro* é a pele suína, devido à semelhança histológica dessa quando comparado a outros modelos (Moser *et al*., 2001). A célula de difusão de Franz é constituída de um compartimento doador e um receptor, separados pela pele (Figura 3). A formulação é colocada no compartimento doador, sobre o tecido, e o compartimento receptor é preenchido com uma solução (fluido receptor), na qual o composto de interesse apresente condições sink (Vargas *et al.*, 2012).

Figura 3. Representação esquemática da célula de difusão tipo Franz.

A administração de fármacos pela via tópica, através de formulações convencionais, pode apresentar limitações devido à baixa taxa de permeação apresentada. Nesse sentido, a absorção de moléculas pode ser aumentada através

de estratégias de desenvolvimento de formulação. Dentre as alternativas, destacam-se a adição de promotores de permeação às formulações como, por exemplo, azona, terpenos, ácidos graxos, dimetilsulfóxido e alcoóis. Esses compostos provocam uma desorganização dos lipídios do estrato córneo (camada mais superficial da pele), diminuindo a resistência da pele à difusão dos fármacos (Martins e Veiga, 2002). Porém o uso dessas substâncias pode ser irritante ou mesmo tóxica (Hadgraft e Lane, 2005). Neste contexto, o desenvolvimento de sistemas de liberação de fármacos, como as nanoemulsões, especialmente no caso de compostos ativos de baixa hidrossolubilidade é uma alternativa farmacotécnica para aumentar as taxas de penetração/permeação de compostos lipofílicos na pele (Friedman, Schwarz e Weisspapir, 1995; Balamohan, Anjali e Ravindran, 2013).

As características físico-químicas de diâmetro de partícula e carga de superfície apresentada pelas nanoemulsões propiciam uma maior interação dessas nanoestruturas com os componentes da pele, tendo influência na velocidade e extensão de permeação das substâncias veiculadas (Benita, 1999). O reduzido diâmetro de gotícula desses sistemas apresenta-se como vantagem em relação aos sistemas convencionais de uso tópico, uma vez que permite a aplicação de um depósito uniforme na pele, resultando em uma elevada superfície de contato do sistema com o órgão. Além disso, os componentes das nanoemulsões (óleos e tensoativos) podem promover a penetração de moléculas através da fluidização dos componentes da pele (Sonneville-Aubrun, Simonnet e L`alloret, 2004). No estudo de Schwars e colaboradores (2012) foi avaliada a penetração do fármaco antiviral aciclovir a partir de nanoemulsões múltiplas A/O/A. Para investigar o perfil da permeação através da pele de orelha suína foram utilizadas células de difusão de Franz. Com os resultados obtidos no estudo, os autores detectaram maiores teores de aciclovir no fluido receptor quando o fármaco foi incorporado em nanoemulsões, comparado com o fármaco solubilizado em solução aquosa.

Diferentes composições de fase oleosa são investigadas no desenvolvimento de nanoemulsões de uso tópico. Com esse intuito, Borges e colaboradores (2013) avaliaram a permeação de dapsona, fármaco utilizado no tratamento da hanseníase e outras doenças da pele, a partir de sistemas nanoemulsificados contendo diferentes fases oleosas através da epiderme de orelha de porco. Os autores

prepararam formulações contendo miristato de isopropila ou metilpirrolidona, sendo que foi observada uma maior permeação do fármaco quando este foi incorporado em nanoemulsões contendo o miristato de isopropila. Além dos componentes oleosos, estudos do efeito de nanoemulsões carregadas na permeação cutânea de ativos têm sido conduzidos por diversos pesquisadores. Uma vez que a pele apresenta cargas negativas, nanoemulsões carregadas positivamente parecem ter efeito superior na permeação de fármacos quando comparados com nanoemulsões carregadas negativamente (Piemi *et al.*, 1999; Fasolo, Bassani e Teixeira, 2009; Hoeller, Sperger e Valenta, 2009).

Piemi e colaboradores (1999) realizaram estudos de permeação *in vitro* com os antifúngicos ceconazol e miconazol (ambos na forma de nitrato), incorporados em nanoemulsões carregadas negativamente ou positivamente. As nanoemulsões carregadas positivamente mostraram-se mais eficientes em termos de permeação em relação às carregadas negativamente, para ambas as moléculas, demonstrando que a presença da carga positiva promoveu a penetração. No estudo de Hoeller, Sperger e Valenta (2009) o efeito de cargas em nanoemulsões, contendo acetato de fludrocortisona ou pivalato de flumetasona, foi avaliado na presença de lecitina (carregado negativamente) e fitoesfingosina (carregado positivamente). Os autores também avaliaram o efeito da presença dos tensoativos não-iônicos laurato de sacarose ou polissorbato 80. Os resultados desse estudo mostraram que a nanoemulsão carregada positivamente em presença de polissorbato 80 promoveu as maiores taxas de permeação do acetato de fludrocortisona. O efeito positivo dos compostos catiônicos sobre a permeabilidade na pele pode ser atribuído a presença de resíduos de proteínas carregados negativamente na superfície da pele em pH fisiológico (4,5 – 6,0) (Rojanasakul *et al*., 1992). Sendo assim, os pesquisadores acreditam que ocorra interação destas cargas com as cargas positivas das formulações.

As mucosas também são consideradas como potenciais locais para a liberação de compostos ativos. Entre as várias vias, a mucosa bucal apresenta excelente acessibilidade, com grande extensão e tecido relativamente imóvel, sendo interessante para administração local e sistêmica (Reddy, Chaitanya e Rao, 2011). Estudos têm mostrado a potencial aplicação de nanoemulsões na liberação de

compostos ativos em mucosas nasal, vaginal e bucal (Bachhav e Patravale, 2009; Campos *et al.*, 2012; Sood, Jain e Gowthamarajan, 2014). Por exemplo, Campos e colaboradores (2012) avaliaram a atividade *in vitro* da nistatina incorporada em nanoemulsão contra a candidíase, e observaram um aumento da atividade antifúngica com a formulação.

Métodos *in vitro* têm sido utilizados na triagem de moléculas em estudos pré-clínicos, bem como para elucidar mecanismos de transporte através da mucosa. A mucosa bucal suína pode ser utilizada como modelo para avaliação da permeabilidade, uma vez que a mesma apresenta epitélio não queratinizado com estrutura e composição similares à da mucosa bucal humana (Squier e Kremer, 2001). Assim como a mucosa bucal, a mucosa do esôfago também é coberta por um epitélio escamoso, estratificado e não queratinizado. Além disso, ambos os epitélios, bucal e esofágico, apresentam composição lipídica semelhante, com níveis elevados de glicosilceramidas e baixas quantidades de ceramidas. Desta forma, devido à fácil obtenção, a mucosa de esôfago suíno é preferida como modelo de membrana para os estudos de permeação (Caon e Simões, 2011; Patel, Liu e Brown, 2011).

No entanto, para adequar as formulações ao uso tópico, nanoemulsões podem ser espessadas com polímeros tais como os carbômeros, quitosona e derivados de celulose (Rowe *et al*., 2009). Depois de espessadas, normalmente as nanoemulsões apresentam-se como fluido não-newtoniano e com comportamento plástico ou pseudo-plástico, tornando-se ideais para a aplicação tópica (Vargas *et al.*, 2012) Ainda, polímeros mucoadesivos podem fornecer uma ferramenta importante para melhorar a biodisponibilidade dos compostos, melhorando, com isso, o tempo de permanência no local de liberação dos ativos (Silva *et al*., 2012).

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CAPÍTULO I

Avaliação da atividade anti-herpética *in vitro* de isoflavonóides da *Glycine max* (soja)

O primeiro capítulo experimental teve como objetivo avaliar a atividade anti-herpética dos isoflavonóides da soja: daidzeína, genisteína, gliciteína e cumestrol, bem como investigar o mecanismo de ação dos compostos bioativos. Para a realização do estudo foram utilizadas células Vero e GMK AH1, permissivas aos vírus HSV-1 e HSV-2, respectivamente. Previamente aos ensaios antivirais, a citotoxicidade dos compostos foi avaliada através da metodologia da sulforrodamina B, sendo os resultados analisados por regressão não-linear para determinar a concentração que inibe em 50% a viabilidade celular (CC_{50}). Após essa etapa, concentrações não tóxicas às células foram utilizadas para avaliar a potencial atividade anti-herpética dos isoflavonóides, através do ensaio da redução do número de placas de lise. Os resultados mostraram um interessante efeito antiviral dos isoflavonóides genisteína e cumestrol. Esses compostos inibiram a replicação de duas cepas do HSV-1 (KOS e 29R, cepa sensível e resistente ao aciclovir, respectivamente) e a cepa 333 do HSV-2, contudo a daidzeína e a gliciteína não apresentaram atividade. Possíveis mecanismos de ação foram avaliados usando diferentes metodologias: avaliação virucida, pré-tratamento, efeito na infecção inicial (avaliação do efeito na adsorção e penetração dos vírus nas células), efeito da adição dos compostos em diferentes tempos, capacidade de inibir a produção de proteínas virais pela técnica do western blotting e de redução das áreas das placas de lise. A análise dos resultados mostrou que o cumestrol afetou as fases iniciais da infecção viral para todos os vírus, e que ambos os compostos foram capazes de reduzir a expressão de proteínas do HSV-1, através da inibição de genes imediatamente iniciais (α), genes iniciais (β) ou de genes tardios (ɣ). Além disso, os compostos mostraram serem capazes de reduzir as áreas das placas virais do HSV-2, sugerindo o efeito dos isoflavonóides na redução da disseminação do vírus célula a célula. Em resumo, os resultados sugerem que a genisteína e o cumestrol apresentam efeitos inibitórios sobre a replicação do HSV, interferindo em diferentes passos do ciclo de replicação dos vírus. Nesse capítulo inicial, foi demonstrado que a genisteína e o cumestrol podem ser considerados potenciais agentes alternativos no tratamento das infecções herpéticas.

Antiherpes evaluation of soybean isoflavonoids

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Abstract

Antiviral effects of soybean isoflavonoids have been recently investigated, especially for genistein. It has been reported that this isoflavone was able to inhibit Herpes Simplex Virus (HSV) replication, which is associated with skin and epithelial mucosa infections. The treatment of these infections with antiherpes drugs has induced the emergence of resistant viral strains. Based on this evidence, the aim of this study was to investigate the anti-HSV effects of soybean isoflavonoids: daidzein, genistein, glycitein, and coumestrol. Genistein and coumestrol inhibited HSV-1 (KOS and 29R strains, which are acyclovir-sensitive and acyclovir–resistant strains, respectively) and HSV-2 (333 strain) replication, whereas no antiviral effects were detected for daidzein and glycitein. The mechanisms of action were evaluated by different methodological strategies. Coumestrol has affected the early stages of viral infection, and both compounds were able to reduce HSV-1 protein expression, as well as HSV-2 cell-to-cell spread.

Keywords: soybean isoflavonoids, genistein, coumestrol, antiherpes, HSV-1, HSV-2

Introduction

Soybean (*Glycine max*) isoflavonoids are considered phytoestrogens because they are structurally similar to 17β-estradiol. Consequently, they can interact with estrogen receptors and mediate estrogenic responses, exerting estrogenic or antiestrogenic activity (Cornwell, Cohick and Raskin, 2004). These effects have been extensively explored for hormone replacement therapy, as well as for anticarcinogenic effects (Gaete *et al*., 2011; Lagari and Levis, 2014; Nadal-Serrano *et al*., 2013). Among the soy bioactive compounds, isoflavones are the most investigated group, especially genistein, daidzein, and glycitein. Genistein is the most abundant and is the major subject of studies considering health-promoting compounds from soybean (Pavese, Farmer and Bergan, 2010; Nagaraju, Zafar and El-Rayes, 2013). In addition to estrogenic activity, genistein also inhibits DNA topoisomerase and protein tyrosine kinase (PTK), which has been related to its anticancer activity (Zhang *et al*., 2013). Biological properties of coumestrol, another isoflavonoid found in soybeans, have also been investigated (Argenta *et al*., 2011; Liu *et al*., 2013; Seo *et al*., 2014). This molecule is a soy isoflavone-like compound with a coumestan skeleton presenting a more potent estrogen receptor affinity than other soy isoflavonoids (Fang *et al*., 2003). Furthermore, studies have reported that coumestrol has cytotoxic effects against several cancer cell lines (Cho *et al*., 2014; Lee *et al*., 2013).

Despite the fact that several flavonoids present antiviral effects against a wide range of viruses, the antiviral activity evaluation of soy isoflavonoids has not been sufficiently investigated. Some reports have shown that genistein was able to reduce the infectivity of some viruses (Akula *et al*., 2002; Kolokoltsov *et al*., 2012; Vela *et al*., 2008). Regarding antiherpes activity, Yura, Yoshida, and Sato (1993) demonstrated the antiherpes (anti-HSV-1) effects of genistein, attributing them to tyrosine kinase inhibition. Lyu, Rhim and Park (2005) also showed antiherpes effects of this isoflavone (anti-HSV-2). Following these findings, our research group investigated the *in vitro* antiherpes effects of topical formulations containing the isoflavone genistein. Our results showed that such formulations improved the antiviral activity of genistein against HSV-1 and HSV-2 (Argenta *et al*., 2014).

HSV-1 and HSV-2 are enveloped viruses with double-stranded DNA genome. During productive infection, their replication cycle includes virion attachment and penetration with subsequent expression of viral immediate early (α) genes that mainly regulate viral replication, such as ICP27; early (β) genes that synthesize and package DNA, such as UL42; and late (ɣ) seneg, most of which are virion proteins, such as gB and gD glycoproteins. The inhibition of any of these stages blocks HSV replication and, therefore, could be considered potential targets for antiherpes therapy (Coleman and Shukla, 2013). HSV is usually associated with skin and epithelial mucosa infections, causing various diseases, such as gingivostomatitis, herpes labialis, meningitis, and ocular and genital infections (Brady and Bernstein, 2004). Although there are several drugs available for the treatment of HSV infections, their prolonged use has induced the emergence of resistant viral strains (Hodge and Field, 2013).

Considering the reported anti-HSV activity of genistein, this study attempted to investigate the antiherpes effects of the soybean isoflavonoids: genistein, daidzein, glycitein, and coumestrol (Figure 1) against HSV-1 (KOS and 29R strains, which are acyclovir-sensitive and acyclovir–resistant strains, respectively) and HSV-2 (333 strain), as well as evaluate the mechanism of action of the most active compounds.

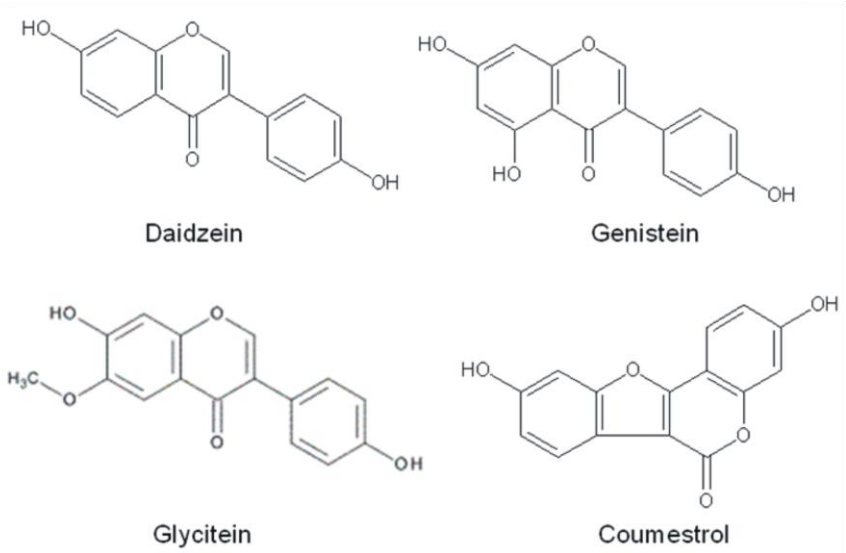

Figure 1. Soybean isoflavonoids

Materials and methods

Soy isoflavonoids

Daidzein, genistein and glycitein were obtained from Cayman Chemical (Ann Arbor, Michigan, USA) and coumestrol was purchase from Sigma (St. Louis, MO, USA). All compounds were dissolved in 1% dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and further diluted in culture medium prior to use. The stock solutions (isoflavonoids in DMSO) were stored at -20°C.

Cells and viruses

The cell lines used were Vero (ATCC: CCL81) and GMK AH1 (Department of Clinical Virology, Göteborg University, Sweden) grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), and maintained at 37 ^{0}C in a humidified 5% CO_2 atmosphere. HSV-1 (KOS and 29R strains, Faculty of Pharmacy, University of Rennes I, Rennes, France), and HSV-2 [333 strain (Department of Clinical Virology, Göteborg University, Sweden)] were propagated in Vero and GMK AH1 cells, respectively. Viral stocks were stored at – 80 ^{0}C and titrated based on plaque forming units (PFU) counted by plaque assay as previously described (Burleson, Chambers, and Wiedbrauk, 1992).

Antiherpes evaluation

Preliminary, the cytotoxicity of isoflavonoids was determined by sulforhodamine B (SRB) assay (Vichai and Kirtikara, 2006). Briefly, Vero and GMK AH1cells (2.5 x 10^4 cells per well) were seeded into 96-well microplates and allowed to adhere for 24 h at 37°C. Afterwards, different concentrations of the samples were dispensed into the wells and incubated for 48 h. The cells were stained with SRB and the absorbances were measured at 510 nm (Molecular Devices SpectraMax M2 microplate reader), and the concentration of each sample that reduced cell viability by 50% (CC_{50}) was calculated, when compared to untreated controls.

Subsequently, the potential antiherpes activity was evaluated by the plaque number reduction assay as previously described by Silva *et al*. (2010). Confluent

monolayers of Vero or GMK AH1 cells were infected with 100 PFU per well of HSV-1 (KOS and 29R strains) or HSV-2 (strain 333), respectively, for 1 h at 37^{o}C. After, the cells were washed with phosphate buffer saline (PBS) and overlaid with CMC medium (MEM containing 1.5% carboxymethylcellulose) in the presence or absence of different concentrations of the samples, and the plates were incubated for further 48 h. Cells were then fixed and stained with naphthol blue black and viral plaques were counted. The IC_{50} of each sample was defined as the concentration that inhibited 50% of viral plaque number when compared to untreated controls. Acyclovir (ACV) was purchased from Sigma (St. Louis, MO, USA) and used as positive control. The IC_{50} and CC_{50} values were estimated by non-linear regression of concentration-response curves generated from the obtained data using the GraphPad Prism® software, version 5.01. The selectivity index (SI = CC_{50}/IC_{50}) of each tested sample was also determined.

Evaluation of the mechanism of action

Virucidal assay

This assay was conducted as described by Silva *et al.* (2010). Mixtures of genistein or coumestrol, at different concentrations, with 4x10^{4} PFU of HSV-1 (KOS and 29-R strains) or HSV-2 (strain 33) in serum-free MEM were co-incubated for 15 min at 37 ^{o}C. Samples were then diluted to non-inhibitory concentrations (1:500) to determine the residual infectivity by plaque reduction assay as described above.

Pretreatment

This assay was performed as described by Bertol *et al.* (2011). Briefly, confluent Vero or GMK AH1 cell monolayers were pretreated with different concentrations of genistein or coumestrol for 3 h at 37 ^{o}C. After, cells were washed with PBS and infected with HSV-1 (KOS and 29R strains) or HSV-2 (strain 333) for 1 h at 37 ^{o}C. The infected cells were washed with PBS and overlaid with CMC medium, and treated as described above for the viral plaque number reduction assay.

Simultaneous treatment

Confluent Vero or GMK AH1 cell monolayers were infected with HSV-1 (KOS and 29R strains) or HSV-2 (strain 333), respectively, with the simultaneous addition of the samples, and incubated for 1 h at 37 ^{0}C. After, cells were washed with PBS and overlaid with CMC medium. Further procedures were the same as described above for the viral plaque number reduction assay (Bertol *et al*., 2011).

Adsorption and penetration assays

Adsorption and penetration inhibition assays were performed following the procedures described by Silva et al. (2010). In the adsorption assay, pre-chilled Vero or GMK AH1 cell monolayers were exposed to 100 PFU per well of HSV-1 (KOS and 29R strains) or HSV-2 (strain 333), in the presence or absence of the samples. After incubation for 2 h at 4 ^{0}C, samples and unabsorbed viruses were removed by washing with cold PBS, and cells were overlaid with CMC medium. Further procedures were the same as described above for the viral plaque number reduction assay. In the penetration assay, viruses (100 PFU per well) were adsorbed for 2 h at 4 ^{0}C on pre-chilled cells. After the removal of unbound viruses, the temperature was shifted to 37 ^{0}C for 5 min to allow penetration. Then, the cells were treated with different concentrations of pre-warmed samples, and incubated for 1 h at 37 ^{0}C. Unpenetrated viruses were inactivated with citrate-buffer (pH 3.0). Cells were washed with PBS and covered with CMC medium. Dextran sulfate (DEX-S) was purchased from Sigma (St. Louis, MO, USA) and used as positive control.

Viral plaque size reduction assay

The effect of samples on HSV cell-to-cell spread was investigated as described by Silva *et al.* (2010) and Bertol *et al.* (2011). Briefly, different concentrations of genistein, coumestrol or ACV (control positive), were added to Vero or GMK AH1 cells 1 h after their infection with 100 PFU per well of HSV-1 or HSV-2, respectively, and the plates were incubated throughout the entire period of plaque development. Results were obtained by analyses of the images of 20 viral plaques formed in the absence (viral control) or the presence of different concentrations of each sample concentration. Images were captured using a digital camera coupled to

the Olympus IX71 inverted microscope and the area of each plaque was determined by using Cell^P Olympus software. The percentages of inhibition were calculated based on the reduction of viral plaque number, when compared to viral controls.

Time-of-addition assay

Time-of-addition study was performed using the virus yield reduction assay as reported by Silva *et al.* (2010) with minor modifications. Briefly, confluent Vero or GMK AH1 cell monolayers were inoculated with 100 PFU per well of HSV-1 (KOS strain) and incubated at 37 ^{0}C for 1 h. After removing viruses, cells were maintained at 37 ^{0}C and treated with genistein (40 µM), coumestrol (40 µM) or ACV (20 µM) at 1, 2, 3, 4, 6, 8, 10, 12, 16, 18 and 24 h post-infection (p.i.). After 48 h of incubation, this assay followed the procedures described earlier for viral plaque number reduction assay. ACV was used was control positive.

Western blotting analysis

In order to understand whether genistein and coumestrol were able to interfere with HSV-1 (KOS strain) protein synthesis, western blotting experiments were performed as described by Cardozo *et al.* (2011). Briefly, Vero cell monolayers were inoculated or not with HSV-1 at a MOI of 0.2. Plates were incubated for 1h at 37 ^{o}C to ensure synchronous viral replication. Then, infected cells were treated with three different concentrations (20, 40 and 60 µM) of genistein or coumestrol or 20 µM of ACV (control positive), and incubated for 18 h. Next, cells were lysed and protein quantification was carried out. Each sample was separated electrophoretically on a 10% SDS–polyacrylamide gel and electroblotted onto nitrocellulose membranes. Membranes were blocked and then incubated overnight with either anti-ICP27 (1:1000, Millipore, Billerica, MA), or anti-UL42 (1:1000, Millipore), or anti-gD (1:1000, Santa Cruz Biotechnology). The anti-β-actin antibody was used as a control for total protein loading. After washing, the membranes were incubated with the respective secondary antibodies for 1 h. The immunoblots were developed and detected using Pierce ECL Western Blotting substrate (Thermo® Scientific, Rockford, IL,USA) according to the manufacturer`s. The images were detected with Bio-Rad ChemiDoc™ MP System and the digitalized using the Image Lab program, version 4.1.

Statistical analysis

The mean values ± standard deviations are representative of three independent experiments. For the determination of CC_{50} and IC_{50} values, nonlinear regressions of concentration-response curves were used. ANOVA/Tukey/SNK tests were applied to test significance between samples and controls.

Results and discussion

Antiherpes activity

In this study, the antiherpes effects of soy isoflavonoids (daidzein, genistein, glycitein, and coumestrol) were investigated against HSV-1 and HSV-2 replication. Daidzein and glycitein showed no antiviral effects. Conversely, genistein and coumestrol presented HSV inhibitory activity at noncytotoxic concentrations (Table 1). Genistein showed stronger inhibitory effects against HSV-1 (29R strain) and HSV-2 replication (SI values around 7.0) than against HSV-1 (KOS strain) replication (SI = 3.88). The same performance was observed with coumestrol, since its inhibitory effects were stronger against HSV-1 (29R strain) (SI = 31.52) and HSV-2 (SI = 28.14) replication, as compared to those against HSV-1 (KOS strain) replication (SI = 9.06). Comparing the tested isoflavonoids, coumestrol showed higher SI values for all HSV tested. Nonetheless, both samples inhibited HSV-1 (29R strain), which is resistant to acyclovir, suggesting that the targets of these compounds are probably different from those of acyclovir.

Table 1. Cytotoxicity (CC_{50}) on Vero and GMK AH1 cells and inhibitory effects of HSV-1 and HSV-2 replication (IC_{50}), respectively, of soy isoflavonoids.

Compounds	CC_{50} (μM)[a]		HSV-1 (KOS strain)		HSV-1 (29R strain)		HSV-2 (333 strain)	
	Vero cells	GMK AH1 cells	IC_{50} (μM)[b]	SI	IC_{50} (μM)[b]	SI	IC_{50} (μM)[b]	SI
Coumestrol	105.3 ± 22.33	>1000	11.62 ± 1.16	9.06	3.34 ± 0.68	31.52	35.53 ± 3.67	>28.14
Daidzein	>1000	>1000	NA	-	NA	-	NA	-
Genistein	54.41 ± 5.39	98.17 ± 14.78	14.02 ± 0.97	3.88	7.76 ± 0.76	7.01	14.12 ± 2.59	6.95
Glycitein	>1000	>1000	NA	-	NA	-	NA	-
Acyclovir	>1000	>1000	2.44 ± 21.95	>1818	NA	-	3.30 ± 0.50	>303

Notes: Values represent the means ± standard deviations of three independent experiments. [a]Cytotoxicity was determined by sulforhodamine B assay on Vero and GMK AH1cells. [b]Antiviral activity was determined by viral plaque number reduction assay. ACV was used as positive control.

Abbreviations: CC_{50}, concentration at which cell viability was reduced by 50%; HSV, Herpes Simplex Virus; IC_{50}, concentration that reduced by 50% the viral plaque number when compared to viral controls; SI= CC_{50}/ $IC_{50;}$ NA, no activity.

Mechanism of antiherpes activity

Genistein and coumestrol action mechanisms were elucidated by different methodological strategies. In theory, an antiviral compound can protect cells against virus infection in several ways, for example, by viral direct inactivation or by interfering with virus replication cycle (Cardozo *et al*., 2011). First, to elucidate whether genistein and coumestrol presented virus-inactivating activity, a virucidal assay was performed. Preincubation of virus suspensions with samples (at concentrations 500 times higher than the IC_{50} values) at 37^{o}C showed that these substances have no virucidal activity (data not shown). To explore the effects of these compounds directly on the host cells, a pretreatment assay was performed. However, this strategy did not affect HSV replication (data not shown), which suggests that these isoflavonoids did not present prophylactic effects *in vitro*.

As previously explained, during productive infection, the viral replication cycle includes virion attachment and penetration with subsequent expression of viral glycoproteins (Coleman and Shukla, 2013). The inhibition of any of these stages could be considered potential targets for the tested compounds. The next step was to evaluate the effects of these compounds in the early stages of viral infection. Genistein or coumestrol were simultaneously added to Vero or GMK AH1 cell monolayers with HSV-1 (KOS and 29R strains) or HSV-2 (strain 333), respectively. Genistein did not inhibit virus replication; however, coumestrol seemed to be able to interfere with the early events. Coumestrol simultaneous treatment resulted in the inhibition of HSV replication with IC_{50} values of approximately 59 µM, 28 µM, and 147 µM for HSV-1 (KOS strain), HSV-1 (29R strain) and HSV-2 (strain 333), respectively (Table 2). To confirm these findings, the effects of these substances on viral adsorption and penetration were individually investigated. Genistein had no effect on the adsorption and penetration steps (data not shown), while coumestrol affected both steps. The lack of inhibition in early events of HSV infection by genistein has also been demonstrated in other studies (Yura, Yoshida and Sato, 1993; Qie, Marcellino and Herold, 1999).

Coumestrol and DEX-S (positive control) inhibited attachment and penetration of all viruses tested (Table 2). Regarding coumestrol, the IC_{50} values obtained from

the attachment assay were approximately 33 μM and 22 μM against HSV-1 (KOS strain) and HSV-1 (29R strain), respectively, and 97 μM against HSV-2 (333 strain). The IC_{50} values obtained from the penetration assay were approximately 65 μM and 24 μM against HSV-1 (KOS strain) and HSV-1 (29R strain), respectively, and 108 μM against HSV-2 (333 strain).

Such results showed IC_{50} values obtained from HSV-1 (KOS and 29R strains) more promising than those obtained for HSV-2, indicating that coumestrol was more effective in inhibiting the initial steps of HSV-1 infection. In addition, IC_{50} values obtained from the simultaneous, adsorption, and penetration assays were higher than the IC_{50} values found in p.i. treatment, which suggests that coumestrol affects different steps of viral replication. As in simultaneous and adsorption assays, samples were added simultaneously with the viruses and the HSV inhibition effects could be due to virucidal action. No effect on the direct inactivation of viruses was detected; therefore, virucidal effects were discarded.

To clarify which steps of HSV infection are targeted by the samples, a time-of-addition study was performed. As shown in Figure 2, anti-HSV-1 (KOS strain) activity of genistein and coumestrol was maintained approximately at 34% and 56%, respectively, when added from 10h p.i. These results suggest that the tested compounds exert effects on the virus cycle steps after viral attachment and penetration into the cells.

Table 2. Effects of coumestrol in early stages of infection.

	IC_{50}[a]					
	Simultaneous treatment		Viral attachment step		Viral penetration step	
HSV	Coumestrol (μM)	DEX-S (μg/mL)	Coumestrol (μM)	DEX-S (μg/mL)	Coumestrol (μM)	DEX-S (μg/mL)
HSV-1 (KOS strain)	59.30 ± 0.98	0.13 ± 0.01	33.22 ± 2.87	0.12 ± 0.02	64.65 ± 3.31	1.56 ± 0.29
HSV-1 (29R strain)	27.65 ± 4.56	0.16 ± 0.02	22.15 ± 2.76	0.16 ± 0.03	24.16 ± 3.87	1.15 ± 0.34
HSV-2 (333 strain)	147.45 ± 18.03	0.14 ± 0.06	97.279 ± 9.24	0.03 ± 0.01	107.63 ± 8.82	2.17 ± 0.42

Notes: Values represent the means ± standard deviations of three independents experiments. [a]Antiviral activity was determined by viral plaque number reduction assay. DEX-S was used as positive control. **Abbreviations**: IC_{50}, concentration that reduced by 50% the viral plaque number when compared to viral controls; HSV, Herpes Simplex Virus; DEX-S, Dextran sulfate.

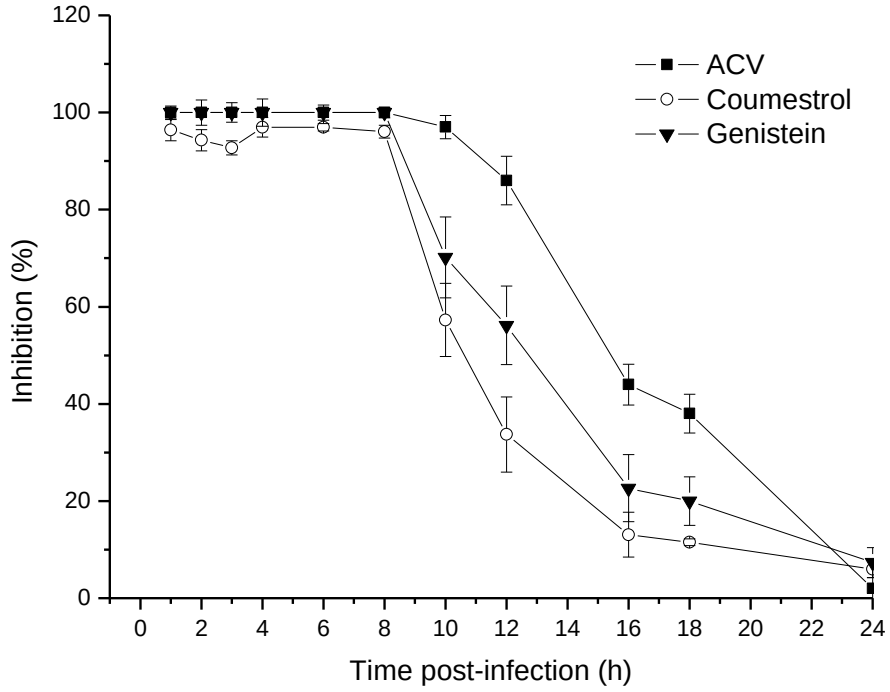

Figure 2. Time-of-addition assay. Vero cells were treated with 40 µM of acyclovir (ACV), coumestrol or genistein at different periods of time after HSV-1 (KOS strain) infection. Each point represents the mean values ± SD of two independent experiments. ACV was used as positive control.

Western blotting analyses were carried out to evaluate the effects of genistein and coumestrol on protein expression during HSV-1 (KOS strain) replication. Figure 3 shows the western blotting results after 18h of treatment with these isoflavonoids (20, 40, and 60 µM), or ACV (20 µM). Genistein completely inhibited UL42 and gD proteins at all tested concentrations and reduced the expression of ICP27 protein more strongly at 60 µM, as compared to the viral control. Coumestrol, in turn, reduced the expression of UL42 and gD proteins at all tested concentrations, but did not reduce the expression of the ICP27 protein.

After penetration, HSV-1 expresses immediate early genes (α) about 2–3 h p.i., early genes (β) about 7 h p.i., and late genes (ɣ) about 12 h p.i. (Whitley, Kimberlin and Roizman, 1998). Western blotting results allowed us to detect a significant reduction of (β) UL42 and (ɣ) gD proteins expression, which were more strongly affected after genistein treatment. The β genes encode mainly enzymes involved in the synthesis and replication of viral DNA, and most of the

encoded products by ɣ gene are structural capsid and tegument proteins, and others that form the virion particles, such as the envelope glycoproteins (Whitley, Kimberlin and Roizman, 1998). In view of these facts, the tested compounds could interfere in DNA synthesis and viral protein production, since both inhibited the expression of (β) UL42 and (ɣ) gD proteins. Moreover, a concentration-dependent inhibition of (α) ICP27 protein by genistein was also observed. Fontaine-Rodrigues and Knipe (2008) demonstrated that ICP27 is required for the efficient expression of HSV late proteins. Therefore, these results suggest that the alteration of the immediate early protein (ICP27) by genistein could affect the expression of UL42 and gD. In contrast, coumestrol did not reduce the expression of ICP27 protein, and it was not sufficient to completely inhibit the UL42 and gD expression.

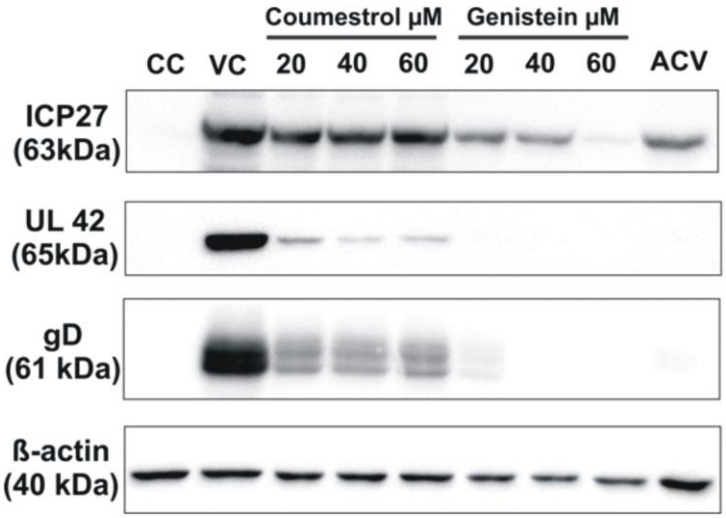

Figure 3. Effects of coumestrol and genistein on HSV-1 (KOS strain) protein expression. Confluent Vero cells were infected with HSV-1 (MOI 0.2) and treated with 20, 40 and 60 µM of samples. Lysates were collected after 18 h of incubation, run on SDS-10% PAGE gel and analyzed using specific antibodies for viral (ICP27, UL42, gD) proteins. Equal protein loading was confirmed by probing for B-actin. **Abbreviations**: CC (cell control) = Vero cells uninfected and untreated; VC (viral control) = Vero cells infected but not treated. Equal protein loading was confirmed by probing for B-actin.

After epidermis/mucosa cells infection, HSV can move between cells without diffusing through the extracellular environment; hence, the inhibition of HSV intercellular spreading is an attractive target for new antiviral drugs (Sattentau, 2008). In the plaque area reduction assay, the HSV-1 plaque areas were not reduced (data not shown). Conversely, both isoflavonoids were able to reduce HSV-2 plaque areas, as compared to untreated controls ($p<0.05$) (Figure

4). Such results suggest that tested compounds reduced the transmission of progeny virions of HSV-2 (333 strain) from an infected cell to an uninfected one by inhibiting cell-to-cell spread.

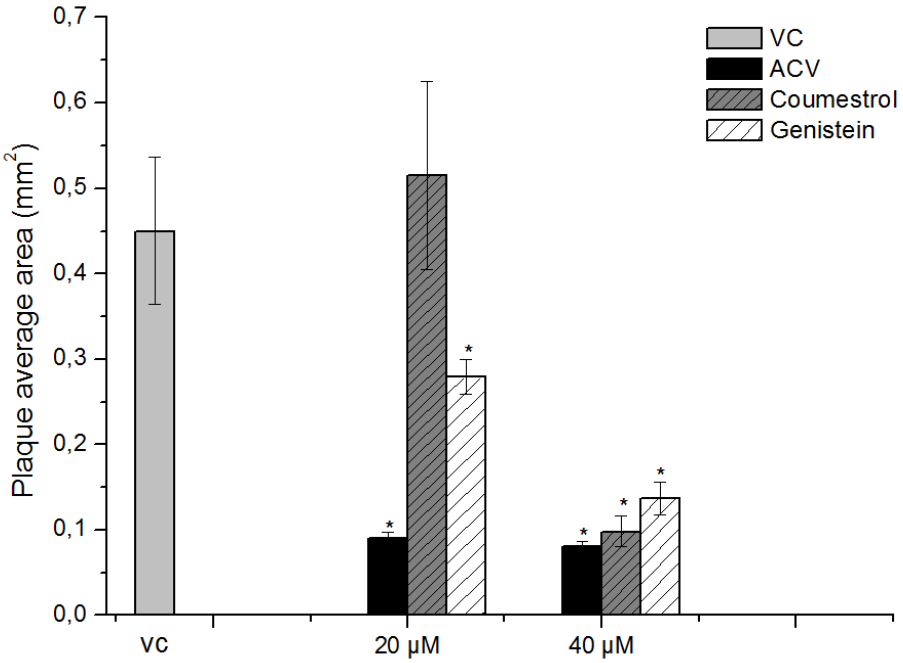

Figure 4. Inhibition of HSV-2 (333 strain) cell to cell spread by acyclovir (ACV), coumestrol and genistein at 20 and 40 µM. Results were expressed as average areas of viral plaques developed in sample-treated cells and compared to infected and untreated controls (=viral control). ACV was used as positive control. (*) indicates significant statistical differences between the tested sample and viral control ($p< 0.05$).

Therefore, the overall results show, for the first time, the effects of coumestrol against HSV types 1 and 2, as well as genistein activity against an acyclovir resistant strain of HSV-1. In addition, we demonstrated the possible mechanism of action of these soy isoflavonoids against HSV.

The tyrosine kinase inhibitory activity of genistein is involved in cellular and viral processes mediated by tyrosine phosphorylation (Guo *et al*., 2013; Ito *et al*., 2014; Qian *et al.,* 2014). Since HSV-1 infection induces the phosphorylation of tyrosine residues in fibroblast growth factor receptor on the cell surface (Baird *et al*., 1990), the inhibitory activity of tyrosine kinase could be responsible for the

anti-HSV activity of genistein. Indeed, Yura, Yoshida and Sato (1993) demonstrated that, after genistein treatment, the phosphorylation of tyrosine residues in specific viral polypeptides was markedly reduced (Yura, Yoshida and Sato, 1993). The authors also observed a significant reduction in HSV-1 gC expression, which is a late gene product, following treatment with 50 µM of genistein.

Given that herpes infections are always accompanied by an inflammatory process (Ma and He, 2014), the combination of antiherpes and anti-inflammatory agents in topical pharmaceutical forms is a therapeutic strategy for HSV infection treatment. Indeed, in a randomized study, Hull *et al*. (2014) showed the efficacy of a topical association of an antiviral drug (acyclovir) and an anti-inflammatory agent (hydrocortisone) for the treatment of HSV infections. The efficacy of this combined therapy was observed through a higher reduction in the cumulative lesion area as compared with acyclovir alone. This therapeutic approach can be explored for genistein and coumestrol, since, beside the antiherpes effects demonstrated here, both compounds presented anti-inflammatory activity (Jantaratnotai *et al*., 2013).

Conclusions

In summary, our findings suggest that coumestrol and genistein present inhibitory effects on HSV replication, interfering with various steps of the HSV replication cycle. Although only coumestrol has affected the early stages of viral infection, both compounds were able to reduce HSV-1 protein expression at different levels, as well as HSV-2 cell-to-cell spread. Here, we report a little investigated biological activity for soybean isoflavonoids, demonstrating that genistein and coumestrol could be regarded as potential alternative antiherpes agents.

Acknowledgements

This study was supported by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) - Rede Nanobiotec-Brazil (grant number 902/2009) and the State Foundation FAPERGS – PRONEM (grant number 11/2206-7).

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CAPÍTULO II

Otimização de nanoemulsões de uso tópico contendo genisteína através de um planejamento fatorial completo do tipo 2^3

No segundo capítulo experimental, avaliamos o efeito da combinação de diferentes componentes da fase interna de nanoemulsões, utilizando experimento fatorial completo do tipo 2^3, sobre diferentes parâmetros. Os fatores avaliados foram: tipo de óleo (óleo de rícino e miristato de isopropila), tipo de co-tensoativo iônico (oleilamina e ácido oléico) e tipo de fosfolipídeo (diestearolilfosfatidilcolina –DSPC e dioleilfosfatidilcolina –DOPC), tendo como respostas de saída as propriedades físico-químicas das nanoemulsões e a quantidade de genisteína retida em pele de orelha suína a partir das mesmas. As 8 formulações obtidas pelo arranjo experimental foram preparadas pelo procedimento da emulsificação espontânea. Análises de variância mostraram efeito de interação de 3^a ordem para as respostas de tamanho de partícula, índice de polidispersão e retenção cutânea e, portanto, a escolha das nanoemulsões foi baseada nesses resultados. A combinação miristato de isopropila/DOPC/oleilamina (NE3) apresentou o menor diâmetro médio e promoveu maior retenção de genisteína na pele, já a combinação miristato isopropila/DSPC/oleilamina (NE1) mostrou o menor índice de polidispersão. A partir dessas duas formulações avaliou-se o perfil de distribuição da genisteína nas diferentes camadas da pele de orelha suína. A retenção da genisteína na pele a partir de uma solução controle (genisteína em propilenoglicol) foi inferior, comparado as nanoemulsões, nas 3 camadas (estrato córneo, epiderme e derme). Os resultados mostraram maior taxa de retenção nas camadas mais profundas da pele (epiderme e derme) para as duas formulações, sendo observado maior retenção da genisteína a partir da nanoemulsão contendo DOPC (NE3). Por fim, a atividade anti-herpética frente ao HSV (tipo 1 e 2) da genisteína (em solução), bem como das nanoemulsões selecionadas contendo essa isoflavona foi avaliada. A atividade anti-herpética da genisteína incorporada nas nanoemulsões foi superior, uma vez que o CI_{50} foi reduzido em torno de 2,6 vezes, indicando que as nanoemulsões preparadas nesse estudo podem intensificar a atividade anti-herpética dessa isoflavona e representar, portanto, uma alternativa promissora no tratamento do herpes simples.

Factorial design applied to the optimization of lipid composition of topical antiherpetic nanoemulsions containing isoflavone genistein

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Publicado em:

International Journal of Nanomedicine, v. 9, p. 4737-47, 2014.

Abstract

The aim of this study was to optimize topical nanoemulsions containing genistein by means of a 2^3 full-factorial design based on physicochemical properties and skin retention. The experimental arrangement was constructed using oil type (isopropyl myristate or castor oil), phospholipid type (distearoylphosphocholine–DSPC or dioleylphosphocholine- DOPC) and ionic co-surfactant type (oleic acid or oleylamine) as independent variables. The analysis of variance showed effect of third order for particle size, polidispersity index and skin retention of genistein responses. Nanoemulsions composed of isopropyl mysristate/DOPC/oleylamine showed the smallest diameter and highest genistein amount in porcine ear skin whereas the formulation composed of isopropyl mysristate/DSPC/oleylamine exhibited the lowest polidispersity index. Thus, these two formulations were selected for further studies. Formulations presented positive ζ-potential values (>25mV) and genistein content close to 100% (at 1mg/mL). The incorporation of genistein in nanoemulsions significantly increases the retention of this isoflavone in epidermis and dermis, especially when the formulation composed by isopropyl miristate/DOPC/ oleylamine was used. These results were supported by confocal images. Such formulations exhibited antiherpetic activity *in vitro* against HSV-1(strain KOS) and HSV-2 (strain 333). Taken together, the results show that the genistein-loaded nanoemulsions developed in this study are promising in herpes treatment.

Keywords: genistein**,** nanoemulsion, factorial design, skin retention, antiherpetic activity

Introduction

Herpes simplex virus (HSV) types 1 and 2 cause various human diseases, such as gingivostomatitis, herpes labialis, meningitis, and ocular and genital infections (Brady and Bernstein, 2004). These viruses are able to establish and maintain latent infections that can be reactivated by different stimuli, such as stress, ultraviolet light, hormones, and immunosuppression (Simmons, 2002). Several drugs are currently available for the treatment of HSV infections. However, the emergence of viral strains resistant to available nucleoside analog drugs has stimulated the search for novel antiherpetic drugs (Mundinger and Efferth, 2008).

Studies exploring the evaluation of the antiherpetic activity of natural compounds, such as flavonoids, have been described (Field, 2001; Chiang *et al*., 2002; Zhong *et al*., 2013). For instance, Lyu *et al.* (2005) evaluated the anti-herpetic action of five different classes of flavonoids. Among the flavonoids tested, genistein – the main soybean isoflavone – showed an important inhibition of virus-induced cytopathic effect. The antiviral activity of genistein has been attributed to its tyrosine kinase-inhibitor effect (Akiyama *et al*., 1987). This kind of inhibition is one of the mechanisms responsible for reducing the infectivity of several viruses, including HSV (Andres, Donovan and Kuhlenschmidt, 2009). In vitro experiments suggested that genistein reduced the HSV-1 replication by specifically inhibiting tyrosine residues' phosphorylation of HSV-1 polypeptides (Yura, Yoshida and Sato, 1993).

In the topical treatment of herpes labialis, the bioactive compounds need to reach the stratum basale of the epidermis since this is the main site for HSV replication (Hukkanen *et al*., 2009). However, human skin is an efficient and selective barrier against the entrance of chemical/biological agents, and therapeutic agents must overcome its integrity, which mainly resides in the outermost layer of skin, the stratum corneum. This layer contains relatively low concentrations of water, being composed of corneocytes surrounded by lipids, which are arranged in highly organized lamellar bilayers (Elias *et al*., 1981; swartzendruber *et al*., 1989). Thus, several research articles have focused on the

evaluation of methods to overcome this resistance and increase stratum corneum permeability, thereby improving drug diffusion through the skin.

Various factors may play a role in the skin penetration of active compounds, including the influence of drug partitioning and its ability to diffuse through the skin layers. Skin permeation enhancement can be achieved by altering the thermodynamic properties of the drug and/or changing the barrier properties of the stratum corneum (Kaplun-Frischoff and Elka, 1997). A longstanding pharmaceutical approach to overcome this barrier effect is the use of penetration enhancers. Different chemicals have been used in this field, such as oils, fatty acids, and surfactants. These compounds may act through different mechanisms, including exerting different levels of interactions with stratum corneum cells, affecting cohesion between corneocytes, and modifying the resistance of the lipid bilayer (Willians and Bary, 2012; Barel, Paye and Maibach, 2009). The use of penetration enhancers in the design of colloidal carriers has been a useful strategy to improve the skin's percutaneous absorption of drugs (Kumar *et al*., 2009; Harwansh *et al*., 2011; Barakat, Fouad and Elmedany, 2012). Such carriers could increase the skin's permeation rate and enhance the topical effect due to the large surface area and low surface tension of these systems (Hoeller, Sperger and Valenta, 2009; Gulbake, Jain and Kjain, 2012; Fernandez- Campos *et al*., 2013).

The development of nanoemulsions aimed to improve the skin delivery of drugs and has attracted much interest. Nanoemulsions are composed of vegetal, synthetic, or semisynthetic oils that are very often stabilized by phospholipids as surfactant. Phospholipids are endogenous components of the human skin that have been extensively used as skin penetration enhancers, due to their high biocompatibility (Morganti, 2001). The incorporation of cosurfactants that may improve the permeation/retention of compounds through the skin has also been investigated. For instance, positively-charged surfactants may also have an effect on the permeation/retention of drugs, due to the possibility of increasing interaction with epithelial cells, which carry a negative charge in the outer cell membranes. Thus, different variables may have an effect on permeation. A factorial design can be used for planning formulation studies, providing maximum

information with a small number of experiments (Montgomery, 2008). Such design could provide a better combination of the formulation's composition, and the skin permeation can be used as a response.

Previous studies by Silva *et al*. (2009) and Vargas *et al*. (2012) have showed the feasibility of incorporating the isoflavone genistein into topical nanoemulsions. Following up on these results, here we applied a 2^3 factorial design to optimize the composition of topical nanoemulsions containing genistein. Our first aim was to evaluate simultaneously the effect of different oils (isopropyl myristate [IM] or castor oil [CO]), surfactants (dioleylphosphaditylcholine [DOPC] or distearoylphosphatidylcholine [DSPC]), and cosurfactants (oleic acid [OA] or oleylamine [OLA]) on the physicochemical properties of nanoemulsions and the retention of genistein into the porcine ear skin. Next, the distribution of genistein in skin layers was investigated for the optimized formulations, as well as their in vitro antiherpetic activity (anti-HSV-1 and anti-HSV-2).

Material and methods

Materials

CO, IM, genistein, OLA, OA, and the dye Nile Red were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Distearoylphosphocholine (DSPC) and dioleylphosphocholine (DOPC) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Polysorbate 80 was supplied by Vetec Quimica Fina Ltda. (Duque de Caxias, Brazil). Methanol and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® Plus apparatus (Merck Millipore, Billerica, MA, USA). Pig ears were generously donated by Ouro do Sul – Cooperativa dos Suinocultores do Caí Superior Ltda. (Harmonia, Brazil).

High-performance liquid chromatography analysis

The quantification of genistein in different samples (oils, formulations, and skin layers) was assessed by high-performance liquid chromatography (HPLC) using previously validated conditions (Vargas *et al*., 2012). In brief, HPLC apparatus consisted of a Shimadzu LC-10A system (Kyoto, Japan) equipped with

a model LC-20AT pump, a SPD-20AV ultraviolet-visible variable wavelength detector (set at 270 nm), a DGU-20A5 degasser, a CBM-20A system controller, and a SIL-20A injection valve with a 100 µL loop. Genistein was analyzed using a Shim-pack CLC-ODS (M) RP-18 column (5 mm, 250×4 mm inner diameter). The mobile phase consisted of a mixture of methanol/water, with 0.1% trifluoracetic acid (70:30, v/v) in isocratic flow at a flow rate of 1.2 mL/min.

Determination of genistein solubility in oils

The solubility of genistein was determined by adding an excess amount of this isoflavone to IM and CO. The mixtures were kept under moderate magnetic stirring for 24 hours at room temperature to reach equilibrium. After, the samples were centrifuged at 15,000 rpm for 15 minutes, and an aliquot of the supernatant of each sample was weighed and diluted with methanol. Genistein content was assayed by HPLC as described above. The experiments were performed in triplicate.

Experimental factorial design

Formulations were obtained by the combination of different oils (X1), phospholipids (X2), and ionic surfactants (X3), according to a qualitative 2^3 full factorial design (Table 1). The final composition of the formulations (w/w) was 8.0% of the oil (IM or CO), 2.0% of phospholipid (DOPC or DSPC), 0.05% of ionic surfactant (OA or OLA), and 1.0% of nonionic surfactant, polysorbate 80, and Milli-Q water up to 100%. Eight different formulations (NE1–NE8) were prepared in triplicate. Statistical analysis was performed using the Minitab® software at a significance level of $P<0.05$.

Nanoemulsions were prepared by spontaneous emulsification. This method consists of injecting an organic phase containing oil core components into the water phase, under magnetic stirring. Subsequently, the organic solvent (ethanol) was removed by evaporation under reduced pressure, at 40°C–45°C. The final pH of formulations was adjusted to values ranging from 5.0 to 6.0. A control solution was prepared at 1 mg/mL of genistein in propylene glycol. The dependent variables related to the physicochemical properties of formulations were the mean droplet size, the polydispersity index, the ζ potential, and also, the

genistein content. The total amount of genistein retained in the whole skin was also used as an additional dependent variable. The experimental conditions used to evaluate these variables are described in the “Characterization of the nanoemulsions” and “Skin permeation/retention studies” sections.

Table 1.Independent factors and their levels.

Factors		Level	
		Low level (-1)	High level (+1)
X_1	Oil	IM	CO
X_2	Phospholipid	DSPC	DOPC
X_3	Ionic cosurfactant	OLA	OA

Abbreviations: CO, castor oil; IM, isopropyl myristate; DOPC, dioleylphosphadityl-choline; DSPC, distearoylphosphatidylcholine; OA, oleic acid; OLA, oleylamine.

Characterization of the nanoemulsions

The droplet size and the ζ potential of the nanoemulsions were determined by photon correlation spectroscopy and electrophoretic mobility, respectively (3000HS Zetasizer; Malvern Instruments, Malvern, England). The samples were diluted in water, for size, or in 1 mM NaCl solution, for ζ potential measurements. Morphological examination was performed by means of transmission electron microscopy (TEM). The formulations were diluted at a 1:10 ratio, obtaining an oil phase concentration equal to 1%. Specimens for TEM viewing were prepared by mixing samples with one droplet of 2% (w/v) uranyl acetate solution. The samples were then adsorbed to the 200 mesh Formvar-coated copper grids, left to dry, and examined by TEM (JEM-1200 ExII; JEOL Ltd., Tokyo, Japan).

The total genistein content was determined after the dilution of formulations in methanol. To estimate the genistein association, nanoemulsions were directly added to ultrafiltration membranes (100,000 Da cutoff, Ultrafree; Merck Millipore) and centrifuged at 15,000 rpm for 15 minutes. The association

efficiency (%) was estimated by the difference between the total and free-genistein concentrations.

Skin permeation/retention studies

Porcine ears were obtained from a local slaughterhouse. The ears were withdrawn from the animals before the scalding procedure, cleaned, and the full-thickness skin was excised from the outer region of the ear with a scalpel. After the removal of subcutaneous fat, the skin was wrapped in aluminum foil and stored at -20°C for a maximum time period of 1 month. Genistein permeation/retention was assessed using Franz-type diffusion cells, which presented a surface area for diffusion of 1.77 cm^2 and a receptor volume of 10.0 mL. Porcine ear skin with a thickness about 0.6 to 0.9 mm was set between the donor and the receptor compartments, with the dermal side facing downward into the receptor medium. Experiments were performed in sink conditions, due to the presence of 30% ethanol in phosphate-buffered saline in the receptor phase. The bathing solution was kept under a controlled temperature (32°C). Approximately 500 µL of each nanoemulsion were placed in the donor compartment. After 8 hours, samples of the receptor fluids were withdrawn from the compartment, and the skin was removed from the cell and cleaned using a cotton swab embedded with Milli-Q water. The genistein content was assessed by HPLC, as described in the “High-performance liquid chromatography analysis” section, in the receptor fluid and in the whole skin, after discarding the first tape strip.

To evaluate the distribution of genistein into the skin layers from selected formulations (NE1 and NE3), genistein retention was determined in the stratum corneum, epidermis, and dermis. Briefly, the first stripped tape was discarded, while the following 15 tapes were used for genistein assay (stratum corneum). Next, the epidermis was separated from the dermis using a scalpel. The dermis was perforated into tiny pieces, and the layers were placed in the test tube. Results were expressed as mean ± standard deviation of genistein retained per unit of area (µg/cm^2) from five experiments.

Histological and confocal fluorescence microscopy studies

For histological studies, the skin cuts were cleaned with a cotton swab and immersed in formaldehyde solution (at 37%). Subsequently, the cuts were dehydrated, embedded in parafafin, and sectioned, with a thickness of 6 µm. The skin tissue specimens, after staining with hematoxylin and eosin, were photographed by optical microscopy. For confocal fluorescence experiments, a fluorescent dye, Nile Red, was added during the preparation of nanoemulsions by a spontaneous emulsification process. Formulations NE1 and NE3 were prepared containing this fluorescent dye at a final concentration of 0.05% (previously solubilized in ethanol). Approximately 500 µL of each fluorescent nanoemulsion was placed in the donor compartment, and the permeation/retention study was performed under the same experimental conditions described in the "Skin permeation/retention studies" section. After 8 hours, the skin cuts were cleaned and mounted with Tissue-Tek® O.C.T.™ (Sakura Finetechnical Co., Ltd., Tokyo, Japan) onto a metal sample holder and frozen at -20°C. Vertical slices of skin cuts of 40 µm thickness were obtained with a cryostat (CM 1,850; Leica Microsystems, Wetzlar, Germany), and the slides were evaluated using a confocal microscopic Olympus FluoView™ 1,000 (Olympus Corporation, Tokyo, Japan), with a helium–neon red laser exciting at 559 nm. The images were taken at a tenfold magnification.

In vitro evaluation of antiherpetic activity of genistein-loaded nanoemulsions

Cells and viruses

The cell lines used were Vero (CCL81; American Type Culture Collection, Manassas, VA, USA) and green monkey kidney (GMK) AH1 (Department of Clinical Virology, Gothenburg University, Gothenburg, Sweden). They were grown in Eagle's minimum essential medium (MEM) (Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (Cultilab) and maintained at 37°C in a humidified 5% CO_2 atmosphere chamber. The HSV-1 (KOS strain) (Faculty of Pharmacy, University of Rennes, Rennes, France) and HSV-2 (333 strain) (Department of Clinical Virology, Gothenburg University) were propagated in Vero and GMK AH1 cells, respectively. Stock viruses were prepared, titrated based on plaque-forming units, counted by plaque assay, as

previously described, and stored at -80°C (Burleson, Chambers and Wiedbrauk, 1992).

Cytotoxicity assay

The cytotoxicity of genistein (dissolved in 1.0% dimethylsulfoxide) and the nanoemulsions was determined by sulforhodamine B assay (Vichai and Kirtikara, 2006). Briefly, the cells were trypsinized, seeded into 96-well microplates, and allowed to adhere for 24 hours, at 37°C in a 5% CO_2 atmosphere chamber. Afterwards, different concentrations of the samples were dispensed into the wells and incubated for 48 hours. The cells were stained with sulforhodamine B, and absorbance was measured at 510 nm using a microplate reader, and for each sample, the concentration at which cell viability was reduced by 50% (CC_{50}) was calculated, comparing with the untreated controls.

Antiherpetic activity

The potential antiherpetic activity of genistein and genistein-loaded nanoemulsions was evaluated by the plaque number reduction assay, as previously described by Silva *et al*. (2010). Confluent monolayers of Vero or GMK AH1 cells were infected with 100 plaque-forming units per well of HSV-1 (KOS strain) or HSV-2 (strain 333), respectively, for 1 hour at 37°C. After, the cells were washed with phosphate-buffered saline and overlaid with agarose gel (0.6%) in the presence or absence of different concentrations of genistein or genistein-loaded nanoemulsions, and the plates were incubated for 48 hours. Cells were then fixed and stained with naphthol blue–black, and viral plaques were counted. The IC_{50} of each sample was defined as the concentration that inhibited 50% of viral plaque number when compared with untreated controls. The IC_{50} and CC_{50} values were estimated by linear regression of the concentration–response curves generated from the obtained data. The selectivity index (CC_{50}/IC_{50}) of each tested sample also was determined. All experiments were performed in triplicate.

Statistical analysis

The mean and standard deviations were calculated for all experiments. The statistical analysis was according to paired *t*-test or analysis of variance ($P<0.05$) in a factorial experimental design, using Minitab 15 software.

Results

Solubility determination in tested oils

Genistein solubility in the oil cores used for the preparation of nanoemulsions was estimated by adding an excess of this isoflavonoid in IM or CO. Genistein was much more soluble in CO (3.32±0.25 mg/g) than in IM (0.34±0.01 mg/g).

Nanoemulsion optimization by factorial design

Table 2 shows the experimental arrangement based on the 2^3 full factorial design as well as the measured responses (droplet size, polydispersity index, ζ potential, genistein content, and skin genistein retention) for the eight formulations obtained by means of spontaneous emulsification. Analysis of variance of responses was performed (Table 3). For mean droplet size (Y1), polydispersity index (Y2), and genistein retention (Y5), the analysis of variance for these responses showed statistical significance for the effects of third-order interactions. The mean droplet size varied, from approximately 160 nm to 420 nm. The combination of X1, X2, and X3 over Y1 response allowed the selection of NE3 (IM/DOPC/OLA) due to the lowest droplet size (161±6 nm). This droplet size value was significantly different from other all-lipid combinations. The CO/DSPC/OA (NE6) combination led to the highest mean droplet size (426±82 nm). With regard to the polydispersity index, the combination of IM/DSPC/OLA (NE1) exhibited the lowest value (0.07±0.02), which was significantly different from the other formulations. As a general rule, formulations presented a polydispersity index lower than 0.3, except for NE6 (CO/DOPC/OLA), for which the highest value, of 0.58±0.08, was observed. Finally, concerning the skin retention of genistein, the values ranged from 0.56 µg/cm² to 1.86 µg/cm². The use of IM (NE1 to NE4) led to a higher retention in the skin; however, NE3 (IM/DOPC/OLA) presented the highest skin retention of genistein (1.86±0.32 µg/cm²). The value was significantly lower for NE1, NE2, and NE4, without

significance among them. Considering the effect of two-way interaction over the Y4 response (genistein content), NE5 and NE6 presented no statistical differences between them (P=0.05), and NE6 presented the lowest content of genistein (P<0.05) in relation to the other nanoemulsions. ζ- potential response (Y3) was significantly affected by oil type and ionic surfactant but not by interactions among them. The NE3 and NE7 nanoemulsions showed higher values (P<0.05) in modulus for ζ-potential response when compared with the other formulations (higher than 30 mV).

Table 2. Experimental arrangement based on the 2^3 full factorial design and measured responses for eight experiment runs.

Code	Experimental arrangement			Y1	Y2	Y3	Y4	Y5
				Z-ave (nm) ± SD	PI ± SD	ζ-potential (mV) ± SD	GEN content (mg/ml) ± SD	GEN retained (μg/cm²) ± SD
NE1	IM	DSPC	OLA	213.60 ± 18.07	0.07 ± 0.02*	+25.20 ± 10.85	0.96 ± 0.041	1.41 ± 0.08
NE2	IM	DSPC	OA	236.90 ± 19.27	0.17 ± 0.04	-17.95 ±0 2.59	0.94 ± 0.047	1.13 ± 0.16
NE3	IM	DOPC	OLA	161.19 ± 06.64*	0.24 ± 0.01	+32.92 ± 04.91	0.95 ± 0.042	1.86 ± 0.32*
NE4	IM	DOPC	OA	287.79 ± 33.70	0.30 ± 0.04	-18.35 ± 07.61	0.97 ± 0.013	1.06 ± 0.10
NE5	CO	DSPC	OLA	225.40 ± 17.23	0.19 ± 0.02	+23.97 ± 03.19	0.83 ± 0.049*	0.90 ± 0.20
NE6	CO	DSPC	OA	426.40 ± 82.94	0.58 ± 0.08	-15.63 ± 05.44	0.80 ± 0.061*	0.65 ± 0.10
NE7	CO	DOPC	OLA	190.82 ± 03.70	0.14 ± 0.03	+36.00 ± 04.90	0.97 ± 0.048	0.81 ± 0.12
NE8	CO	DOPC	OA	219.02 ± 20.68	0.13 ±0.03	-19.13 ± 04.83	0.97 ± 0.044	0.56 ± 0.08

IM: isopropyl myristate; CO: castor oil; DSPC: distearoylphosphatidylcholine; DOPC: dioleylphosphatidylcholine; OLA: oleylamine; OA: oleic acid; GEN: genistein. *significantly different ($p<0.05$)

Table 3. The analysis of variance for responses.

Responses	p-values		
	Main effects	2-Way Interactions	3-Way Interactions
ParticleSize (Y1)	< 0.001	0.003	< 0.001
Polidispersity index (Y2)	< 0.001	< 0.001	< 0.001
ZetaPotential (Y3)	0.001	0.381	0.898
GenisteinContent (Y4)	< 0.001	0.011	0.898
SkinRetention (Y5)	< 0.001	0.028	0.001

TEM imaging of selected nanoemulsions

The morphology of the NE1 and NE3 nanoemulsions can be seen in Figure 1. The TEM images showed the typical appearance of oil–water nanoemulsions, with oil droplets displaying a size of close to 150–250 nm.

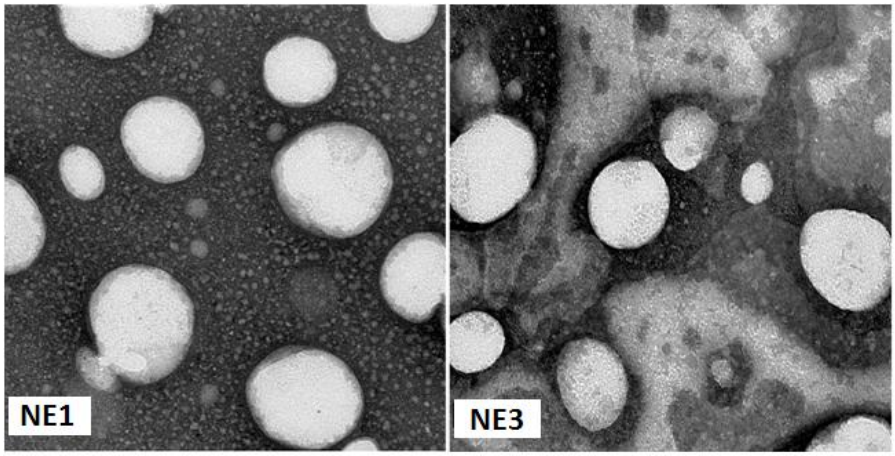

Figure 1 TEM images of the selected nanoemulsions composed of IM/DSPC/OLA (NE1) and IM/DOPC/OLA (NE3). No differences were detected in the morphology of oil droplets of NE1 and NE3. **Note:** Scale bar =200 nm. **Abbreviations:** DOPC, dioleylphosphaditylcholine; DSPC, distearoylphosphatidylcholine; IM, isopropyl myristate; NE, nanoemulsion; OLA, oleylamine; TEM, transmission electron microscope.

Genistein distribution into skin layers from selected nanoemulsions

Figure 2 shows the amount of genistein retained from the formulations, in the porcine ear skin layers. Regardless of the composition, genistein was detected in all layers. A lower amount of genistein was detected in the stratum corneum from nanoemulsions in comparison with the control propylene glycol solution. The incorporation of genistein into nanoemulsions significantly increased the retention of this isoflavone in the epidermis and dermis (P<0.05). In addition, the formulation composed by IM/DOPC/OLA (NE3) led to a significantly higher amount (P<0.05) of genistein in the dermis in comparison with those composed by IM/DSPC/OLA (NE1). Following 8 hours of permeation/retention, genistein content was below the methods lower limit of quantification (0.1 µg/mL) for both NE1 and NE3.

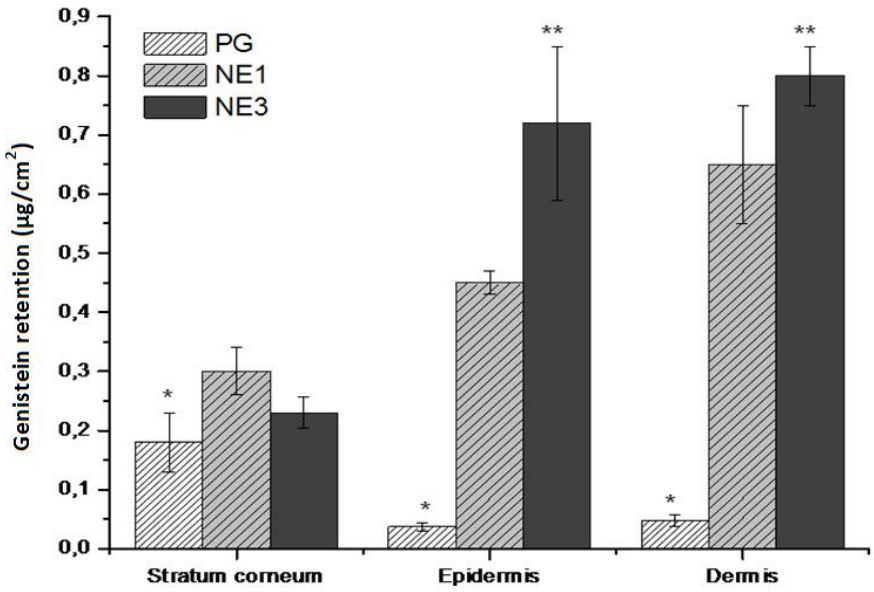

Figure 2 Distribution profile of genistein into porcine ear skin layers after 8 hours of permeation/retention study, from a control solution (PG solution) and NE1 and NE3. **Notes:** A lower amount of genistein was detected in the stratum corneum from nanoemulsions, with a significantly increased retention of this isoflavone into epidermis and dermis. The values are means ± SD of five experiments. *Significantly lower (P<0.05) for PG in stratum corneum, epidermis and dermis as compared to NE1 and NE3; **significantly higher (P<0.05) for NE3 in epidermis and dermis as compared with PG and NE1. **Abbreviations:** PG, propylene glycol; NE, nanoemulsion; SD, standard deviation

Skin distribution of the dye Nile Red from selected formulations

Figure 3 shows a photomicrograph panel of porcine ear skin colored by hematoxylin/eosin, of untreated skin (Figure 3A) or after 8 hours of the permeation/retention study for NE1 (Figure 3B) and NE3 (Figure 3C). Images showed the cells' nuclei stained in blue and the other components stained in red. Regardless of the treatment (NE1 and NE3), the treated skin maintained a structure similar to untreated skin (Figure 3A), presenting stratum corneum with little detachment, a well-defined nucleus and cytoplasm without vacuoles or lyses, absence of intraepidermal clefts, and dermal–epidermal junction without separation of the two layers. In similar experimental conditions, confocal images of vertical skin sections of 40 µm thickness were performed (Figure 3D–F). The images revealed that the Nile Red dye was mainly located in the stratum corneum when this fluorescent dye was dispersed in propylene glycol. Fluorescence was distributed throughout the skin layers (stratum corneum, viable epidermis, and dermis) when this dye was incorporated into the nanoemulsions. The fluorescence intensity seems to be higher after nanoemulsion application when compared with the control solution.

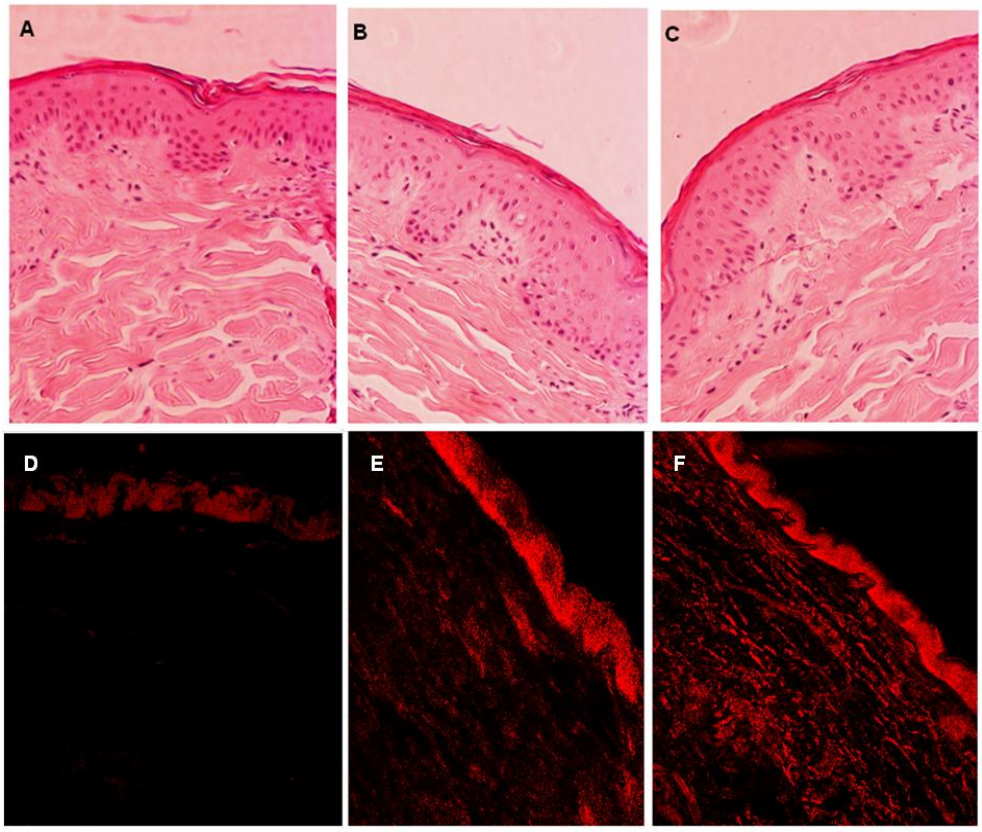

Figure 3 Hematoxylin-/eosin-stained histological (A–C) or fluorescent (D–F) images of full-thickness porcine ear skin treated with a genistein propylene glycol solution (A and D) or with genistein-loaded NE1 (B and E) or NE3 (C and F). **Notes:** Histological images show no skin damage after treatment with the nanoemulsions. The confocal images revealed that the dye Nile Red was mainly located in stratum corneum when dispersed in propylene glycol. However, the fluorescence was distributed throughout the skin layers when the dye was incorporated into nanoemulsions. Images were obtained after 8 hours of permeation/retention studies using a Franz diffusion cell. Images were obtained at ×10 magnification. Nile Red was used as fluorescent dye in confocal images. **Abbreviation:** NE, nanoemulsion.

Antiherpetic activity

Table 4 shows the antiherpetic activity (anti-HSV-1 and anti-HSV-2) of genistein solution (solubilized in dimethyl-sulfoxide) and genistein-loaded NE1 and NE3 nanoemulsions. Genistein was significantly (P<0.05) more cytotoxic for Vero cells (CC_{50} =23.91 µg/mL) in comparison with GMK AH1 cells (CC_{50} =50.36 µg/mL). An increase in cytotoxicity was observed when genistein was incorporated into nanoemulsions, especially when GMK AH1 cells were used. As it can be seen, genistein exhibited an IC_{50} of approximately 13 µg/mL for both viral strains tested

(HSV-1, in the KOS strain, and HSV-2, in the strain 333). The incorporation of genistein into formulations (NE1 or NE3) reduced the IC_{50} values for both viral types of viral plaque formation. Blank nanoemulsions (without genistein) showed no activity against HSV-1 and HSV-2 viruses.

Table 4. Cytotoxicity (CC_{50}) on Vero and GMK AH1 cells and inhibitory effects of HSV-1 and HSV-2 replication (IC_{50}), respectively, of free-genistein and genistein-loaded nanoemulsions (NE1 and NE3).

		Genistein	NE1	NE3
HSV-1	CC_{50} (µg/mL)*	23.91 ± 1.12	16.59 ± 0.94	17.55 ± 2.82
	CI_{50} (µg/mL)#	13.14 ± 1.26	06.75 ± 0.13	06.38 ± 2.63
HSV-2	CC_{50} (µg/mL)*	50.36 ± 7.97	19.88 ± 2.21	21.23 ± 0.61
	CI_{50} (µg/mL)#	12.58 ± 1.62	05.87 ± 0.85	04.82 ± 0.62

Notes: Values represent the mean ± standard deviations of three independents experiments. *Cytotoxicity was determined by sulforhodamine B assay on Vero and GMK AH1cells. #Antiviral activity was determined by plaque number reduction assay. **Abbreviations:** CC_{50}, concentration at which cell viability was reduced by 50%; HSV, herpes simplex virus; IC_{50}, concentration that inhibited 50% of viral plaque number; NE, nanoemulsion; GMK, green monkey kidney.

Discussion

In the present study, we evaluated the effects of lipid components on the properties of nanoemulsions, with the intention of optimizing the composition of the formulations intended for genistein skin delivery. The effects of the oil core, the phospholipid, and the ionic surfactant composition on the different physicochemical properties of nanoemulsions, genistein content, and genistein retention in the skin were evaluated by means of a 2^3 full factorial design. This experimental design enabled all factors to be varied simultaneously, allowing the quantification of the effects caused by independent variables along with the interactions between them (Montgomery, 2008). Main effects alone do not have much meaning when they are involved in significant interactions. These interactions are the key to achieving the optimal conditions.

Among the dependent variables evaluated, the obtained results showed three-way interactions for the droplet size and the polydispersity index of nanoemulsions, as well as for genistein retention in porcine ear skin after 8 hours of permeation/retention kinetics. Such results can be better interpreted using the factorial plots of three-way interactions for these variables (Figure 4).

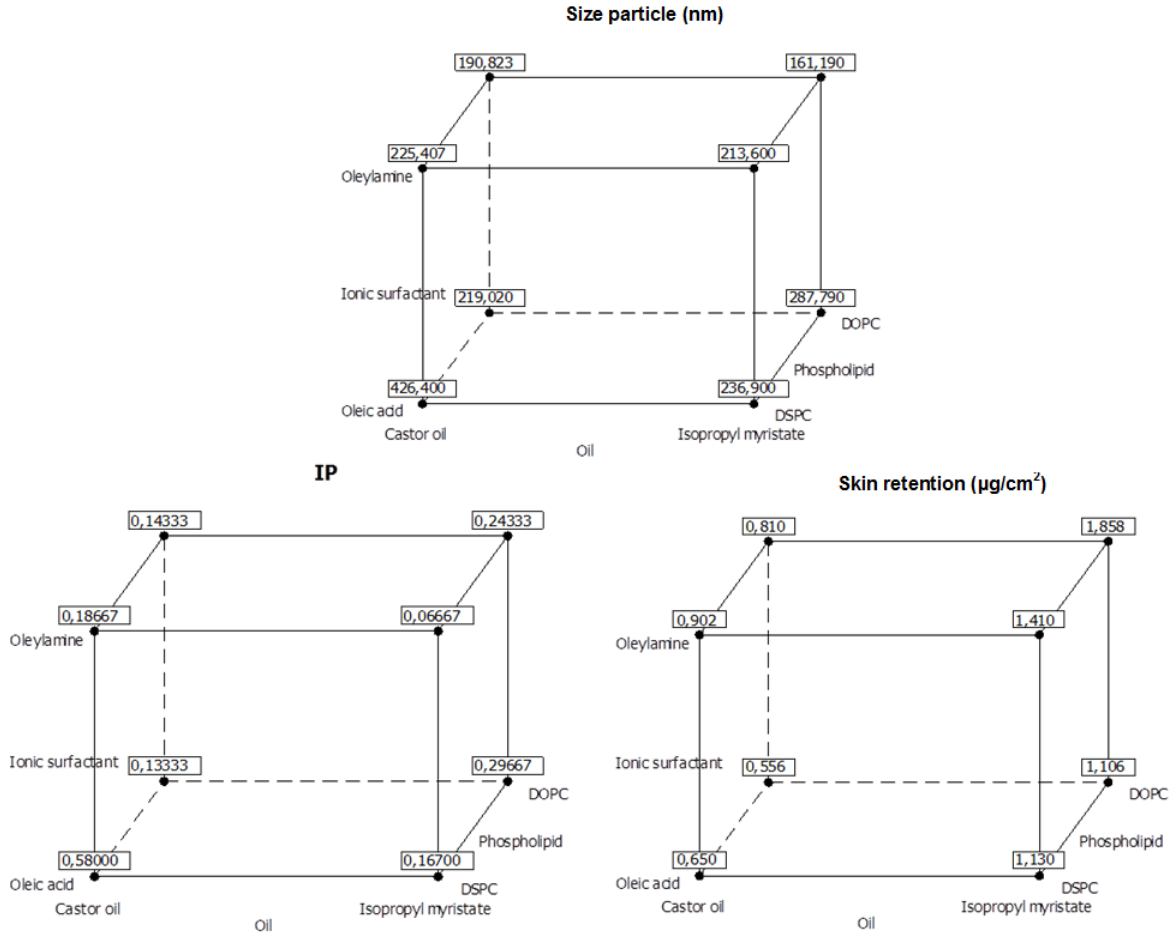

Figure 4. Factorial plots of three-way interactions for droplet size response, polydispersity index response, and genistein retention in the skin. **Notes:** The graphical representation shows that the NE3 presented the lowest droplet size and the highest genistein retention in the porcine ear skin. The NE1 presented the lowest polydispersity index. The plots were obtained from measured responses. **Abbreviations:** DOPC, dioleylphosphaditylcholine; DSPC, distearoylphosphatidylcholine; NE, nanoemulsion; PI, polydispersity index.

The IM/DOPC/OLA (NE3) combination led to the lowest droplet size of the nanoemulsions (161±6 nm). A smaller droplet size can be related to a greater surface to interact with skin, thus improving the permeation/retention of drugs (Abolmaali *et*

al., 2011; Mitri *et al*. 2011). However, the polydispersity index of this formulation was 0.24±0.01, which can be considered a limit value according to the literature. Polydispersity index gives some information about the deviation from the average size, and values up to 0.250 could be acceptable (Muller *et al*., 2004). From the analysis of variance, the replacement of DOPC (NE3) by DSPC (NE1) showed the lowest polydispersity index (0.07±0.02). Thus, the ternary mixture of a high transition temperature phospholipid (DSPC) with IM and OLA yielded more homogeneous systems. No marked differences were detected in the oil droplets of these formulations by TEM; the droplet size was in the 200 nm range, which is in accordance with the photon correlation spectroscopy experiments. Taking into account the physicochemical characterization, NE1 and NE3 were selected for further studies. Similarly, analysis of variance of the data obtained in the experimental design showed three-way interactions for the variable genistein retention in the skin. An overall analysis of the results showed that the use of IM as the oil core instead of CO led to a significant increase of genistein in the skin. One initial explanation is that IM could be considered a better absorption enhancer than CO (Goldberg-Cettina *et al*., 1996; Santos *et al*., 2012). In addition, since the solubility of genistein in the IM was significantly lower (P<0.05) than in CO, as demonstrated in this study, a saturation of the vehicle might lead to an increase in the retention of this isoflavone into the skin, whereas the high solubility of genistein in CO increased the affinity of this isoflavone into the oil core, decreasing the partitioning from the vehicle into the skin. Among the IM-based formulations, the genistein retention in skin was significantly higher (P<0.05) from positively-charged NE3 (composed of IM/DOPC/OLA), followed by NE1 (composed of IM/DSPC/OLA). It is well documented that positively-charged coloidal carriers can interact with negatively charged protein residues of epithelial cells, leading to a disturbance in the lipid structure of the stratum corneum (Rojanasakul *et al*., 1992; Eskandar, Simovic and Prestidge, 2010), which, in turn, can lead to increased drug permeability. Finally, the formulation composed of IM/DOPC/OLA (NE3) led to a significantly higher amount of genistein retained in the skin when compared with the combination containing DSPC (NE1). This result may be attributed to the more fluid interface of

IM/DOPC/OLA nanoemulsions, due the presence of DOPC, which is in the fluid state and could lead to an increase of partitioning of genistein to the skin.

Regardless of the combination of the lipids used, analysis of variance of the ζ-potential of the nanoemulsions showed only significance for the main effect. In other words, there was no interaction between lipid components over this property. ζ-potential was only influenced by adding charged cosurfactants in the formulation composition. Concerning the selected formulations (NE1 and NE3), the binary mixture of these cosurfactants with DOPC or DSPC did not have a significant effect on this parameter. Since the phosphatidylcholine polar head group of DOPC or DSPC did not exhibit charge (due to the coexistence of the negatively charged phosphate group and the positively charged quaternary ammonium group of choline in the polar head group) at the formulation's final pH, the ζ-potential was only influenced by the presence of OLA. It must be pointed out that the bulk pH of positively-charged nanoemulsions presented values of approximately 8.5–9.0 due to the presence of the OLA. For these formulations, final pH was adjusted to 5.0–6.0 to produce theoretically fully ionized OLA molecules at the interface, as previously reported (Rabinovich-Guilatt *et al*., 2004; Martini *et al*., 2008). Furthermore, it was demonstrated that the pH of the vehicle had a significant effect in the cutaneous retention of genistein, which is higher at a pH of 6.0, due the nonionic form of genistein in this medium (Huang *et al*., 2008).

For the optimized formulations, the genistein content values were higher than 95% (0.96±0.041 and 0.95±0.042 for NE1 and NE3, respectively), without any difference among them (P>0.05). Since genistein was not detected in the water phase, after separation of a fraction of the water phase on ultrafiltration membranes, this isoflavone seems to be incorporated into the oily phase of nanoemulsions. This result could be attributed to the affinity of this poorly soluble aglycone (LogP =3.04) (Rothweel, Day and Morgan, 2005) for the lipid components of the inner phase of the nanoemulsions. Regarding the genistein content response, the effect of two-way interaction between the oil (CO or IM) and phospholipid (DSPC or DOPC) factors was observed. Statistical analyses showed that the interaction of CO and DSPC (containing OA or OLA) presented the lowest content of genistein: 0.83±0.049 mg/mL

(for NE5) and 0.80±0.061 mg/mL (for NE6). This result could be related with a decrease of the fluidity of the oil core (in the presence of CO and DSPC) and thus, the solubility of genistein in those formulations.

To have a better insight into the distribution of genistein in the porcine ear skin layers from optimized formulations, stratum corneum, epidermis, and dermis were separated and the amount of this isoflavone determined by HPLC. A higher amount of genistein was detected in the deep layers (epidermis and dermis) when isoflavone was incorporated into nanoemulsions, indicating that the positively-charged optimized formulations were able to overcome the barrier property of the outermost layer of the skin. The skin retention of genistein from other colloidal carriers has been described elsewhere (Kim *et al*.,2012; Kitagawa *et al*., 2010; Zampieri *et al*., 2013). Conversely, the skin retention of genistein from the propylene glycol control solution was higher in the stratum corneum, which demonstrates that skin penetration is hampered by the barrier effect of the stratum corneum. These results were illustrated by the confocal images. A higher fluorescence intensity was detected in the deep layers of porcine ear skin (without a deleterious effect on the skin, according to histological photomicrographs) when compared with images obtained with a propylene glycol solution, in which Nile Red seemed to be located mainly in the upper skin layers.

Finally, our results clearly showed the inhibition of HSV-1 and HSV-2 replication by genistein, which follows in line with data previously reported by Yura et al (1993). Interestingly, the incorporation of genistein into the optimized nanoemulsions (NE1 and NE3) decreased the IC_{50} of the viral plaque formation, against both HSV-1 and HSV-2. This result suggests that the nanoemulsions tested could enhance intracellular genistein uptake. They most probably took advantage from the destabilization of the cell membrane due to the positive charges of the nanoemulsions, which probably increased membrane permeability and allowed the genistein molecules to enter the cells more efficiently (Rehman, Zuhorn and Hoekstra, 2013). For instance, the formulation composed of IM/DOPC/OLA (NE3) showed an IC_{50} up to 2.6-fold lower than free-genistein in HSV-2. This formulation exhibited a selectivity index (that is the ratio between CC_{50} and IC_{50}) higher than 4,

which can be considered promising for candidates as antiviral agents (Amoros *et al*., 1992). The antiherpetic activity of genistein has been related to the protein-tyrosine phosphorylation, which is involved in the regulation of cell growth and in receptor-mediated signal transduction (Andres, Donovan and Kuhlenschmidt, 2009); however, the mechanism needs to be fully elucidated.

Conclusion

The factorial design allowed us to select the combinations of IM, OLA, DSPC, or DOPC as the best inner phases for stable nanoemulsions with higher potential for increasing skin retention of genistein, showing the effect of positively charged nanoemulsions on these properties. A significant increase in the genistein content was detected into viable skin layers (epidermis and dermis) when this isoflavone was incorporated into nanoemulsions, without visible skin damage as it was visualized in the histological images. These results were supported by confocal laser scanning microscopy experiments. Finally, such formulations exhibited in vitro antiherpetic activity against HSV-1 (strain KOS) and HSV-2 (strain 333). Taken together, the results showed that the genistein-loaded nanoemulsions developed are promising for antiherpes treatment.

Acknowledgments

This work was supported by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), Rede Nanobiotec-Brazil (grant number 902/2009) and the State Foundation for Research Support (FAPERGS) – PRONEM (grant number 11/2206-7). DA wishes to thank CAPES for her scholarship.

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CAPÍTULO III

Avaliação da distribuição da genisteína em mucosa de esôfago suíno a partir de nanoemulsões espessadas com hidroxietilcelulose e atividade anti-herpética *in vitro*.

Neste terceiro capítulo, as nanoemulsões selecionadas pelo planejamento fatorial foram espessadas com hidroxietilcelulose (HEC) a fim de adequar a viscosidade das mesmas para o uso tópico. Primeiramente, os hidrogéis obtidos (HNE-DSPC e HNE-DOPC) foram caracterizados quanto aos parâmetros físico-químicos. Independente da composição, as nanoemulsões apresentaram-se monodispersas com diâmetro médio próximo de 200 nm e potencial zeta positivo (> 25 mV). A adição do HEC não alterou significativamente os resultados de tamanho de gotícula e índice de polidispersão. Contudo, após espessamento das formulações, os valores de potencial zeta foram reduzidos (P<0,05). Com relação à reologia, os hidrogéis foram caracterizados como sistemas não-Newtonianos com comportamento pseudoplástico. Posteriormente, foram realizados estudos de liberação da genisteína a partir das formulações através de membrana sintética de celulose. A solução de genisteína em propilenoglicol foi utilizada como controle. A liberação da genisteína foi superior a partir da solução controle (100% liberada em 3 horas de estudo), quando comparada com as formulações (P<0,05). Com relação às formulações, após 10 horas de estudo, a liberação da isoflavona foi claramente influenciada pela composição das nanoemulsões, sendo maior a partir da nanoemulsão contendo DOPC, e pela viscosidade dos hidrogéis derivados, uma vez que a adição do HEC reduziu a cinética de liberação da genisteína de ambas as nanoemulsões. Os estudos de permeação/retenção da genisteína em mucosa esofágica suína foram realizados usando células de difusão de Franz. Os perfis de permeação foram de acordo com os perfis da liberação da genisteína a partir das formulações. No entanto, ao contrário do resultado observado na liberação, a permeação da isoflavona a partir da solução controle foi mais baixa, comparada com as formulações. As imagens de confocal corroboram com esses resultados. A avaliação da atividade antiviral frente a uma cepa do HSV-1, resistente ao aciclovir, mostrou que a genisteína apresentou atividade superior, em torno de duas vezes, quando incorporada nas nanoemulsões. Portanto, nesse capítulo, os resultados apresentam formulações contendo genisteína de consistência adequada para o tratamento tópico e/ou transmucosa do herpes simples.

In vitro evaluation of mucosa permeation/retention and antiherpes activity of genistein from cationic nanoemulsions

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Artigo aceito para publicação no *Journal of Nanoscience and Nanotechnology*, 2015

Abstract

In this report, we described the genistein distribution on excised porcine esophageal mucosa from cationic nanoemulsions, as well as the anti-HSV-1 activity against a viral strain resistant to acyclovir. Cationic nanoemulsions were obtained by spontaneous emulsification procedure. This procedure yielded monodisperse nanoemulsions exhibiting a mean droplet size of approximately 200–300 nm. Genistein was successfully incorporated into cationic nanoemulsions obtained by spontaneous emulsification. Hydroxyethyl cellulose was added at the end of the manufacturing process as a thickening agent (at 3%). Such formulations exhibit a non-Newtonian pseudoplastic behavior. The addition of this polymer significantly reduces the genistein flux through excised porcine mucosa specimens as compared with values elicited by nanoemulsions before thickening. Furthermore, a significant increase of genistein retention in mucosa (3-fold) was observed from formulations as compared to the genistein propylene glycol solution. Formulations exhibited antiherpetic activity in vitro against HSV-1 (strain 29R). Taken together, these results suggest that these formulations have promising potential to be used topically for herpes infections.

Keywords: genistein, nanoemulsion, hydrogel, mucosa permeation, antiherpetic

Introduction

Genistein is an isoflavone found in soybeans that has attracted much attention because of its biological activities (Ajdžanović *et al*., 2014; Saha, Sadhukhan and Sil, 2014). A well-documented literature has showed that genistein inhibits herpes virus (HSV-1 and HSV-2) replication. Such activity has been related to protein-tyrosine phosphorylation, which is involved in the regulation of cell growth and receptor-mediated signal transduction (Yura, Yoshida and Sato, 1993; Lyu Rhim and Park., 2005).

HSV typically infects the mucosal tissues causing various clinical manifestations, such as cold sores, gingivostomatitis, genital ulcers, blindness, or encephalitis (Brady and Bernstein, 2004). HSV-1 often causes oral and ocular infections, whereas HSV-2 is responsible for genital infections. These viruses penetrate to the nervous system where they establish latency in sensory neurons, and their reactivation is responsible for recurrent infections, a period during which the virus may be transmitted to the next host (Cunninghan *et al*., 2006). Reduction in recurrent disease has been obtained using antivirals, especially nucleoside analogs such as acyclovir. However, the emergence of viral strains resistant to available drugs highlights the need for alternative methods/compounds for the management of HSV infections (Field, 2001; Wang and Smith, 2014).

Skin and oral mucosa are the main routes for administering antiherpetic drugs in terms of local treatment (Hasler-Nguyen *et al*., 2009; Chou and Hong, 2014). Concerning the composition of oral mucosa, buccal and esophagus mucosa are covered by a squamous stratified and non-keratinized epithelium with lipid composition; thus, the permeability barrier in these tissues consists of groups of lipid lamellae located in the intercellular spaces of the superficial epithelial layer (Squier and Kremer, 2001). Studies using porcine oral mucosa have been considered a suitable model for *in vitro* permeability studies due its similar structure and composition to that of humans (Caon and Simões, 2011; Padula *et al*., 2013; Santos *et al*., 2014; Ramirez-Rigo *et al*., 2014).

Recently, the skin permeation/retention of genistein from cationic nanoemulsions optimized by means of a 2^3 full factorial design was evaluated. Formulations composed of an oil core of isopropyl myristate, stabilized by a combination of the cationic lipid oleylamine and a phospholipid (dioleylphosphatidylcholine or distearoylphosphatidylcholine), were selected for further studies mainly due to enhanced retention of genistein in the porcine ear skin from such formulations. Increased antiherpetic activity of genistein against HSV-1 and HSV-2 viruses was also shown when incorporated into such formulations (Argenta *et al*., 2014).

Following up on these results, the aim of this study was to evaluate the genistein distribution on excised porcine esophageal mucosa from cationic nanoemulsions, as well as the anti-HSV-1 activity against a viral strain resistant to acyclovir. The effect of the addition of the thickening agent hydroxyethyl cellulose on the properties and genistein distribution in mucosa from formulations was also investigated.

Experimental

Materials

Isopropyl myristate, genistein, oleylamine and Nile red were purchased from Sigma-Aldrich (St Louis, MO, USA). Dioleylphosphocholine (DOPC) and distearoylphosphocholine (DSPC) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Polysorbate 80 was supplied by Vetec Quimica Fina Ltda. (Duque de Caxias, Brazil) and hydroxyethyl cellulose (HEC) was adquire by Delaware (Porto Alegre, Brazil). Methanol and trifluoroacetic acid were purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® Plus apparatus (Merck Millipore, Billerica, MA, USA). Pig esophagus was obtained from Ouro do Sul – Cooperativa dos Suinocultores do Caí Superior Ltda. (Harmonia, Brazil).

High-performance liquid chromatography analysis

The quantification of genistein in formulations and esophagus mucosa was assessed by high-performance liquid chromatography (HPLC) using the chromatographic conditions previously validated by our research group (Vargas *et al*, 2012a; Nemitz *et al*., 2015). In brief, HPLC apparatus consisted of a Shimadzu LC-10A system (Kyoto, Japan) equipped with a model LC-20AT pump, a SPD-20AV ultraviolet-visible variable wavelength detector (set at 270 nm), a DGU-20A5 degasser, a CBM-20A system controller, and a SIL-20A injection valve with a 100 µL loop. Genistein was analyzed using a Shim-pack CLC-ODS (M) RP-18 column (5 mm, 250×4 mm inner diameter). The mobile phase consisted of a mixture of methanol/water, with 0.1% trifluoracetic acid (70:30, v/v) in isocratic flow at a flow rate of 1.2 mL/min.

Preparation of genistein-loaded nanoemulsions and hydrogels

Preparation of the O/W nanoemulsions was carried out by spontaneous emulsification procedure following the method previously described (Argenta *et al*, 2014). Oily phase of nanoemulsions was composed by genistein, isopropyl myristate, phospholipid DSPC or DOPC and oleylamine dissolved in ethanol. This organic phase was poured into the water phase containing polysorbate 80 under constant magnetic stirring, and after 15 minutes the formulations were concentrated by evaporation under reduced pressure at 40-45ºC until concentration of 1mg/mL of genistein. The proportion of water and ethanol used was 2:1, respectively. The final pH of formulations was adjusted with HCl 1N to values of 5.5 to 6.0.

Hydrogels containing genistein-loaded nanoemulsions (HNE-DSPC and HNE-DOPC) were prepared by adding of HEC in the nanoemulsions. The mixture was stirred at room temperature with a magnetic stirrer to form a semisolid consistent formulation. Final composition of the formulations is presented in Table 1.

Table 1. Final composition (%, w/w) of formulations.

	NE-DSPC	HNE-DSPC	NE-DOPC	HNE-DOPC
Genistein	0.10	0.10	0.10	0.10
IM	8.00	8.00	8.00	8.00
Oleylamine	0.05	0.05	0.05	0.05
DSPC	2.00	2.00	-	-
DOPC	-	-	2.00	2.00
Polysorbate 80	1.00	1.00	1.00	1.00
Water q.s.p	100	100	100	100
HEC	-	3.00	-	3.00

Isopropyl myristate (IM); Distearoylphosphocholine (DSPC); Dioleylphosphocholine (DOPC); Hydroxyethyl cellulose (HEC)

Characterization of formulations

Aliquots of the nanoemulsions and hydrogels were diluted in water (1:1000) to determine the mean droplet size and the polydispersity index (PI) by photon correlation spectroscopy (PCS) at 25°C and angle of the laser beam of 90^0. The determination of the ζ- potential was performed by eletrophoretic mobility, after formulations dilution in 1mM NaCl (1:1000). All analyses were carried out in triplicate using a Malvern Nano-ZS90® (Malvern Instruments, England). To determine the genistein content, aliquots of genistein-loaded nanoemulsion before and after incorporation into hydrogels were appropriately diluted in methanol, filtered and analyzed by liquid chromatography using a method previously reported (Vargas *et al.* 2012a).

Rheological behavior of hydrogels

Rheological characterization of nanoemulsion-based hydrogels was carried out using a Brookfiel LVLD-II+ rotational viscometer at room temperature with a shear rate from 0.4 to 4.0 rpm and decreasing it back to 0.4 rpm, respecting the limits of values of torque (above 10% and less than 100%). Spindle SC-29 was lowered perpendicularly into the gel placed in a Brookfiel dispositive to evaluate small quantities taking care that the spindle does not touch the bottom of the dispositive.

Samples containing 10 g of formulation were analyzed for one minute in each speed. The values of shear stress (Dm/cm^2) were plotted in function of shear rates to obtain the rheograms.

Release study

The genistein release from formulations was evaluated through the cellulose ester membrane filters (50 nm pore diameter, Millipore) in a Franz-type diffusion cell with a diffusion area of 1.77 cm^2. The receptor compartment was filled with 10 mL of phosphate-buffer pH 7.4 (PBS) containing 30% of ethanol to maintain the sink conditions. The solubility of genistein in the receptor fluid was previously determined as 214.8 µg/mL (Vargas *et al*., 2012b). The bathing solution was kept under a controlled temperature (32^{o}C ± 1°C) and stirred at 650 rpm. Approximately 500 µg of each formulation were placed in the donor compartment and samples of 1.0 ml of receptor fluid were withdrawn in different time intervals to estimate genistein content released from formulations. The same volume of fresh receptor fluid was added to receptor compartment to maintain a constant volume. At the end of the experiment, the amount of genistein that across the membrane was analyzed by HPLC as described above. Results are expressed as mean ± standard deviation of four independent experiments.

Esophageal mucosa permeation/retention study

Porcine esophagi, obtained from a local slaughterhouse, were opened longitudinally and the loose connective membrane was cut with a scalpel to separate the mucosa from the muscular layer followed by washing of the separated mucosa with PBS. Mucosa membrane was then mounted and tightly attached between donor and acceptor compartment of Franz type diffusion cells. Experimental conditions were the same as described for release study. Samples of receptor fluid were withdrawn in time intervals of 1, 2, 4, 5, 6, 7 and 8 hours and analyzed to estimate the amount of genistein permeated. After 8 hours, the mucosa was removed from cells, cleaned using a cotton swab embedded with Milli-Q water and cut in small pieces. Genistein was then extracted with methanol using ultrassom bathing (30

min.). The genistein content was assessed by liquid chromatography as previously described, in the receptor fluid as well as in the mucosa tissue.

Histological and confocal fluorescence microscopy studies

Histological analyses of the mucosa were made after treatment with formulations, using mucosa untreated as control. After permeation kinetic (8 hours), the mucosa were cut, cleaned with a cotton swab and immersed in formaldehyde (37%) solution for its preservation. Subsequently, the cuts were dehydrated, embedded in paraffin and sectioned with a thickness of 6 µm. The color was made by hematoxylin-eosin. The tissue specimen were photographed and analysed by optical microscopy. Photographs were taken with an optical increase of 20-fold. For confocal fluorescence experiments a confocal laser scanning microscope (Leica TCS SP5, DMI6000B Inverted Microscope) was used to collect images from porcine mucosa treated with the formulations. In this experiment, the fluorescent dye Nile Red was added during the preparation of nanoemulsions NE-DSPC and NE-DOPC at the concentration of 0.05%. Approximately 500 µL of each fluorescent formulation (nanoemulsions and hydrogels) was placed in the donor compartment, and the permeation/retention study was performed under the same experimental conditions described for mucosa permeation/retention assay. After 8 hours, the mucosae were clean, mounted with Tissue-tec O.C.T. ® (Sakura Finetechnical, Tokyo, Japan) onto a metal sample holder and freezing at -20°C. Cryostat (Leica CM 1850, Nussloch, Germany) was used to cut vertical slices of 40 µm thickness, which were subjected to fluorescent microscopy using an Olympus FluoView™ 1000 (Olympus Corporation, Tokyo, Japan) with a helium-neon red laser exciting at 559 nm. The images were taken at a ten-fold magnification. The fluorescence intensity was evaluated by Image J software.

In vitro evaluation of anti-HSV-1 (29R strain)

Cells and virus

Cell line used was Vero (ATCC: CCL81) (Department of Clinical Virology, Göteborg University, Sweden) grown in Eagle's minimum essential medium (MEM;

Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA). Cell culture was maintained at 37°C in a humidified 5% CO_2 atmosphere chamber. The virus strain used was HSV-1 29R (acyclovir–resistant) (Faculty ofPharmacy, University of Rennes I, Rennes, France). Viral stock was stored at – 80 ^{0}C and titrated based on plaque forming units (PFU) counted by plaque assay as previously described (Burleson, Chambers and Wiedbrauk, 1992).

Antiherpes evaluation

The cytotoxicity of genistein (dissolved in 1.0% dimethylsulfoxide) and the genistein-loaded nanoemulsions was determined by sulforhodamine B assay (Vichai and Kirtikara, 2006). Cells were seeded into 96-well microplates, and allowed to adhere for 24 hours, at 37°C in a 5% CO_2 atmosphere chamber. Afterwards, different concentrations (30 µg/mL to 0.23 µg/mL) of the samples were dispensed into the wells and incubated for 48 hours. Cells were stained with sulforhodamine B, and absorbance was measured at 510 nm using a microplate reader. The concentration of each sample that reduced cell viability by 50% (CC_{50}) was calculated. Antiherpes activity of formulations was evaluated by the plaque number reduction assay (Silva *et al.*, 2010). Confluent monolayers of Vero cells were infected with 100 plaque-forming units per well of HSV-1 (29 strain) for 1 hour at 37°C. After, the cells were washed with phosphate-buffered and overlaid with agarose gel (0.6%) in the presence or absence of different concentrations of genistein or genistein-loaded nanoemulsions, and the plates were incubated for 48 hours. Cells were then fixed and stained with naphthol blue–black, and viral plaques were counted. The IC_{50} of each sample was defined as the concentration that inhibited 50% of viral plaque number when compared with untreated controls. The IC_{50} and CC_{50} values were estimated by linear regression of the concentration–response curves generated from the obtained data. All experiments were performed in triplicate.

Statistical analysis

All tests were repeated and expressed as the mean ± SD. The statistical analysis was according to ANOVA/Tukey (P<0.05).

Results

Characterization of formulations

Table 2 shows the physicochemical properties of formulations. Nanoemulsions containing DOPC exhibited a smaller droplet size (153 nm) than DSPC-based ones (225 nm), $p<0.05$. All formulations presented a polydispersity index lower than 0.25. Droplet size and polydispersity index remained similar after the addition of HEC. Regardless of the phospholipid used, ζ-potential was positive (≈+30 mV). A significant ($p<0.05$) reduction of ζ-potential (+23 mV for HNE-DSPC and +26 mV for HNE-DOPC) was detected for formulations after the addition of HEC. Genistein content was higher than 95 % (for an initial content of 1 mg/ml).

Table 2. Physicochemical properties of formulations and genistein content.

	NE-DSPC	HNE-DSPC	NE-DOPC	HNE-DOPC
Droplet size (nm ± SD)	225.06 ± 2.80	240.27 ± 11.99	153.27 ± 08.67	160.40 ± 22.00
Polydispersity index (± SD)	0.08 ± 0.02	0.105 ± 0.020	0.25 ± 0.01	0.24 ± 0.018
ζ- potential (mV± SD)	+32.12 ± 4.57	+23.42 ± 2.0	+31.08 ± 0.16	+26.25 ± 2.0
Genistein content (mg/mL ± SD)	0.96 ± 0.04	1.0 ± 0.03	0.95 ± 0.042	0.95 ± 0.014

Notes: PI, polidispersity index; data shown are the mean ± SD of three replicates

Rheological characterization

Figure 1 depicts the rheograms of genistein-loaded cationic nanoemulsions after thickening with HEC. Formulations exhibit flow curves typical of non-Newtonian fluids, given that viscosity decreases with increasing shear rate. The viscosity of nanoemulsion-loaded hydrogels decreased in comparison with blank gel. Under the conditions used in this study, HEC hydrogel exhibited thixotropic behavior.

Different rheological flow models (Binghan, Ostwald, Casson and Herchel-Bulkley) were used to predict flow behavior based on the shear stress-shear rate data. The best-fit model selected on the basis of coefficient of determination values was found to be the Ostwald model ($T = K\dot{\gamma}^n$, where K represents the consistency and n the flow index) for all hydrogels (Table 3).The flow index values (n), obtained by Ostwald equation, were lower than 1 (0.49, 0.48 and 0.36 for HEC gel, HNE-DSPC and HNE-DOPC, respectively). Since n=1 corresponds to Newtonian flow, and n>1 or n< 1 indicates, respectively, shear thickening or shear thinning, HNE-DSPC and HNE-DOPC exhibit non-Newtonian flow with pseudoplastic behavior. These results indicate that the rheological behavior of blank HEC gel is similar to nanoemulsions-loaded hydrogels.

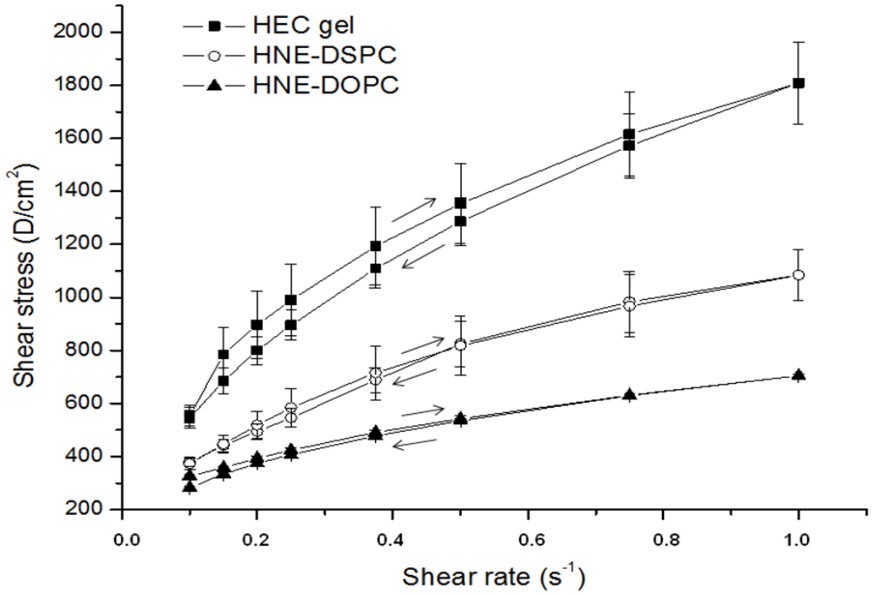

Figure 1. Rheological profiles of control gel (HEC gel) and nanoemulsion-loaded hydrogels (HNE-DSPC and HNE-DOPC). Data shown are the mean ± SD of three replicates.

Table 3. Coefficients of determination (r^2) for flow models in shear stress-shear rate curve of hydrogels.

	Coefficient of determination (r^2)		
	HEC gel	HNE-DSPC	HNE-DOPC
Binghan	0.9468	0.9663	0.9791
Ostwald	0.9838	0.9938	0.9994
Casson	0.9672	0.9879	0.9964
Herchel-Bulkley	0.9815	0.9608	0.9525

Genistein release study

Genistein release over time was investigated through cellulose esters membranes using Franz-type cells (Figure 2). Genistein was fully released from a propylene glycol solution after 3 hours (103.45 ± 5.22%). A progressive release of genistein from nanoemulsions was observed, achieving 97.82 ± 1.66% for NE-DOPC and 61.47 ± 2.87% for NE-DSPC after 10 hours. The amount of genistein released was higher than that from hydrogels (49.44 ± 3.54% for HNE-DOPC and 40.86 ± 5.25% for HNE-DSPC).

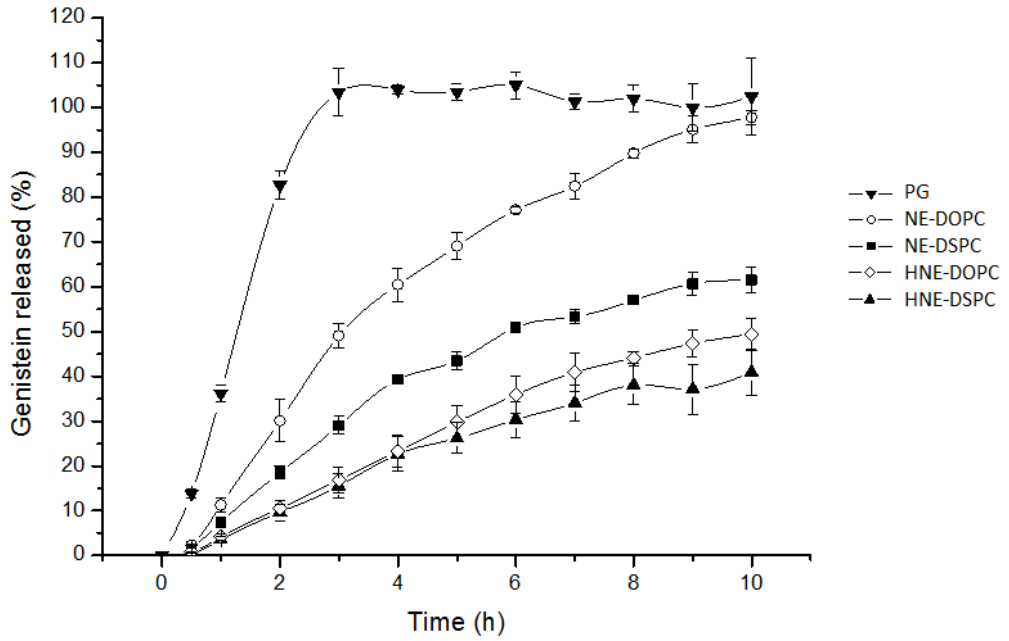

Figure 2. Release profile of genistein from propylene glycol (PG) nanoemulsions (NE-DSPC and NE-DOPC) and hydrogels (HNE-DSPC and HNE-DOPC) through synthetic cellulose membranes. Data shown are the mean ± SD of four replicates.

Mucosa permeation/retention assay

Genistein permeation/retention studies were evaluated by using Franz-type diffusion cells to compare the effect of the composition of nanoemulsions and the addition of HEC on genistein distribution through porcine esophageal mucosa. Figure 3 shows the transmucosal delivery profile of genistein from formulations. A reduction in the permeation rate was observed for formulations containing HEC. As can be seen in Table 4, genistein permeation from nanoemulsions (NE-DSPC and NE-DOPC) was approximately 50 μg/cm^2 after 8 hours, whereas these values were reduced approximately 2-fold when HEC was added to the formulations. As can be seen in Table 4, the genistein flux across mucosa was faster from nanoemulsions (up to 7.46 ± 2.17 μg/cm²/h for NE-DSPC and 8.26 ± 0.63 μg/cm²/h for NE-DOPC) and hydrogels (4.55 ± 0.49 μg/cm²/h for HNE-DSPC and 4.40 ± 0.21 μg/cm²/h for HNE-DOPC) in comparison with a control solution (1.47± 0.01 μg/cm²/h). The lag time (the drug's first detection) was similar for all tested formulations, ranging from 3.44 to 3.67 hours.

Figure 4 shows the retention of genistein in mucosa tissue. After 8 hours of kinetics, the amount of retained genistein was higher from nanoemulsions (8.04 ± 0.89 µg/cm² for NE-DSPC and 9.72 ± 1.29 µg/cm² for NE-DOPC) and HNE-DOPC hydrogel (6.85 ± 0.99 µg/cm²) than from the control solution (4.24 ±1.08 µg/cm²). Despite the higher amount of retained genistein from HNE-DSPC (5.36 ± 1.0 µg/cm²), the result was not statistically different (*P*>0.05) from control solution.

Table 4. Genistein permeation parameters through esophageal mucosa.

Formulations	Lag time (h)	Flux (µg/cm^2 /h)	Q_8 (µg/cm^2)
PG	3.50	1.47 ± 0.01	7.93 ± 0.15
NE-DSPC	3.44	7.46 ± 2.17	49.16 ± 4.59
NE-DOPC	3.60	8.26 ± 0.63	53.79 ± 1.82
HNE-DSPC	3.66	4.55 ± 0.49	20.99 ± 2.30
HNE-DOPC	3.67	4.40 ± 0.21	23.23 ± 4.14

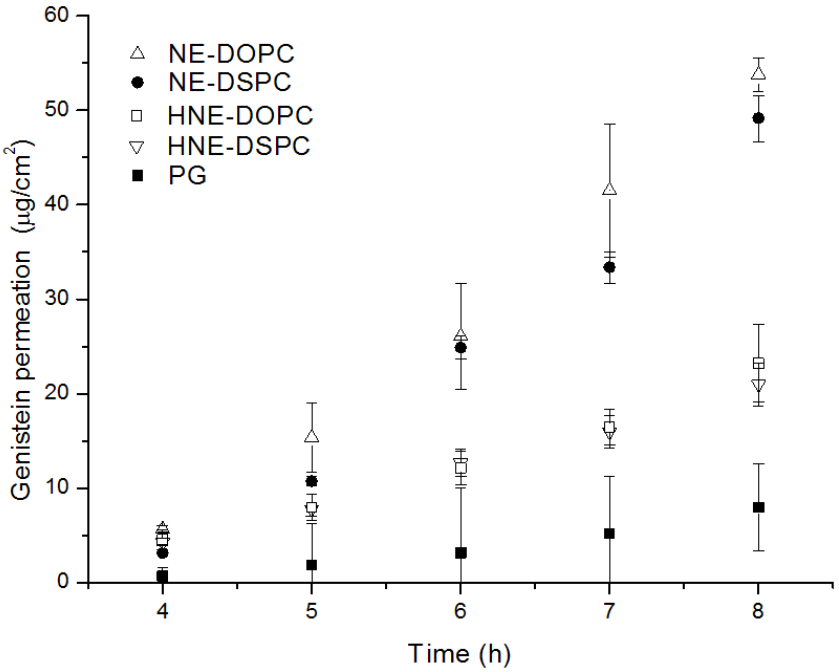

Figure 3. Genistein permeation profile from propylene glycol (PG), nanoemulsions (NE-DSPC and NE-DOPC) and hydrogels (HNE-DSPC and HNE-DOPC) through porcine esophageal mucosa after 8h of kinetic study. The values are means ± SD of 5 experiments.

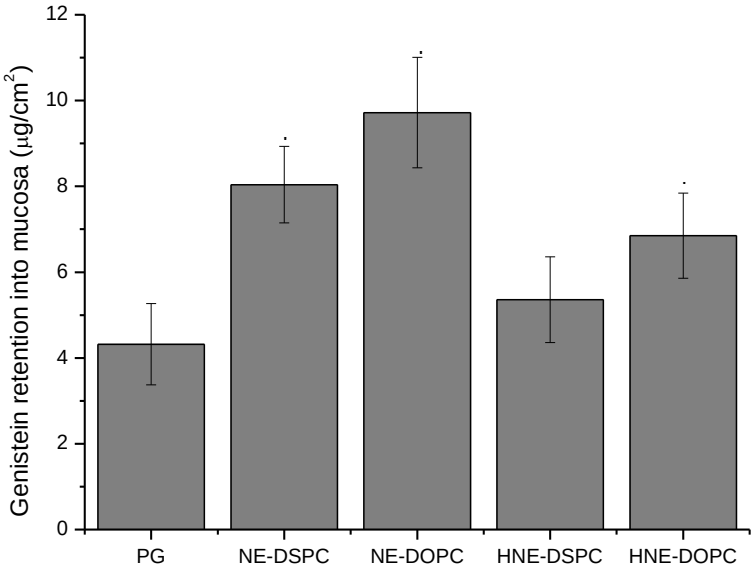

Figure 4. Genistein retention (μg/cm^2) from propilene glycol (PG), nanoemulsions (NE-DSPC and NE-DOPC) and hydrogels (HNE-DSPC and HNE-DOPC) into porcine esophageal mucosa after 8 h of permeation assay. The values are means ± SD of 5 experiments, *Significantly higher (P<0.05) as compared with PG.

Histological and confocal analysis

Figure 5A-5E shows the histological structure of pig esophageal mucosa by using hematoxicilin-eosin staining after treatment with genistein-loaded nanoemulsions before and after adding hydroxyethyl cellulose. The images revealed the presence of the three layers of the epithelia: the basal layer, stained more darkly; the middle layer, with larger and compacted cells; and the superficial stratum, with less compacted cells and surrounded by mucus. Confocal images (Figure 5F-5J) show the distribution of fluorescent dye (Nile Red) on esophageal mucosa after permeation/retention studies from genistein-loaded nanoemulsion before and after thickening. When compared with genistein in propylene glycol, a higher amount of fluorescence is visibly observed after treatment with nanoemulsions and hydrogels. However, the measure of fluorescence intensity showed statistical difference only for nanoemulsion NE-DOPC (Figure 6).

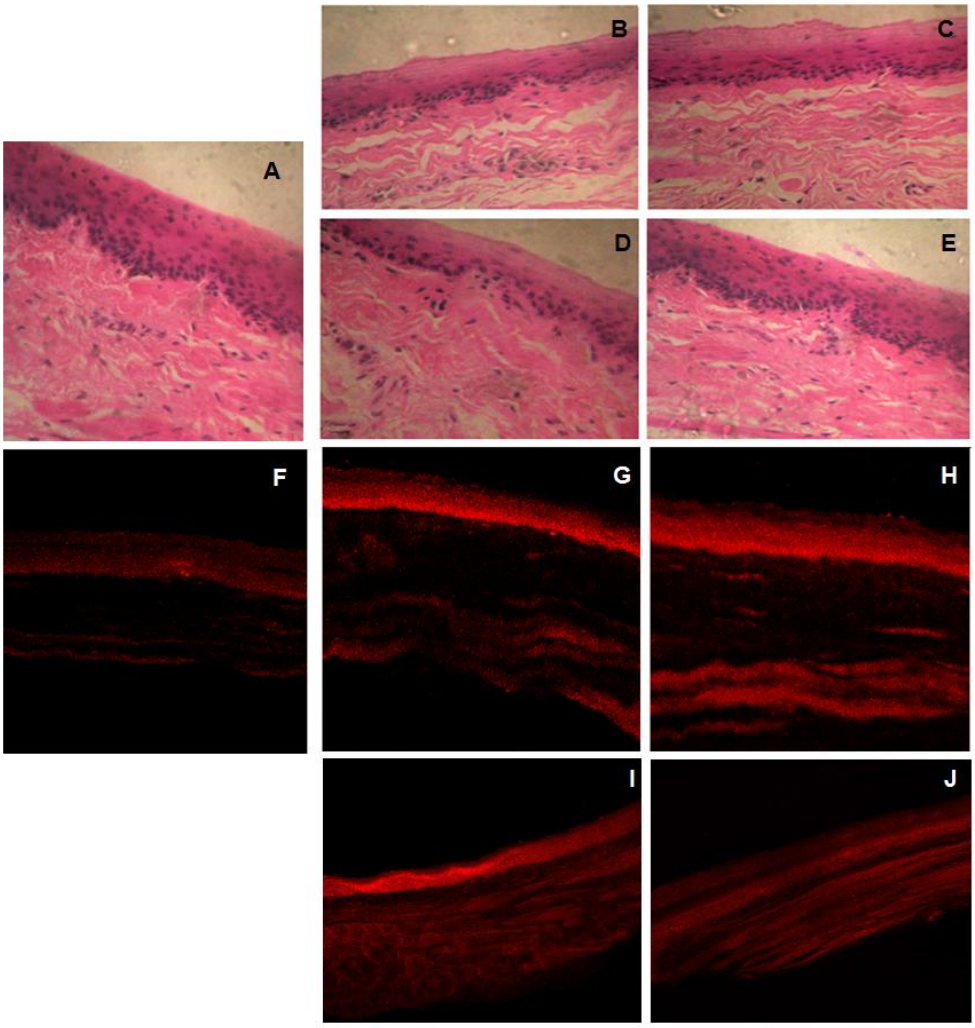

Figure 5. Hematoxycilin/eosin histological (A-E) and fluorescent (F-J) images of porcine esophageal mucosa treated with genistein propylene glycol solution -PG (A,F), NE-DSPC (B,G) and NE-DOPC (C, H) or hydrogels HNE-DSPC (D,I) and HNE-DOPC (E,J). Images were obtained after 8 hours of permeation/retention studies using a Franz diffusion cell. Images were obtained 10-fold of magnification. Nile red was used as fluorescent dye in confocal images.

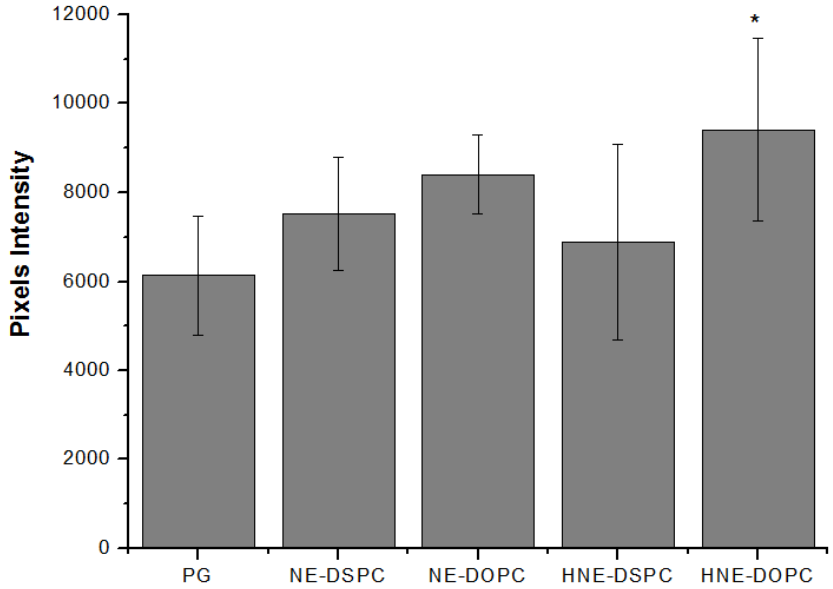

Figure 6. Fluorescence intensity of Nile red in porcine esophageal mucosa after treatment with genistein propylene glycol solution-PG, genistein–loaded nanoemulsions (NE-DSPC and NE-DOPC) and genistein–loaded nanoemulsions hydrogels (HNE-DSPC and HNE-DOPC). *Significantly higher (P<0.05) as compared with PG.

Antiherpetic activity

Table 5 shows the antiherpetic activity (anti-HSV-1 29R) of genistein solution and genistein-loaded NE-DSPC and NE-DOPC nanoemulsions. Genistein-loaded nanoemulsions were more cytotoxic for vero cells (CC_{50} of approximately 5.3 µg/mL for NE-DSPC and 5.4 µg/mL for NE-DOPC) than for free genistein solution (CC_{50} = 14.70 µg/mL). An approximately two-fold reduction of IC_{50} values was observed when genistein was incorporated into nanoemulsions for HSV-1 29R virus. Blank nanoemulsions (without genistein) showed no activity.

Table 5. Cytotoxicity (CC_{50}) on Vero cells and inhibitory effects of HSV-1 29R replication (IC_{50}) of free-genistein and genistein-loaded nanoemulsions (NE-DSPC and NE-DOPC).

	CC_{50} (µg/mL)*	CI_{50} (µg/mL)[#]
Genistein	14.70 ± 1.44	2.13 ± 0.21
NE-DSPC	5.33 ± 1.45	1.07 ± 0.22
NE-DOPC	5.41 ± 0.45	1.20 ± 0.28

Values represent the mean ± standard deviations of three independents experiments. *Cytotoxicity was determined by sulforhodamine B assay on Vero cells. [#]Antiviral activity was determined by plaque number reduction assay. **Abbreviations**: CC_{50}, concentration at which cell viability was reduced by 50%; IC_{50}, concentration that inhibited 50% of viral plaque number.

Discussion

In this report, we designed genistein-loaded cationic nanoemulsions containing a thickening agent in an attempt to enable mucosal delivery of this isoflavone intended for the local treatment of herpes infections. Genistein was efficiently incorporated into the oil core of nanoemulsions due to the poor water solubility of this isoflavone. Considering that the final genistein content (1 mg/ml) is higher than its solubility into the oil, a part of genistein can be adsorbed at the oil/water interface (Argenta *et al*., 2014) To adjust the nanoemulsions viscosity, HEC was selected as suitable non-ionic thickening agent due its high water solubility, which leads to produce a homogenous dispersion with bioadhesive and film forming properties (Ivarsson and Wahlgren, 2012). The addition of HEC polymer at the final concentration of 3% in nanoemulsions did not markedly change the formulations' mean droplet size and polydispersity index. A slight increase was detected, albeit without statistical significance ($p>0.05$). Formulations exhibit a positive ζ-potential due to the presence of the cationic lipid oleylamine located at the oil-water interface. A significant reduction of ζ-potential was detected after thickening with HEC, even if it is a nonionic polymer. These findings can be due the effect of the adsorption of the polymer at the oil/water interface of nanoemulsions (Hezaveh and Muhamad, 2012). Regardless of the formulation, genistein content was close to 100% (for a content of

1mg/mL). Overall, the results suggest that the structure of the cationic nanoemulsions was maintained after thickening with HEC polymer. These results follow in line with earlier literature regarding the incorporation of colloidal carriers in hydrogels (Joshi and Patravale; 2008; Mou *et al*., 2008; Junyaprasert *et al*., 2009).

The rheological characterization of hydrogels showed a non-Newtonian behavior, since the shear stress was not directly proportional to the shear rate and pseudoplastic flow, i.e., characterized by a decrease in viscosity with an increase in shear rate. There was a reduction of viscosity in the formulations as compared with HEC hydrogel, especially in hydrogels containing the DOPC phospholipid, which can be related to possible interactions of nanoemulsions with the polymer network, which is in agreement with previous studies concerning the dispersion of colloidal carriers into hydrogels (Ratsimbazafy *et al.,*1999; Lim and Lawrence, 2004). A thixotropic behavior was observed for HEC hydrogel, once the ascending and descending flow curves do not overlap. On the opposite, the results observed for HEC hydrogels containing both DSPC- and DOPC-based nanoemulsions are almost overlapped, suggesting an effect of the oil droplets on the structural organization of the hydrogel polymer network (Sharma *et al*., 2014). However, the incorporation of genistein-loaded nanoemulsions into hydrogel did not change the pseudoplastic rheological behavior of the HEC gel, since the formulations showed higher r^2 for the same mathematical model (Ostwald model) with flow index values lower than 1, indicating characteristics of non-Newtonian flow with pseudoplasitc behavior (Alves, Pohlmann and Guterres, 2005).

A lower release of genistein was observed for nanoemulsions either before or after the addition of HEC in comparison with a propylene glycol solution through cellulose membranes. Concerning the nanoemulsions, genistein release was clearly influenced by the type of phospholipid used. Genistein release from NE-DOPC formulation was higher than from NE-DSPC formulation. This difference could be explained by differences in the transition phase temperature of phospholipids located at the oil-in-water interface: T_m=55°C, for DSPC and T= -21°C for DOPC (Garcia *et al*., 2006). DOPC seems to favor genistein release from formulations, probably due to

the lower resistance of the fluid-state hydrocarbon chains to genistein diffuse from the oily core and/or interface of nanoemulsions (Garcia *et al*., 2006; Babu *et al*., 2009). Indeed, since the transition temperature of DOPC is negative, the conformation and motion of its hydrocarbon chains move more freely in the temperature of experiments (32 ^{o}C), which may lead a more fluid oil-water interface. In addition, NE-DOPC exhibits a smaller droplet size as compared with NE-DSPC, suggesting a higher specific surface may also contribute in faster genistein release.

A delay in genistein release was observed from both nanoemulsions when the HEC thickening agent was used in the formulations. Flux values were significantly lower for HNE-DSPC and HNE-DOPC. Thus, hydrogels can retain the nanoemulsions in the polymer network, increasing the resistance to genistein release (Hezaveh and Muhamad, 2012). Therefore, in the experimental conditions used in this study, genistein release seemed to be more influenced by the thickening of the nanoemulsions in the aqueous phase (due to the addition of HEC) than by the nature of the phospholipid used in the nanoemulsion formulation.

To get a better understanding of genistein's ability to accumulate in the tissues where herpes viruses establish infections, genistein permeation/retention in excised porcine esophageal mucosa from formulations was estimated by using Franz diffusion cells. Esophageal and buccal epithelium have a qualitatively and quantitatively similar lipid composition. Moreover, the esophageal has a more uniform thickness, as compared to buccal epithelium (Squier and Kremer, 2001). These facts encouraged us to use esophageal mucosa as a model for studies of mucosal permeation/retention.

The permeation process begins with release of genistein from formulations, followed by its permeation/retention in mucosa tissue. As showed in the Figure 2, there was a delay of genistein release from hydrogels, when compared to nanoemulsions. Similar profile behavior was observed in the genistein permeation through porcine esophagus mucosa.

Regardless of the formulation, genistein was detected and quantified in receptor fluid from 4 to 8 hours of kinetics. A higher amount of genistein was detected in the acceptor phase from nanoemulsions as compared with derivative hydrogels, without statistical differences ($p>0.05$) between them. Lower levels of genistein were detected when this isoflavone was dispersed in a control propylene glycol solution, demonstrating the effect of the formulations on genistein permeation.

Genistein permeation from PG presented a different profile compared with release studies. The release of genistein was faster from propylene glycol solution than from formulations, probably due the lower viscosity and resistance to diffusion process of isoflavone through solution. However, the genistein permeation from PG was slowest. Thus, despite the known enhancer property of propylene glycol (Touitou, Godin and Weiss, 2000), under the contidions used in this study, PG was less effective in to enhance the permeation/retention of genistein than nanoemulsions and hydrogels. The higher effects of formulations can be explained by possible interactions of positively-charged cosurfactant oleylamine with negative charge on the mucosal surface, leading to a disturbance in the tissue lipid structure, and promoting the genistein permeation/retention (Argenta *et al*., 2014). It is also important to mention that, in our previous studies, genistein was not capable of permeating through the skin from nanoemulsions (Argenta *et al*., 2014), illustrating the effect of the histological/anatomical characteristics of the administration site on genistein transport.

Finally, genistein retention in mucosa from formulations was evaluated after 8 hours of kinetics. A higher amount of genistein was detected in mucosa from nanoemulsions as compared with the formulations containing HEC ($p<0.05$). A plausible explanation for this reduction could be related to lesser contact between the mucosa and the oil droplets of the nanoemulsion, most likely caused by the droplets being trapped inside the HEC polymer network. In addition, the results also showed low levels of genistein in the mucosa when this isoflavone was dispersed in propylene glycol. The fluorescence observed in the mucosa after treatment with nanoemulsions and hydrogels was visibly more intense than after genistein in propylene glycol solution treatment, although statically analysis shows higher

fluorescence intensity only for hydrogel HNE-DOPC. A histological evaluation of the mucosa was performed at the end of the permeation/retention studies in an attempt to evaluate potentially deleterious effects of the different formulations on the mucosa, notably related to the presence of the cationic lipid oleylamine. Nanoemulsion components could interact with mucosa components, changing their structure and increasing the drugs' mucosa penetration (Sintov *et al*., 1999; Bhaskar *et al*., 2009). However, regardless the formulation, no evidence of tissue damage was detected in this study using the hematoxylin-eosin histological evaluation.

The increased viral resistance to available drugs has motivated the search for compounds with different mechanisms of action, along with technological strategies to promote higher permeation/retention of such compounds (Hasler-Nguyen *et al*., 2009). We have recently demonstrated the potential antiherpes activity of genistein-loaded nanoemulsions against HSV-1 (KOS strain) and HSV-2 (333 strain) (Argenta *et al*., 2014). Here, we investigated the activity against a virus resistant to acyclovir [(HSV-1) 29R strain)], a drug used in herpes treatment. Indeed, the incorporation of genistein into the cationic nanoemulsions decreased the IC_{50} of the viral plaque formation, against HSV-1 29R up to 1.77–fold (NE-DSPC) and 2.0-fold (NE-DOPC) in all cases lower than that observed for free genistein. In addition, both nanoemulsions exhibited a selectivity index (i.e., the ratio between CC_{50} and IC_{50}) higher than 4 (4.98 and 4.50 for NE-DSPC and NE-DOPC, respectively), which can be considered promising for candidates as antiviral agents (Amoros *et al*., 1992). These results indicate that genistein-loaded nanoemulsions NE-DSPC and NE-DOPC have potential application in the treatment of HSV-1 resistant strain to analog nucleoside drugs, such as acyclovir.

Conclusions

In this study, topical cationic genistein-loaded nanoemulsions were successfully obtained into a more convenient topical dosage form containing a thickening polymer. Our results showed an enhanced genistein permeation/retention through porcine esophageal mucosa from formulations. Therefore, due to the potential benefits reported in the literature for genistein, coupled with our studies

showing antiherpetic activity, the application of genistein-loaded nanoemulsions hydrogel developed here seems to be a suitable alternative for topical and/or transmucosal treatment of herpes simplex.

Acknowledgements

This work was supported by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) - Rede Nanobiotec-Brazil (grant number 902/2009) and the State Foundation for Research Support (FAPERGS) – PRONEM (grant number 11/2206-7). DA wishes to thank CAPES for her scholarship.

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CAPÍTULO IV

Nanoemulsões catiônicas contendo cumestrol: estudos de permeação/retenção em mucosa íntegra e lesada e atividade anti-herpética

O quarto capítulo apresenta os resultados das propriedades físico-químicas de nanoemulsões catiônicas contendo cumestrol, antes e após, espessadas com o polímero de hidroxietilcelulose (HEC). Nessa última parte do trabalho, foi avaliada a distribuição do cumestrol em mucosa íntegra e lesada de esôfago suíno, bem como a atividade anti-herpética in vitro do cumestrol incorporado nas nanoemulsões. As formulações contendo o fosfolipídeo DSPC (NE-COU/DSPS e HNE-COU/DSPC) apresentaram maior tamanho de gotícula e menor índice de polidispersão, comparada com as nanoemulsões contendo DOPC (NE-COU/DOPC e HNE-COU/DOPC). Todas as formulações apresentaram potencial zeta positivo devido à presença do lipídio catiônico oleilamina (> 18 mV). Com exceção dos valores de potencial zeta, as características das nanoemulsões não foram alteradas de forma significativa após a adição do agente espessante ($p<0,05$). Com relação aos estudos de permeação em mucosa íntegra, o fluxo de permeação do cumestrol foi maior a partir das formulações contendo DOPC (nanoemulsão e hidrogel). Esse mesmo comportamento foi observado em mucosa lesada, porém o fluxo de permeação, bem como a quantidade total de cumestrol permeado foi superior para todas as formulações, quando comparado com os resultados obtidos com a mucosa íntegra. A retenção do cumestrol também foi superior a partir das formulações contendo DOPC, porém não foi observada diferença nas quantidades retidas entre a mucosa íntegra e lesada. Por fim, a atividade anti-herpética do cumestrol foi intensificada após a incorporação da molécula na nanoemulsão contendo DOPC frente aos vírus HSV-1(cepa KOS) e HSV-2 (cepa 333). A presença do DSPC na nanoemulsão catiônica não intensificou a atividade antiviral do cumestrol. Os resultados obtidos nesse capítulo sugerem que a incorporação do cumestrol nas formulações desenvolvidas, especialmente as contendo DOPC, são promissoras no tratamento de infecções herpéticas estabelecidas em mucosas.

Antiherpes and porcine mucosa distribution of coumestrol from positively-charged nanoemulsions

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Abstract

This study described the coumestrol distribution from nanoemulsions on porcine esophagus mucosa in two different conditions (full and injured tissue), as well as the *in vitro* anti-herpes effects. Cationic nanoemulsions were obtained by spontaneous emulsification procedure. This procedure yielded monodisperse nanoemulsions exhibiting a mean droplet size of approximately 200–300 nm, as attested by transmission electron microscopy. Hydroxyethyl cellulose was added as a thickening agent. Dioleylphosphaditylcholine (DOPC)-based formulations promoted higher coumestrol permeation/retention in excised porcine mucosa as compared with formulations containing distearoylphosphatidylcholine (DSPC) in both mucosa conditions. These results were supported by confocal fluorescence images. In addition, the results showed an increase of coumestrol permeation through injured mucosa from all formulations as compared with full mucosa. However, coumestrol retention results were similar in both mucosa conditions. Furthermore, the incorporation of coumestrol into cationic nanoemulsions containing DOPC increased the antiherpes activity against HSV-1 and HSV-2 viruses. Overall, such results showed that the coumestrol-loaded nanoemulsions developed in this study could be considered as a promising formulation for herpes simplex treatment.

Keywords: coumestrol, nanoemulsion, release study, hydroxylethyl cellulose, mucosa permeation, herpes

Introduction

Coumestrol is an isoflavonoid naturally occurring in soybean, alfalfa, and red clover. The activity of coumestrol against herpes simplex virus (HSV) was recently demonstrated (Argenta *et al*., 2015b). Coumestrol inhibited HSV-1 (KOS and 29R strains, which are acyclovir-sensitive and acyclovir–resistant strains, respectively) and HSV-2 (333 strain) replication. In addition, coumestrol has affected the early stages of viral infection, reducing HSV-1 protein expression and HSV-2 cell-to-cell spread.

HSV replicate rapidly in epithelial tissues and therefore are primarily associated with mucocutaneous infections characterized by pain, redness and swelling followed by vesicopustules which rupture and crust over. HSV-1 often causes oral and ocular infections, which may affect the all areas of the mouth, lips and peri-orally, whereas HSV-2 is responsible for genital infections, although, genital herpes can be a consequence of infection by HSV-1, and conversely, HSV-2 can infect oral epithelial (Brady and Bernstein, 2004; Goodyear, 2015). The potential transmission to next hosts in the episodes of reactivation, and the emergence of viral strains resistant to available drugs has motivated the search for new drugs to prevent new infections and/or to reduce the infectivity at mucosa membranes (Hodge and Field, 2013).

Mucosae are considered as potential sites for local and systemic drug administration. For local application of drugs, the oral and the vaginal cavities represent the target of various pathologies of bacterial, fungal, and viral origin, which are usually treated with topical pharmaceutical dosage (Sandri *et al.*, 2004).

Thereby, due the physiological characteristics of mucosa tissues, topical formulations intended to mucosal application may present bioadhesive properties to allow spread and surface contact of formulations when applied on tissue. Since mucosa are protected by mucus layers composed by negatively charged glycoproteins, positively charge colloidal carriers could increase the interaction of formulations in the mucosa membranes, improving the drug`s efficacy (Issa *et al.*,

2005; Neves and Bahia, 2006). These nanoparticles could also improve the permeation across mucosa due to the small size and by the presence of permeation promoters.

In this study, the anti-HSV-1 and anti-HSV-2 activity of coumestrol-loaded cationic nanoemulsions as well as its distribution in excised porcine esophageal mucosa. Since the local treatment of oral herpes includes applying medication on the affected tissues before and/or the blisters appear, coumestrol distribution from formulations thickened with hydroxyethyl cellulose was also evaluated in the impaired epithelial barrier to gain a better insight on the effect of the integrity of these tissues on flavonoid retention.

Experiments

Materials

Coumestrol, isopropyl myristate, oleylamine and Nile red were purchased from Sigma-Aldrich (St Louis, MO, USA). Dioleylphosphocholine (DOPC) and distearoylphosphocholine (DSPC) from Lipoid GmbH (Ludwigshafen, Germany). Polysorbate 80 and hydroxyethyl cellulose (HEC) were supplied by Delaware (Porto Alegre, Brazil). Methanol and trifluoroacetic acid were purchased from Merck KGaA (Darmstadt, Germany). Pig esophagus was obtained from Ouro do Sul – Cooperativa dos Suinocultores do Caí Superior Ltda. (Harmonia, Brazil).

High-performance liquid chromatography conditions

The quantification of coumestrol in formulations and esophagus mucosa was assessed by high-performance liquid chromatography (HPLC) using the chromatographic conditions previously validated (Argenta *et al.*, 2011). The LC system consisted of a Shimadzu LC-20A system (Kyoto, Japan) equipped with a model LC-20AT pump, a SPD-20 AV UV-VIS variable–wavelength detector, SIL-20A auto injector and a degasser module. The data were acquired and processed by Shimadzu LCSolution GPC software (Shimadzu, Kyoto, Japan). The column used was a C18 Phenomenex Gemini (150 x 4.6 mm, i.d., 5µm particle size). The

optimized mobile phase consisted of a mixture of methanol/water with 0.1% trifluoracetic acid (70:30, v/v) in isocratic flow. The LC system was operated at room temperature, sample injection volume of 20µL, flow rate of 0.8mL. min^{-1} and detection at 343 nm.

Nanoemulsions and hydrogels preparation

Nanoemulsions were prepared by spontaneous emulsification procedure following the method previously described (Argenta *et al*., 2014). The oily phase of nanoemulsions was composed by coumestrol, isopropyl myristate, phospholipid DSPC or DOPC and oleylamine dissolved in ethanol. This organic phase was poured into the water phase containing polysorbate 80 under constant magnetic. After, the formulations were concentrated by evaporation under reduced pressure at 40-45ºC and the final pH of formulations was adjusted to values of 5.5 to 6.0.

Hydrogels were prepared by addition of hydroxyethyl cellulose (HEC) to coumestrol-loaded nanoemulsions and the mixture was stirred at room temperature with a magnetic stirrer until to form a semisolid consistent formulation (HNE-DSPC and HNE-DOPC). Final composition of the formulations is presented in Table 1.

Table 1. Final composition (%, w/w) of formulations

	NE-COU/DSPC	HNE-COU/DSPC	NE-COU/DOPC	HNE-COU/DOPC
Coumestrol	0.10	0.10	0.10	0.10
IM	8.00	8.00	8.00	8.00
Oleylamine	0.05	0.05	0.05	0.05
DSPC	2.00	2.00	-	-
DOPC	-	-	2.00	2.00
Polysorbate 80	1.00	1.00	1.00	1.00
Water q.s.p	100	100	100	100
HEC	-	3.00	-	3.00

Isopropyl myristate (IM); Distearoylphosphocholine (DSPC); Dioleylphosphocholine (DOPC); Hydroxyethyl cellulose (HEC)

Characterization of formulations

Nanoemulsions and hydrogels were diluted in water to determine the mean droplet size and the polydispersity index (PI) by photon correlation spectroscopy (PCS) at 25°C. ζ-potential was determined by eletrophoretic mobility, after formulations dilution in 1mM NaCl. All analyses were carried out in triplicate using a Malvern Nano-ZS90® (Malvern Instruments, England). To determine the coumestrol content, aliquots of nanoemulsions and hydrogels were appropriately diluted in methanol, filtered and analyzed by liquid chromatography using a method previously reported.

Morphological examination was performed by means of transmission electron microscopy (TEM). For these analyses, formulations were previously diluted at a 1:10 ratio to obtain an oil phase concentration equal to 1%. Specimens for TEM viewing were prepared by mixing samples with one droplet of 2% (w/v) uranyl acetate solution. Subsequently, the samples were adsorbed to the 200 mesh formvar-coated copper grids, left to dry, and examined by TEM (JEM-1200 ExII, Jeol, Japan).

Esophageal mucosa permeation/retention study

Previously to permeation/retention study, porcine esophagi were opened longitudinally and the loose connective membrane was cut with a scalpel to separate the mucosa from the muscular layer. Mucosa membrane was washed with phosphate-buffer saline pH 7.4 (PBS) and mounted between donor and acceptor compartment of Franz type diffusion cells with a diffusion area of 1.77 cm^2. Experiments with injured party tissue were also performed. For this, the mucosal epithelium was scraped with a scalpel before being placed in the Franz type diffusion cells. To maintain the sink condition, receptor fluid was composed by phosphate-buffer saline pH 7.4 (PBS) containing 40% of ethanol. The bathing solution was kept under a controlled temperature (32 °C ± 1 °C) under stirring. Approximately 400 µg of each formulation were placed in the donor compartment and samples of receptor fluid were withdrawn every hour up to 8 hours and analyzed to estimate the amount of coumestrol permeated though full and injured mucosa. After 8 hours, coumestrol

extraction from tissues followed the procedure described by our research group (Argenta *et al*., 2015a). Coumestrol content was assessed by liquid chromatography as described above, in the receptor fluid as well as in the mucosal tissues.

Histological analysis

Histological analyses of the mucosa were made after treatment with formulations, using full or injured mucosa untreated as control. After 8h of permeation, mucosa were cleaned with a cotton swab and immersed in formaldehyde (37%) solution. Subsequently, the samples were dehydrated, embedded in paraffin and sectioned with a thickness of 6 µm. The color was made by hematoxylin-eosin. Finally, the tissue specimen were photographed and analysed by optical microscopy. Photographs were taken with an optical increase of 20-fold.

Confocal fluorescence microscopy

Fluorescent dye Nile Red was used for confocal experiments and a confocal laser scanning microscope (Leica TCS SP5, DMI6000B Inverted Microscope) was used to collect images from full and injured mucosa treated with the formulations. The dye was added during the preparation of nanoemulsions at the concentration of 0.05%. Permeation/retention study was performed under the same experimental conditions described above. After 8 hours, the mucosa were clean, mounted with Tissue-tec O.C.T. ® (Sakura Fine technical, Tokyo, Japan) onto a metal sample holder and freezing at -20°C. Cryostat (Leica CM 1850) was used to cut vertical slices of 40 µm thickness, which were subjected to fluorescent microscopy using an Olympus FluoView™ 1000 (Olympus Corporation, Tokyo, Japan) with a helium-neon red laser exciting at 559 nm. The images were taken at a twenty-fold magnification and the fluorescence intensity was evaluated by Image J software.

In vitro antiherpes activity of coumestrol-loaded nanoemulsions

Cells and viruses

The cell lines used were Vero (ATCC:CCL81) and GMK AH1 (Department of Clinical Virology, Göteborg University, Sweden). They were grown in Eagle’s

minimum essential medium (MEM; Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (FBS; Cultilab) and maintained at 37^{o}C in a humidified 5% CO_2 atmosphere chamber. The viruses tested were HSV-1 (KOS and 29R strains, Faculty of Pharmacy, University of Rennes I, Rennes, France), and HSV-2 [333 strain (Department of Clinical Virology, Göteborg University, Sweden)] propagated in Vero and GMK AH1 cells, respectively.

Antiherpes evaluation

Preliminary, the cytotoxicity of coumestrol (dissolved in 1.0 % dimethylsulfoxide) and nanoemulsions was determined by sulforhodamine B (SRB) assay (Vichai and Kirtikara, 2006). Briefly, Vero and GMK AH1 cells were tripsinized, seeded into 96-well microplates and allowed to adhere for 24 h at 37°C in a 5% CO_2 atmosphere chamber. Afterwards, different concentrations of the samples were dispensed into the wells and incubated for 48 h. Then, cells were stained with SRB and the absorbances were measured. Concentration of each sample that reduced cell viability by 50% (CC_{50}) was calculated comparing to the untreated controls by non-linear regression analysis using GraphPad Prism® software, version 5.01.

After cytotoxicity determination, the potential antiherpes activity of coumestrol-loaded nanoemulsions was evaluated by the plaque number reduction assay as previously described by Argenta and co-workers (2014). Confluent monolayers of Vero or GMK AH1 cells were infected with HSV-1 (KOS and 29R strains) or HSV-2 (333 strain), respectively, for 1 h at 37^{o}C. After, cells were washed with PBS and overlaid with agarose gel (0.6%) in the presence or absence of different concentrations of coumestrol or coumestrol-loaded nanoemulsions, and the plates were incubated for 48 h. Cells were then fixed and stained with naphthol blue black and viral plaques were counted. The IC_{50} of each sample was defined as the concentration that inhibited 50% of viral plaque number when compared to untreated controls and estimated by non-linear regression analysis using GraphPad Prism® software, version 5.01. The selectivity index (SI = CC_{50}/IC_{50}) of each tested sample also was determined. All experiments were performed in triplicate.

Statistical analysis

The mean and standard deviations were calculated for all experiments. The statistical analysis was according to ANOVA/Tukey (P<0.05).

Results

Characterization of nanoemulsions and hydrogels

Table 2 shows the physicochemical characterization of formulations. Formulations containing DSPC exhibited a droplet size of approximately 270 nm whereas formulations containing DOPC showed droplet size of approximately 200 nm. Formulations containing DSPC presented lower PI values in comparison with those obtained with DOPC. Regardless of the phospholipid used, ζ-potential was positive (approximately 30mV). Coumestrol content was higher than 90 % (for an initial content of 1mg/ml).

Table 2. Physicochemical properties of formulations and coumestrol content.

	NE-COU/DSPC	HNE-COU/DSPC	NE-COU/DOPC	HNE-COU/DOPC
Droplet size (nm ± SD)	269.73 ± 18.03	277.33 ± 22.76	200.05 ± 3.46	193.40 ± 13.15
PI ± SD	0.08 ± 0.01	0.144 ± 0.01	0.25 ± 0.02	0.28 ± 0.01
ζ- potential (mV± SD)	+20.47 ± 2.68	+18.60 ± 1.58	+27.23 ± 3.52	+20.65 ± 2.75
COU content (mg/mL ± SD)	0.90 ± 0.02	1.00 ± 0.06	0.90 ± 0.07	1.04 ± 0.08

Morphological analysis was carried out in order to characterize the surface morphology of the nanoemulsions before and after dispersion with HEC gel. TEM images allowed the observation of the droplet structure in the expected nanometric range for all formulations, showing the efficiency of the preparation method used (Figure 1).

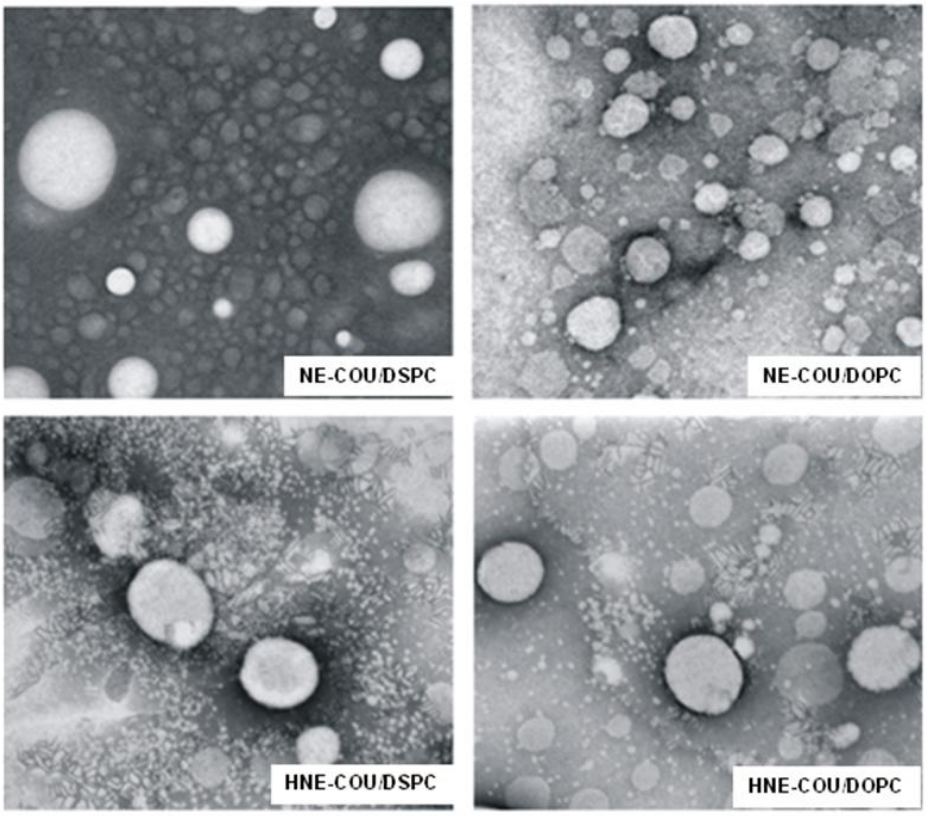

Figure 1. TEM images of nanoemulsions (NE-COU/DSPC and NE-COU/DOPC) and hydrogels - nanoemulsions thickened with hydroxyehyl cellulose (HNE-COU/DSPC and HNE-COU/DOPC). No differences were detected in the morphology of oil droplets of formulations.

Mucosa permeation/retention assay

Permeation/retention studies were performed using porcine esophagus mucosa to compare the effect of the composition of nanoemulsions and the addition of HEC on coumestrol distribution through full and injured mucosa. Transmucosal delivery profiles of coumestrol from formulations are presented in Figure 2A-2B and the coumestrol permeation parameters are presented in Table 3.

After 8 h of kinetic, coumestrol permeation through full mucosa was higher from nanoemulsion NE-COU/DOPC and its derived hydrogel HNE-COU/DOPC than from formulations containing the phospholipid DSPC (NE-COU/DSPC and HNE-COU/DSPC). Similar permeation profile was observed using injured mucosa. As can be seen in Table 3, flux of coumestrol across full mucosa was faster from

nanoemulsion NE-COU/DOPC (4.30 ± 0.42 µg/cm^2/h) than others formulations (approximately 1.90, 2.20 and 3.10 µg/cm^2/h for NE-COU/DSPC, HNE-COU/DSPC and HNE-COU/DOPC, respectively). Concerning the permeation across injured mucosa, the flux of coumestrol was significantly faster from NE-COU/DOPC (7.12 ± 1.51 µg/cm^2/h) and HNE-COU/DOPC (7.16 ± 0.36 µg/cm^2/h) as compare to NE-COU/DSPC (3.28 ± 0.41 µg/cm^2/h) and HNE-COU/DSPC (3.30 ± 0.39 µg/cm^2/h). Moreover, amount of cumulative coumestrol was significantly higher using injured mucosa permeation. Regression analysis showed r^2 values higher when the amount of permeated coumestrol was plotted against the time (zero order model kinetic), r^2> 0.97 for coumestrol permeation through full mucosa and r^2> 0.95 for coumestrol permeation through injured mucosa.

Table 3. Permeation parameters of the nanoemulsions and hydrogels through full and injured mucosa.

	J (μg/cm^2/h)		Q_8 (μg)		r^2	
	Full mucosa	Injured mucosa	Full mucosa	Injured mucosa	Full mucosa	Injured mucosa
NE-COU/DPSC	1.90 ± 0.17	3.28 ± 0.41	15.24 ± 1.59	24.92 ± 2.99	0.997	0.978
NE-COU/DOPC	4.30 ± 0.42*	7.12 ± 1.51**	31.74 ± 5.39	49.51 ± 15.70	0.994	0.970
HNE-COU/DSPC	2.17 ± 0.31	3.30 ± 0.39	17.86 ± 5.03	24.91 ± 2.93	0.972	0.958
HNE-COU/DOPC	3.06 ± 0.45	7.16 ± 0.36**	25.47 ± 6.09	47.22 ± 3.33	0.992	0.973

J:flux; Q_8: amount of coumestrol released in 8 h; r^2: determination coefficient from coumestrol release plotted against the time (zero order model). Notes: Data shown are the mean ± SD of five replicates.*significantly higher for coumestrol permeation through full mucosa; **significantly higher for coumestrol permeation through injured mucosa.

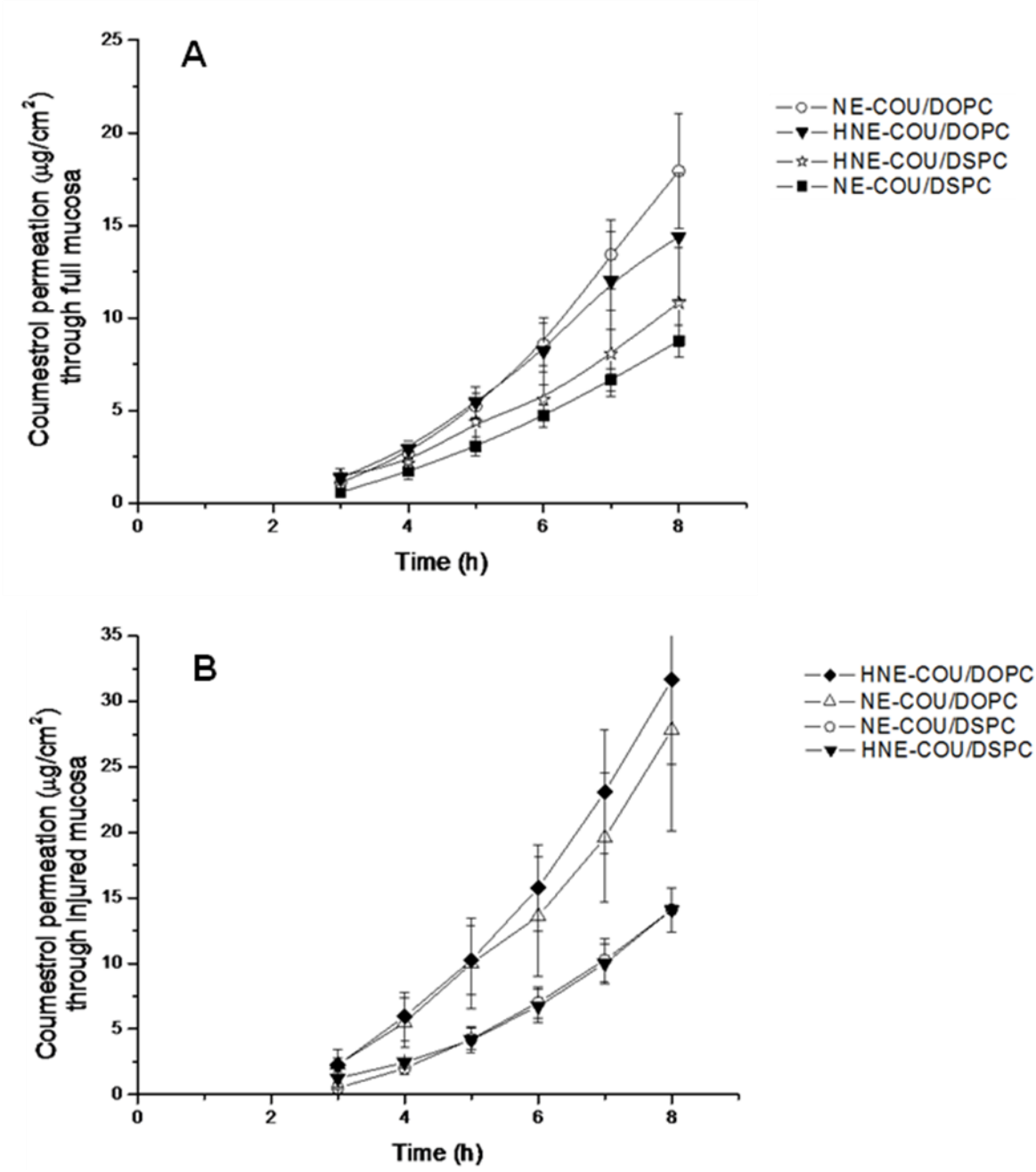

Figure 2. Coumestrol permeation profile from nanoemulsions (NE-COU/DSPC and NE-COU/DOPC) and hydrogels (HNE-COU/DSPC and HNE-COU/DOPC) through porcine esophageal full (A) and injured (B) mucosa after 8h of kinetic study. The values are means ± SD of 5 experiments.

Figure 3 shows the retention of coumestrol in mucosa tissues. After 8 hours of permeation, the amount of coumestrol retained was higher from nanoemulsion and hydrogel containing DOPC (5.12 ± 0.63 µg/cm² for NE-COU/DOPC and 4.71 ± 0.6 µg/cm² for HNE-COU/DOPC) than from those containing DSPC (2.39 ± 0.16 µg/cm² for NE-COU/DSPC and 2.69 ± 0.24 µg/cm² for HNE-COU/DSP). Coumestrol retention into injured mucosa was also higher from formulations containing DOPC (4.72 ± 0.51 µg/cm² for NE-COU/DOPC and 4.99 ± 0.40 µg/cm² for HNE-COU/DOPC) compared to those containing DSPC (2.43 ± 0.60 µg/cm² for NE-COU/DSPC and 2.39 ± 0.65 µg/cm² for HNE-COU/DSPC). In addition, the profiles of coumestrol retention from formulations were similar in both mucosa conditions.

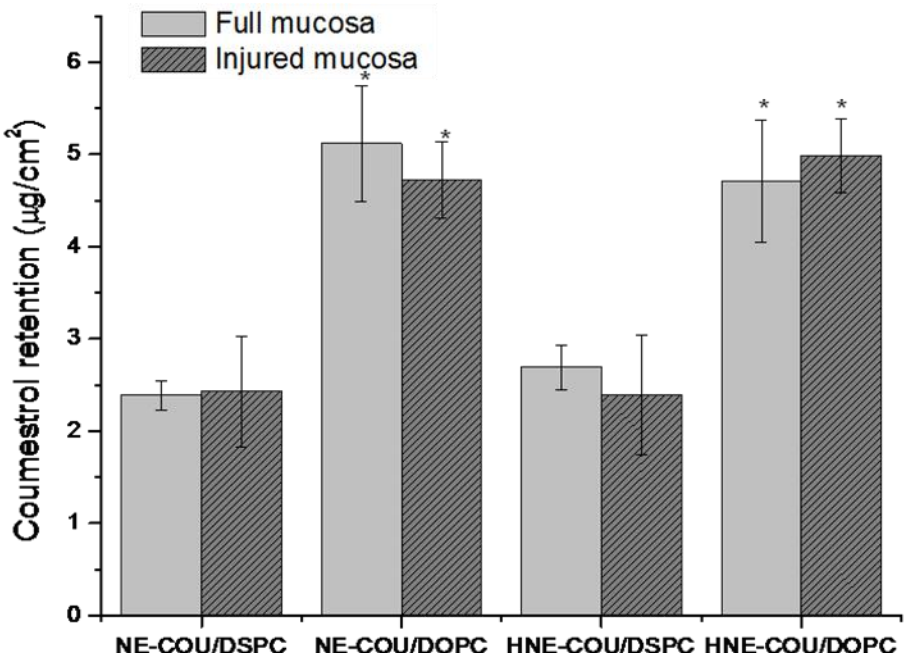

Figure 3. Coumestrol retention (µg/cm^2) from nanoemulsions (NE-COU/DSPC and NE-COU/DOPC) and hydrogels (HNE-COU/DSPC and HNE-COU/DOPC) into porcine esophageal full and injured mucosa after 8 h of permeation assay. The values are means ± SD of 5 experiments, *Significantly higher (P<0.05) as compared with NE-COU/DSPC and HNE-COU/DSPC.

Histological analysis

Figure 4 shows the histological structure of pig esophageal full and injured mucosa after treatment with coumestrol-loaded nanoemulsions before and after adding HEC. Three layers of the mucosal epithelia can be observed in the images of full mucosa (Figure 4A-4E): basal layer, stained more darkly; the middle layer, with larger and compacted cells; and the superficial stratum, with less compacted cells and surrounded by mucus. Concerning injured mucosa, the tissue damage, caused by gentle scraping, induced removal of the superficial epithelium layer of the mucosa (Figure 4F-4J). The contact of the formulations with full mucosa caused a visibly thickening of the superficial layer, especially in the presence of phospholipd DOPC (Figure 4B-4E). This effect was less remarkable in injured mucosa (Figure 4G-4J).

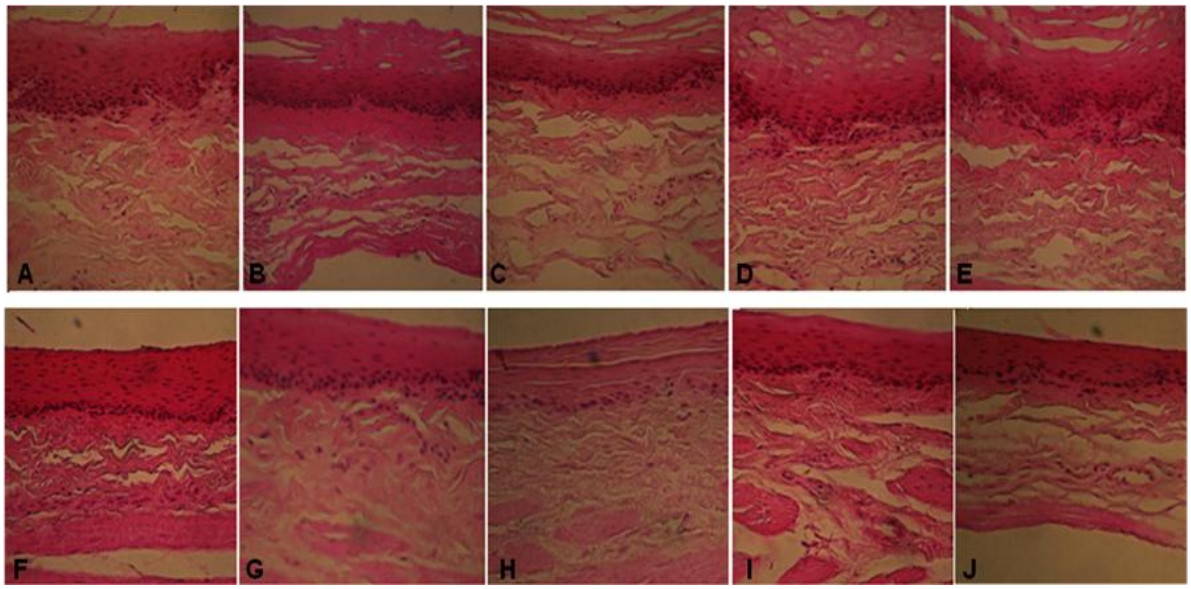

Figure 4. Histological images obtained after 8 hours of permeation in full mucosa (A-E) or injured mucosa (F-J) after treatment with nanoemulsions [NE-COU/DSPC (A,E) and NE-COU/DOPC (C,G)], and hydrogels [HNE-COU/DSPC (B,F) and HNE-COU/DOPC (D,H)]. Untreated full mucosa (A) and untreated injured mucosa (B) were used as controls. Images were obtained 40-fold of magnification.

Confocal images analysis

Figure 5 shows the distribution of fluorescent dye (Nile red) on full and injured mucosa after permeation from coumestrol-loaded nanoemulsion before and after thickening. Regardless mucosa condition used the fluorescence of Nile red is visibly distributed to tissues after treatment with all formulations. Figure 6 shows the measure of fluorescence intensity of Nile Red. As can be seen, the fluorescence intensity was higher after treatment with NE-COU/DOPC and HNE-COU/DOPC ($p<0.05$) when compared with NE-COU/DSPC and HNE-COU/DSPC in both mucosa conditions (full and injured mucosa). In addition, fluorescence intensity presented similar measures after thickening of the nanoemulsions ($p>0.05$).

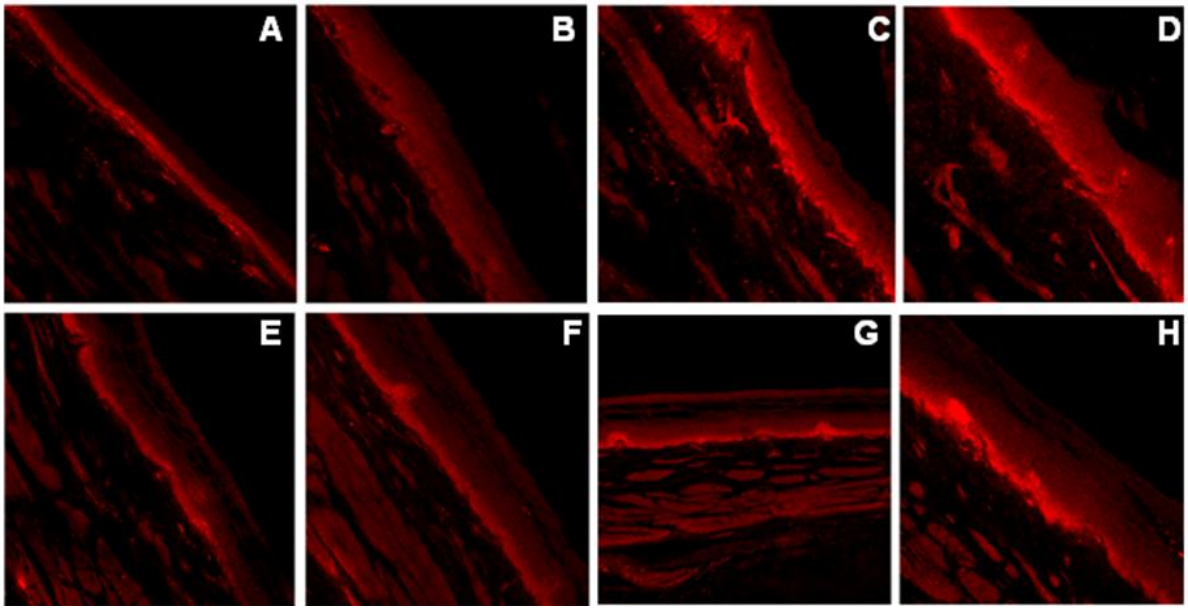

Figure 5. Confocal images obtained after 8 hours of permeation in full mucosa (A-D) or injured mucosa (E-H) using Nile red as fluorescent dye. The confocal images were obtained after treatment with nanoemulsions [NE-COU/DSPC (A,E) and NE-COU/DOPC (C,G)], and hydrogels [HNE-COU/DSPC (B,F) and HNE-COU/DOPC (D,H)]. Images were obtained 20-fold of magnification.

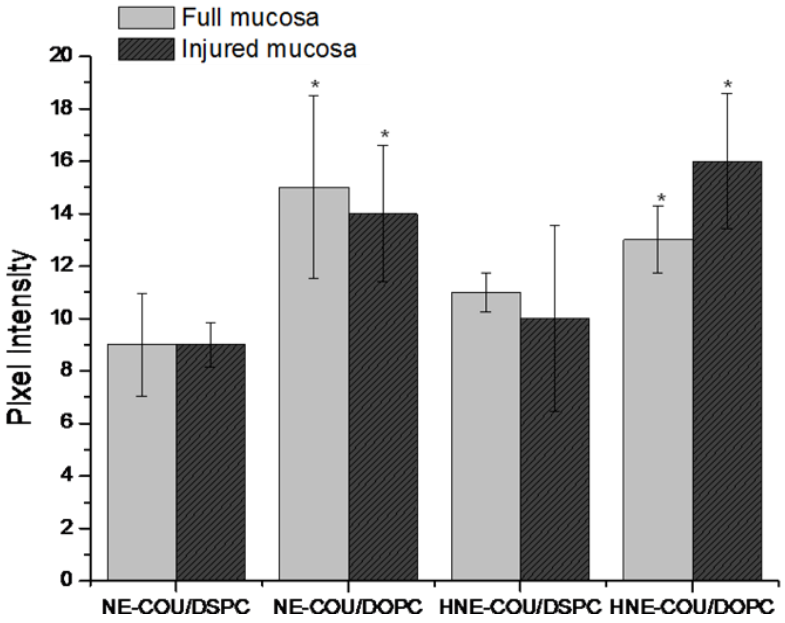

Figure 6. Fluorescence intensity of Nile red in porcine esophageal mucosa after 8h of treatment with coumestrol–loaded nanoemulsions (NE-COU/DSPS and NE-COU/DOPC) and coumestrol–loaded nanoemulsions hydrogels (HNE-COU/DSPC and HNE-COU/DOPC). *Significantly higher (P<0.05) as compared with NE-COU/DSPC and HNE-COU/DSPC.

Antiherpes activity

Table 4 shows the antiherpes effect of coumestrol solution and coumestrol-loaded nanoemulsions (NE-COU/DSPC and NE-COU/DOPC) against HSV-1 (KOS and 29R strain) and HSV-2 (333 strain) viruses. An increase in cytotoxicity was observed when coumestrol was incorporated into nanoemulsions, especially when GMK AH1 cells were used, demonstrated by CC_{50} and selectivity index values. As can be seen in Table 4 coumestrol-loaded nanoemulsion NE-COU/DOPC presented IC_{50} values lower than free coumestrol against HSV-1 (KOS strain) and HSV-2. Contrary, coumestrol-loaded nanoemulsion NE-COU/DSPC showed higher IC_{50} values than free coumestrol for both HSV-1 strains and similar IC_{50} value for HSV-2. Blank nanoemulsions (without coumestrol) showed no activity.

Table 4. Cytotoxicity (CC_{50}) on Vero and GMK AH1 cells and inhibitory effects of HSV-1 and HSV-2 replication (IC_{50}), respectively, of free coumestrol and coumestrol-loaded nanoemulsions (NE-COU/DSCP and NE-COU/DOPC).

Samples	CC_{50} (µg/mL)[a]		HSV-1 (KOS strain)		HSV-1 (29R strain)		HSV-2 (333 strain)	
	Vero cells	GMK AH1 cells	IC_{50} (µg/mL)[b]	SI	IC_{50} (µg/mL)[b]	SI	IC_{50} (µg/mL)[b]	SI
Coumestrol	25.03 ± 5.31	>500	3.33 ± 1.11	7.52	0.98 ± 0.09	25.54	9.50 ± 1.00	>52.63
NE-COU/DSPC	07.91 ± 1.98	10.97 ± 1.04	5.50 ± 1.10	1.44	5.09 ± 1.12	1.55	9.59 ± 1.69	1.14
NE-COU/DOPC	07.86 ± 1.69	12.25 ± 0.30	2.04 ± 0.29	3.85	1.45 ± 0.08	5.42	5.83 ± 1.36	2.10

Notes: Values represent the means ± standard deviations of three independent experiments. [a]Cytotoxicity was determined by sulforhodamine B assay on Vero and GMK AH1cells. [b]Antiviral activity was determined by viral plaque number reduction assay. **Abbreviations**: CC_{50}, concentration at which cell viability was reduced by 50%; HSV, Herpes Simplex Virus; IC_{50}, concentration that reduced by 50% the viral plaque number when compared to viral controls; SI= CC_{50}/ IC_{50}.

Discussion

Antiherpes effects of soy isoflavonoids were recently investigated by our research group (Argenta *et al*., 2015b). Here, permeation/retention studies of coumestrol-loaded nanoemulsions containing HEC were performed using full and injured mucosa tissues. Nanoemulsion NE-COU/DSPC and its derivative hydrogel HNE-COU/DSPC exhibited higher droplet size and lower PI as compared to formulations containing DOPC. These results are in agreement with physicochemical characteristics previously described for genistein-loaded nanoemulsions and hydrogels (Argenta *et al*, 2015a). Regardless the formulation, ζ-potential presented positive values, indicating that this parameter was influenced by the cationic lipid oleylamine. DOPC and DSPC phospholipids did not exhibit charge, due the coexistence of the negatively-charged phosphate group and the positively-charged quaternary ammonium group of choline in the polar head group. Morphological analysis showed that under the conditions applied in this study, the oil droplet structure of nanoemulsions was maintained after addition of thickening agent HEC.

Considering that HSV infection is followed by inflammatory response and mucosa lesion (Hull *et al.*, 2014), in this study we evaluated the coumestrol permeation and retention through full mucosa as well as injured mucosa. Regardless mucosa condition, the flux of coumestrol through tissue was higher from formulations containing DOPC. The influence of type of phospholipid used was also observed in coumestrol retention, since formulations containing DOPC promoted higher coumestrol retention into full and injured mucosa. Indeed, the fluid-state hydrocarbon chain of DOPC could promote a higher interaction between the oil-water interface with mucosa components, increasing the coumestrol permeation/retention. In accordance with these results, the fluorescence intensity of Nile red dye was higher after mucosa treatment with formulations containing DOPC. This dye is intensely fluorescent and it is used as a sensitive vital stain for the detection of cytoplasmic lipid droplets (Greenspan, Mayer and Fowler, 1985). Due its lipophilic characteristic, in this study, Nile red was added in the oily phase of nanoemulsions. Coumestrol permeation through mucosa seems be predominantly influenced by the nature of phospholipid, since coumestrol flux and coumestrol permeated amount from nanoemulsions and its derived hydrogels were similar ($p>0.05$). Coumestrol

permeation was higher when tissue was damaged. The injury of mucosa could remove parts of the surface layer of mucosa, decreasing tissue barrier, and leading an increased of coumestrol permeation. However, despite the differences of permeation, coumestrol retention was similar in both mucosa conditions (full and injured mucosa). Indeed, confocal analysis supported the coumestrol permeation/ retention results, since measure of dye fluorescence intensity was higher from formulations containing DOPC, without difference between full and injured mucosa.

A histological analysis of the mucosa were performed to evaluate the effects of the coumestrol-loaded nanoemulsions before and after thickened on the full and injured mucosa. A thickening of superficial stratum of full mucosa was observed after 8 hours of contact with formulations. This effect could be the result of interactions of nanoemulsions components with mucosa, since the same mucosa thickening extension was observed with hydrogels. Probably, the positive charge of nanoemulsions interacts with negatively charged groups (such as carboxyl and sulphate) on the mucin and cell surface of mucosa epithelium, causing such effects (Neves and Bahia, 2006; Gabal *et al*., 2014).

HSV-1 and HSV-2 infections result in a lifelong latent infection after peripheral replication in mucosal tissues, and recurrent infections episodes are frequently associated with significant impact on physical, emotional, and social well-being of patients. Thus, the search for new antiviral molecules and/or therapeutic strategies is subject of several researches (Hull *et al.*, 2014; Uyangaa, Patil and EO, 2014). Coumestrol showed a potential anti-herpetic activity. Moreover, the incorporation of this isoflavonoid into nanoemulsions (NE-COU/DOPC) seems be an interesting pharmaceutical strategy to topical application, once the coumestrol activity against HSV-1 (KOS strain) and HSV-2 (333 strain) was higher. Such results suggest an enhanced intracellular absorption when coumestrol was incorporated into nanoemulsions.

Conclusions

Here, we demonstrated that the incorporation of coumestrol into positively-charged nanoemulsions improved the antiherpes activity of this isoflavonoid. A higher coumestrol retention was detected in the porcine esophageal mucosa, especially for DOPC-based formulation. An increase in the permeation was observed when these tissues were injured. Overall, such results showed that the coumestrol-loaded nanoemulsions developed in this study could be considered as a promising formulation for herpes simplex treatment.

Acknowledgments

The authors wish to thank the Brazilian Federal Agency (CAPES-Rede Nanobiotec) and the State Foundation for Research Support (FAPERGS) for their financial support. DA wishes to thank CAPES for her scholarship

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Entre os isoflavonóides mais estudados da soja destacam-se a genisteína, daidzeína e gliciteína, pertencentes à subclasse isoflavona e o cumestrol pertencente à subclasse cumestano. Estudos têm demonstrado que a isoflavona genisteína apresenta atividade anti-herpética contra os vírus HSV-1 e HSV-2 (Yura, Yoshida e Sato,1993; Lyu, Rhim e Park, 2005). Esses vírus causam uma dermatose infecto-contagiosa crônica e recorrente, sendo que o tratamento tópico da doença baseia-se em sucessivas aplicações de formulações contendo fármacos antivirais, como o aciclovir e penciclovir, que levam ao progressivo desaparecimento das lesões e ao alívio da dor (Hasler-Nguyen *et al*., 2009). No entanto, o surgimento de cepas resistentes e a inexistência de tratamento definitivo, têm motivado a busca por novas moléculas e/ou estratégias terapêuticas para o tratamento do herpes simples (Field, 2011). Neste contexto, a presente tese apresenta os isoflavonóides genisteína e cumestrol como potenciais agentes alternativos no tratamento tópico das infecções herpéticas.

Entretanto, a reduzida hidrossolubilidade dos isoflavonóides dificulta a sua incorporação em formas farmacêuticas. Neste sentido, o nosso grupo de pesquisa demonstrou a viabilidade de incorporação da genisteína e do cumestrol em nanoemulsões de uso tópico (Silva *et al*., 2009; Vargas *et al*., 2012), sendo que as mesmas encontram-se preferencialmente dispersas e/ou adsorvidas na interface das gotículas da fase interna oleosa, estabilizada por lecitina ou pela combinação lecitina/ polissorbato 80. Os estudos de retenção/permeação desses sistemas usando pele de orelha suína indicam um baixo fluxo dos isoflavonóides através da pele, apontando para uma potencial aplicação local. Dando continuidade aos estudos, o desenvolvimento de sistemas nanoemulsionados contendo genisteína ou cumestrol com ênfase no desenvolvimento de produtos para o tratamento do herpes simples foi o objetivo geral dessa tese.

A primeira etapa do trabalho consistiu na avaliação do potencial efeito anti-herpético de isoflavonóides da soja, bem como na investigação dos mecanismos envolvidos na ação antiviral dos compostos bioativos. Entre os compostos avaliados, a genisteína e o cumestrol mostraram atividade frente aos vírus HSV-1 e HSV-2, sendo que o cumestrol apresentou maiores valores de índice de seletividade (IS). O cálculo do IS, calculado a partir da razão entre citotoxicidade e atividade antiviral

(IS=CC_{50}/CI_{50}), é um requisito importante na previsão da aplicabilidade do composto como potencial agente terapêutico, sendo que valores de IS acima de 4 indicam potencial atividade antiviral (Amoros *et al*., 1992). Nesse estudo os valores de IS dos compostos foram maiores para o vírus HSV-1 da cepa 29R, a qual é resistente ao aciclovir (IS de 31, 52 e 7,01 para o cumestrol e genisteína, respectivamente). Esses resultados demonstram que a genisteína e o cumestrol são moléculas promissoras uma vez que, os estudos buscam por compostos alternativos que apresentem atividade anti-herpética, principalmente frente a cepas resistentes aos fármacos disponíveis atualmente para o tratamento do herpes.

A atividade anti-HSV-1 e anti-HSV-2 *in vitro* da genisteína foi previamente relatada na literatura (Yura, Yoshida e Sato, 1993; Lyu, Rhim e Park, 2005), porém o efeito dessa isoflavona frente a uma cepa do HSV-1 resistente ao aciclovir (fármaco de escolha no tratamento das infecções), foi demonstrado pela primeira vez nesta tese. Assim como, até o momento, não há relatos na literatura da atividade anti-herpética do cumestrol, sendo essa demonstrada pela primeira vez nesse estudo. O mecanismo de ação dos isoflavonóides foi investigado utilizando diferentes metodologias. Os resultados mostraram que os compostos não apresentam efeito virucida, nem efeito profilático com o pré-tratamento de 3 horas. Com relação aos efeitos dos compostos durante as etapas iniciais da infecção, somente o cumestrol parece interferir na adsorção, bem como na penetração dos vírus nas células. No entanto, as concentrações efetivas foram maiores que as encontradas no ensaio da redução das placas de lise (pós-infecção), o que indica que outros mecanismos também estão envolvidos na ação antiviral desse composto. De fato, a genisteína e o cumestrol foram capazes de inibir a expressão de proteínas do vírus HSV-1 (cepa KOS). A análise do ensaio do western blotting, apontou diferentes perfis de redução da expressão das proteínas para os compostos. A genisteína afetou a expressão de genes α, β e ɣ, o que significa que a isoflavona parece ter ação na expressão das proteínas imediatas (α), precoces (β), bem como tardias (ɣ). Já o cumestrol, não afetou a produção de proteína imediata. Ambas as moléculas reduziram as áreas das placas virais do HVS-2, sugerindo o efeito na redução da disseminação viral de uma célula infectada para outra não infectada e, conseqüente redução das lesões provocadas pelas infecções herpéticas.

Com relação a genisteína, os estudos encontrados na literatura mostram que essa isoflavona inibe a fosforilação de enzimas tirosina quinase (Akiyama *et al*., 1987; Markovits *et al*., 1989; Dixon e Ferreira, 2002). Nesse sentindo, Yura, Yoshida e Sato (1993) sugerem que essa atividade pode ser relacionada com a atividade antiviral da molécula, uma vez que a genisteína inibe a fosforilação de polipeptídeos do vírus HSV-1 produzidos na fase tardia (gC), o que mostra similaridades com esse estudo, já que foi demonstrado nessa tese que a genisteína reduz a expressão da proteína gD da fase tardia. Em outro estudo, Nicola e colaboradores (2005), mostraram que o tratamento das células epiteliais humanas com a genisteína acarretou em redução da entrada dos vírus HSV-1 no núcleo das células, porém não no citosol, o que também está de acordo com os resultados encontrados nesse trabalho, pois a genisteína não foi capaz de impedir os eventos iniciais da infecção viral, apresentando atividade nas etapas subsequentes.

Em conjunto, os resultados indicam que a genisteína e o cumestrol apresentam efeito anti-herpético *in vitro* frente ao HSV-1 e HSV-2, podendo apresentar diferentes mecanismos de ação, o que é de interesse na busca por agentes antivirais, uma vez que, a resistência viral faz com que diferentes estratégias de tratamento sejam necessárias no combate aos vírus. Ainda, ambos os isoflavonóides mostraram atividade contra a uma cepa resistente ao aciclovir, indicando que seus efeitos são diferentes da ação do fármaco, o qual já tem mostrado ser ineficaz em alguns tratamentos (Hodge e Field, 2013).

Visando o tratamento tópico das infecções herpéticas, após avaliação do potencial efeito anti-herpético dos isoflavóides da soja, a segunda etapa do trabalho consistiu no desenvolvimento e otimização de sistemas nanoemulsionados contendo o isoflavonóide majoritário da *Gycine max*, genisteína, através de um planejamento fatorial qualitativo completo do tipo 2^3, bem como a avaliação da atividade anti-herpética *in vitro* das formulações selecionadas frente aos vírus HSV-1 (cepa KOS) e HSV-2 (cepa 333). Para a análise do experimento fatorial foram avaliados os fatores: tipo de óleo (óleo de rícino e miristato de isopropila), tipo de co-tensoativo iônico (oleilamina e ácido oléico) e tipo de fosfolipídeo (diestearolilfosfatidilcolina – DSPC e dioleilfosfatidilcolina –DOPC), tendo como respostas de saída as propriedades físico-químicas das nanoemulsões (diâmetro médio de partícula, índice

de polidispersão, potencial zeta e teor de genisteína) e a quantidade de genisteína retida em pele de orelha suína a partir das mesmas.

As oito formulações obtidas pelo arranjo experimental foram preparadas pelo procedimento da emulsificação espontânea. As análises de variância mostraram efeito de interação de 3^{a} ordem para as respostas de tamanho de partícula, índice de polidispersão e retenção cutânea. A escolha das formulações foi baseada nesta análise. A combinação miristato de isopropila/ DOPC/oleilamina apresentou o menor diâmetro médio e promoveu maior retenção de genisteína na pele, enquanto a substituição do DOPC pelo DSPC conduziu a um menor índice de polidispersão. Portanto, essas formulações foram selecionadas para dar continuidade aos estudos. O menor tamanho de gotícula pode promover maior interação com a pele e com isso acarretar em maior retenção/permeação do composto (Abolmaali *et al*., 2011; Mitri *et al*., 2011). De fato, a retenção da genisteína nas camadas de pele suína foi significativamente superior a partir da formulação que apresentou menor tamanho de partícula. Já valores baixos de índice de polidispersão indicam sistemas mais homogêneos e, portanto, formulações mais estáveis (Müller *et al*., 2004). A resposta de maior interesse no estudo é a retenção cutânea, uma vez que, os sistemas desenvolvidos buscam melhorar e intensificar os efeitos da genisteína na pele. Portanto, a associação do miristato de isopropila, que tem propriedades promotoras (Goldberg-Cettina *et al*., 1995; Santos *et al*., 2012), da carga positiva conferida pela oleilamina, a qual é capaz de interagir com as cargas negativas presentes na pele (Rojanasakul *et al*., 1992; Eskandar, Simovic e Prestidge, 2010) e um fosfolipídio de cadeias hidrocarbonadas fluidas (DOPC; temperatura de transição de fase = -21 ^{o}C), capaz de conferir uma interface mais fluida (Hoeller, Sperger e Valenta, 2009), promoveu significativo aumento da retenção cutânea da genisteína.

A partir das duas formulações selecionadas, avaliamos o perfil de distribuição da genisteína nas diferentes camadas da pele de orelha suína. A retenção da genisteína na pele a partir de uma solução controle (genisteína em propilenoglicol) foi avaliada a fim de investigar o efeito promotor dos sistemas estudados. Os resultados obtidos mostraram uma menor retenção da isoflavona a partir da solução controle nas três camadas da pele, o que foi correlacionado com as imagens de confocal. Com relação às nanoemulsões, observou-se maior taxa de penetração nas

camadas mais profundas da pele (epiderme e derme) para as duas formulações, sendo observada uma maior retenção da genisteína a partir da nanoemulsão contendo DOPC em ambas as camadas. Esses resultados evidenciam o efeito promotor dos sistemas nanoemulsionados na liberação tópica da genisteína, com destaque para a formulação que apresenta o fosfolipídio DOPC. Provavelmente, o menor tamanho de gotícula, juntamente com a presença das cadeias hidrocarbonadas fluidas do DOPC (Lim e Lawrence, 2004) promoveram maior interação com os componentes da pele, e consequentemente maior retenção da genisteína. A retenção da isoflavona nas camadas mais profundas da pele tem grande relevância nesse estudo, uma vez que o melhor efeito dos fármacos antivirais aplicados topicamente está relacionado à concentração que alcança à camada basal da epiderme, que é o principal local de replicação do HSV (Hasler-Nguyen *et al*., 2009). Ainda, avaliamos o potencial efeito das formulações sobre a estrutura da pele através de análises histológicas. As imagens obtidas não revelaram mudanças estruturais, sendo assim, nas condições experimentais avaliadas, as formulações parecem não causar danos aos tecidos.

Como os vírus herpéticos estabelecem infecções em pele e mucosas, avaliou-se a retenção/permeação da genisteína incorporada nas nanoemulsões em mucosa de esôfago suíno. O uso da mucosa de esôfago em substituição a mucosa bucal se deve pelo fato de apresentar estrutura e composição semelhantes à bucal, ser um tecido de fácil obtenção e por apresentar maior área de tecido (Caon e Simões, 2011).

A fim de adequar a viscosidade das formulações para o uso tópico, as nanoemulsões catiônicas contendo genisteína foram espessadas com polímero de hidroxietilcelulose. Por ser um polímero não-iônico, a hidroxietilcelulose é compatível com muitos materiais que apresentam natureza iônica (Braun e Rosen, 2000), como é o caso das nanoemulsões do estudo, por isso esse polímero foi escolhido para a obtenção de hidrogéis. A análise dos resultados da caracterização físico-química mostrou que o espessamento das formulações com hidroxietilcelulose a 3% não alterou significativamente ($p>0,05$) o tamanho médio de gotícula e o índice de polidispersão dos sistemas nanoemulsificados. As formulações apresentaram um potencial zeta positivo devido à presença do lipídio catiônico oleilamina, porém com

uma redução significativa nos valores, quando comparada as nanoemulsões. Provavelmente, a redução do potencial zeta está relacionada a adsorção da rede polimérica no entorno da interface óleo/água das gotículas. Uma vez que o potencial zeta mede a diferença de tensão elétrica entre a superfície de um colóide e o meio dispersante, a presença da rede polimérica pode interferir na medida desse parâmetro (Schaffazick *et al*., 2003; Hezaveh e Muhamad, 2012). A caracterização reológica dos hidrogéis mostrou que os mesmos apresentam características de fluxo não-Newtoniano e comportamento do tipo pseudoplástico. Diversos sistemas coloidais tais como soluções poliméricas e formas semissólidas espessadas com polímeros de metilcelulose, carboximetilcelulose e gomas (Genaro, 2000) apresentam esse comportamento, o qual é caracterizado pela diminuição da viscosidade quando uma tensão de cisalhamento é aplicada, ou seja, o material começa a fluir com a aplicação de uma tensão (Almeida e Bahia, 2003).

Os estudos de liberação mostraram a influência da composição das formulações na liberação da genisteína através de membranas sintéticas de celulose. A liberação da isoflavona foi superior a partir da solução de genisteína em propilenoglicol, quando comparado com as formulações. Esse resultado pode ser relacionado com a menor viscosidade da solução e, consequente menor resistência de liberação da genisteína através da mesma. Com relação às nanoemulsões, a taxa de liberação da isoflavona foi maior quando essa foi incorporada na nanoemulsão contendo DOPC, sugerindo a influência do menor tamanho de partícula da nanoemulsão e da presença das cadeias fluidas do DOPC, o qual poderia conferir menor resistência à liberação da isoflavona. No entanto, a taxa de liberação a partir das formulações espessadas foi menor, sem diferença estatística entre os hidrogéis ($p>0,05$), demonstrando o efeito predominante da rede polimérica ao prolongar a liberação.

Os resultados da permeação/retenção da genisteína através da mucosa de esofâgo suíno mostraram perfis semelhantes ao estudo de liberação para as nanoemulsões e hidrogéis, porém diferente para a solução controle. Apesar de o propilenoglicol ser considerado um promotor de permeação, a permeação da genisteína a partir da solução foi mais baixa, comparada com as formulações. Esse resultado sugere que o efeito da propriedade de barreira da mucosa foi maior para a

permeação a partir da solução controle do que para as formulações. Possíveis interações entre as cargas positivas das formulações com as cargas negativas encontradas na superfície da mucosa podem ter efeitos na desorganização do tecido, promovendo a permeação e a retenção da genisteína de forma mais intensa. A visualização das imagens do confocal parecem corroborar com tais resultados. As análises histológicas mostraram que a estrutura da mucosa manteve-se similar a mucosa controle (não tratada), indicando que o contato com as formulações, nas condições empregadas no estudo, não causaram danos aos tecidos.

A atividade anti-herpética da genisteína incorporada nas nanoemulsões também foi avaliada frente a uma cepa resistente ao aciclovir (HSV-1 29R). Assim como para as atividades *in vitro* contra HSV-1 (cepa KOS) e HSV-2 (cepa 333), a incorporação da genisteína nas nanoemulsões aumentou a atividade antiviral da molécula.

O cumestrol, outro isoflavonóide da soja, apresentou potencial atividade anti-herpética. Dessa forma, esse composto foi incorporado nas nanoemulsões selecionadas na segunda parte do estudo. A avaliação da permeação/retenção do cumestrol em mucosa íntegra e lesada de esôgfago suíno e a antividade antiviral desse isoflavonóide a partir das formulações foi demonstrada no quarto capítulo do trabalho. Os resultados da caracterização físico-química das nanoemulsões (cotendo DOPC ou DSPC) estão de acordo com os resultados encontrados para as nanoemulsões contendo genisteína. Portanto, o tamanho médio das gotículas foi maior para a nanoemulsão contendo o fosfolipídeo DSPC, e essa mesma formulação mostrou menor índice de polidispersão. Ambas as formulações apresentaram potencial zeta positivo, indicando a presença do lipídio catiônico na interface da nanoestrutura. Devido a espressão da carga catiônica, as nanoemulsões foram espessadas com o polímero não-iônico hidroxietilcelulose a fim de adequar a viscosidade das formulações para uso tópico. Todas as formulações mostraram teores próximos a 100%. Ainda, as imagens da morfologia, mostram que a adição do polímero manteve a estrutura esférica das gotículas.

Assim, após a eficiente incorporação do cumestrol nas formulações, na sequência, avaliou-se a permeação e retenção dessa molécula utilizando mucosa de

esôfago suíno. Uma vez que as infecções herpéticas geram resposta inflamatória e lesões epiteliais (Goodyear, 2015), além de avaliar o efeito das formulações em mucosa íntegra, nesse capítulo, também foi avaliado o efeito das mesmas em mucosa lesada.

Após 8 horas de estudo, os perfis de permeação do cumestrol através da mucosa íntegra mostraram maior fluxo a partir das formulações contendo DOPC. Contrariamente aos resultados de permeação da genisteína, nesses experimentos, não foram observadas diferenças significativas entre as nanoemulsões e seus hidrogéis derivados. Portanto, a maior viscosidade dos hidrogéis não foi um fator limitante para a permeação do cumestrol. Esse perfil foi mantido após a permeação da molécula através da mucosa lesada, sendo observado um aumento significativo da permeação nessas condições. Esses resultados podem ser relacionados com a remoção parcial da barreira superficial do tecido. Os resultados da retenção do cumestrol nas mucosas (íntegra e lesada) seguiram o perfil dos estudos de permeação, ou seja, a quantidade de cumestrol retida foi superior a partir das formulações contendo DOPC, sem diferença significativa entre as nanoemulsões e seus hidrogéis derivados ($p>0,05$). No entanto, a quantidade total retida foi semelhante em ambas as condiçoes de mucosa utilizadas, íntegra ou lesada. Esses resultados estão de acordo com as medidas de intensidade da fluorescência do corante vermelho do Nilo nas mucosas. Dessa forma, o cumestrol parece ser preferencialmente retido nas camadas mais profundas do tecido, não sendo afetado pela remoção da camada mais superficial (mucosa lesada). Adicionamente, as imagens histológicas das mucosas tratadas com as formulações indicam que as mesmas não causaram danos aos tecidos.

Por fim, a atividade anti-herpética do cumestrol incorporado na nanoemulsão contendo DOPC foi superior ao composto livre frente aos vírus HSV-1 (cepa KOS) e HSV-2 (cepa 333), mas não para o HSV-1 (cepa 29R). Por outro lado, a atividade antiviral da molécula incorporada na nanoemulsão contendo DSPC não foi maior que o cumestrol livre, o que pode ser associado ao menor efeito deste fosfolipídeo sobre a penetração do cumestrol a partir dessa formulação.

O aumento da citotoxicidade após a incorporação da genisteína ou cumestrol nas nanoemulsões foi observada para ambas as células (Vero e GMK AH1). Possíveis interações entre as cargas positivas da oleilamina (amina primária), presente nas formulações com cargas negativas das células pode ser uma explicação para a toxicidade observada (Gershanik *et al.,* 2000). Além disso, pode-se considerar a existência de efeitos citotóxicos físicos das nanopartículas. No entanto, ainda há poucos esclarecimentos sobre a citotoxicidade de sistemas nanoemulsionados *in vitro* para algumas linhagens celulares (Liu *et al*., 2015). Logo, investigações acerca de possíveis interações e como tais interações ocorrem entre as nanogotículas e as células são fundamentais para explicar os efeitos tóxicos, quando existem, desses sistemas. Apesar da citotoxicidade apresentada, nesse estudo, as avaliações histológicas sugerem que as formulações propostas não provocaram danos aos tecidos, sendo seguras para o uso tópico.

Os estudos apresentados nos capítulos III e IV mostram algumas diferenças com relação aos perfis de permeação/retenção para as formulações contendo genisteína e cumestrol. A permeação/retenção da genisteína foi claramente influenciada pela rede polimérica das formulações, enquanto que a permeação/retenção do cumestrol não parece ter sido afetada pelo espessamento com o polímero hidrofílico. Algumas suposições podem ser levantadas para explicar esses resultados. Por exemplo, apesar de ambas as moléculas apresentarem baixa solubilidade em meio aquoso, o log P do cumestrol e da genisteína é próximo de 2,40 e 3,04, respectivamente, o que indica que a genisteína apresenta características mais lipofílicas que o cumestrol (Argenta *et al*., 2011; Rothwell, Day e Morgan, 2005). Logo, por ser menos lipossolúvel, o processo de difusão do cumestrol pela rede hidrofílica dos hidrogéis é mais rápido comparado ao da genisteína. Além disso, diferentes interações entre as moléculas e a interface das nanoemulsões podem ocorrer, levando a diferentes resultados de perfis de permeaçao/retenção na mucosa.

O conjunto dos resultados obtidos indica que a incorporação da genisteína e do cumestrol nas nanoemulsões catiônicas desenvolvidas, bem como o espessamento das mesmas com polímero não-iônico são uma alternativa promissora no desenvolvimento de um sistema de liberação para o tratamento tópico

de infecções herpéticas. Assim como na avaliação da retenção da genisteína em pele, os estudos utilizando mucosa também mostraram o efeito promotor dos sistemas desenvolvidos para a genisteína e o cumestrol. Além da retenção, ambos os isoflavonóides permearam através da mucosa a partir das formulações desenvolvidas. Esse efeito transmucosal pode ser de interesse para aplicação de compostos bioativos, um vez que tanto a genisteína quanto o cumestrol apresentam outras atividades, como antioxidante e estrogênica.

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CONCLUSÕES

A genisteína e o cumestrol apresentaram potencial atividade anti-herpética *in vitro* frente aos vírus HSV-1 (cepas KOS e 29R) e HSV-2 (cepa 333), sendo capazes de interferir em diferentes etapas do ciclo de replicação viral, como na fase inicial da infecção e na expressão de proteínas virais;

- A aplicação de um experimento fatorial completo do tipo 2^3 permitiu selecionar nanoemulsões compostas de um núcleo de miristato de isopropila estabilizado por oleilamina e DSPC ou DOPC com base nas propriedades físico-químicas (menor diâmetro de gotícula e/ou maior homogeneidade), bem como maior retenção da genisteína em pele de orelha suína;

- A adição de HEC a 3%, como agente espessante das nanoemulsões contendo genisteína ou cumestrol, possibilitou a obtenção de formas semissólidas com fluxo do tipo não-Newtoniano, mantendo as propriedades físico-químicas das nanoestruturas obtidas;

- A permeação/retenção da genisteína em mucosa de esôfago suíno a partir das formulações foi superior comparado a uma solução controle de genisteína em propilenoglicol. A presença da DOPC na nanoemulsão promoveu liberação mais rápida da molécula, bem como maior permeação/retenção da mesma. Ainda, foi observado o efeito da rede polimérica na liberação e permeação mais lenta da genisteína;

- A permeação/retenção do cumestrol em mucosa íntegra ou lesada de esôfago suíno foi influenciada pelo tipo de fosfolipídio usado (DSPC ou DOPC), sendo que a permeação foi superior através da mucosa lesada para todas as formulações, quando comparada com a mucosa íntegra. A presença do DOPC nas formulações (nanoemulsão e hidrogel) promoveu maior permeação, bem como maior retenção do cumestrol nos tecidos;

- A incorporação da genisteína e do cumestrol nas nanoemulsões aumenta a atividade anti-herpética frente aos vírus HSV-1 (cepa KOS) e HSV-2 (cepa 333), sugerindo que a formulação promoveu maior penetração intracelular dos isoflavonóides.