UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Maria Júlia Mancim Imbriani

Nanoemulsão contendo hesperitina: desenvolvimento, caracterização e avaliação de seus efeitos anti-inflamatório e pró-osteogênico

Araraquara

2023

UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Maria Júlia Mancim Imbriani

Nanoemulsão contendo hesperitina: desenvolvimento, caracterização e avaliação de seus efeitos anti-inflamatório e pró-osteogênico

Dissertação apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara, para obtenção do título de Mestre em Odontologia, na Área de Periodontia

Orientadora: Profª Drª Denise Madalena Palomari Spolidorio

Coorientadora: Profª Drª Patricia Milagros Maquera Huacho

Araraquara

2023

I32n

Imbriani, Maria Júlia Mancim
Nanoemulsão contendo hesperitina: desenvolvimento, caracterização e avaliação de seus efeitos anti-inflamatório e pró-osteogênico / Maria Júlia Mancim Imbriani. -- Araraquara, 2023
88 f. : il., tabs., fotos

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara
Orientadora: Denise Madalena Palomari Spolidorio
Coorientadora: Patricia Milagros Maquera Huacho

1. Doenças periodontais. 2. Flavonoides. 3. Nanopartículas. 4. Regeneração Óssea. 5. Inflamação. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Odontologia, Araraquara. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

Maria Júlia Mancim Imbriani

Nanoemulsão contendo hesperitina: desenvolvimento, caracterização e avaliação de seus efeitos anti-inflamatório e pró-osteogênico

Comissão julgadora

Dissertação para obtenção do grau de Mestre em Odontologia

Presidente e orientadora: Profª Drª Denise Madalena Palomari Spolidorio

2º Examinador: Prof. Dr. Joni Augusto Cirelli

3º Examinador: Prof. Dr. Vinícius de Paiva Gonçalves

Araraquara, 31 de outubro de 2023.

DADOS CURRICULARES

Maria Júlia Mancim Imbriani

NASCIMENTO: 24/03/1998 – Mococa – São Paulo

FILIAÇÃO: Rita de Cássia da Silva Mancim
Marcelo dos Santos Imbriani

2013 – 2015: Ensino médio (2º grau)
Fundação Universitária Vida Cristã

2016 – 2021: Graduação em Odontologia
Universidade Estadual Paulista Júlio de Mesquita Filho (Unesp) – Araraquara

2021 – atual: Mestrado em Odontologia – Área Periodontia
Universidade Estadual Paulista Júlio de Mesquita Filho (Unesp) – Araraquara

Dedico este trabalho à minha avó Maria Jacinta da Silva Mancim, por ser uma das mulheres mais fortes e corajosas, e ao mesmo tempo mais doce, que já convivi. Sou eternamente grata pela sorte de fazer parte da família que a senhora construiu.

Ao meu avô, Antônio José Mancim (*in memoriam*), que foi um dos homens mais batalhadores que já vi. Me ensinou muito sobre amor, humildade e honestidade. Como foi importante para mim conviver com o senhor, muito do que sou hoje devo ao seu exemplo.

À minha avó Jane dos Santos, que é um exemplo de resiliência e força, me mostrando, mesmo sem saber, que tudo é possível e que nunca devemos desistir daquilo que sonhamos.

E por fim, à minha mãe, Rita Mancim, pessoa ímpar, forte, batalhadora e o melhor exemplo de mulher e mãe que eu poderia ter. Só cheguei aonde estou hoje graças ao seu esforço e dedicação ilimitados. Muito obrigada por nunca medir esforços e sempre me dar o conforto de saber que onde quer que eu estivesse, você estaria ali e tornaria tudo possível.

AGRADECIMENTOS

À Deus por iluminar meus caminhos e pensamentos, sempre me trazendo o conforto de que tudo pode ficar bem.

À minha mãe, Rita, por ser o meu maior exemplo, me incentivar e nunca ter medido esforços para que eu alcance tudo aquilo que desejo.

Aos meus irmãos, Augusto e Rafael, por sempre me darem forças em todos os momentos, e por me mostrarem que independente de onde estivermos sempre teremos uns aos outros.

À toda minha família, que independente de qualquer distância sempre se faz presente e me dá o conforto de saber que sempre terei um lugar cheio de amor me esperando.

À minha orientadora, Profª Drª Denise Madalena Palomari Spolidorio, por acreditar em mim e sempre abrir portas para que eu aprendesse e vivesse experiências incríveis.

À minha coorientadora, Profª Drª Patricia Milagros Maquera Huacho, por toda dedicação em me ensinar durante todos estes anos, por me inspirar tanto na área acadêmica, por dividir comigo seu conhecimento e por todo incentivo.

Ao meu orientador na Université Laval, Prof. Dr. Mahmoud Rouabhia, por ter me acolhido e me orientado de forma ímpar, me inspirando muito profissionalmente e pessoalmente.

Ao meu namorado, Marcus, por me trazer calma e paz no dia a dia, e por estar sempre por perto em todos os momentos.

Aos meus amigos de Araraquara e de Mococa, que me incentivam e apoiam, deixando essa caminhada muito mais leve e feliz.

À minha amiga Letícia Durão, que me permite aprender e ter trocas incríveis, tanto no laboratório como fora dele.

A todos os amigos do laboratório, com quem tive o prazer de conviver e dividir algum momento desde a iniciação científica, Jhonathan, Leo, Juliana, Zé, Paco, Álvaro, Vinícius, Flávia, Gabriel, Cristiano, Vivian (*in memoriam*), Verônica, Maria Fernanda e Vinícius Manzoli, por tornarem o dia a dia mais alegre e por toda troca que tivemos e ainda teremos.

Aos excelentes pesquisadores, Jonatas e Leonardo, e ao Prof. Dr. Marlus Chorilli, da Faculdade de Ciências Farmacêuticas de Araraquara- FCFAr/UNESP, pelo tanto que me ensinaram e por possibilitarem que eu iniciasse este trabalho em seus laboratórios, sempre dispostos a ajudar.

Às amigas que fiz no Canadá, especialmente a Mai, que dividiram momentos inesquecíveis comigo, tornando muito melhores os momentos felizes e mais fáceis os momentos difíceis e de angústia.

À FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Processo nº 2020/ 14155-0, Processo nº 2021/05881-1 e Processo nº 2022/09690-9) pelo apoio financeiro essencial para realização dessa pesquisa.

À Faculdade de Odontologia de Araraquara – UNESP, e todos os professores, técnicos e demais profissionais, por manterem uma faculdade de excelência, permitindo uma formação de ótima qualidade.

Ao Programa de Pós-Graduação em Odontologia, e a todos os professores que colaboram de forma muito importante para a formação do meu mestrado.

“A ignorância gera mais confiança do que o conhecimento: são os que sabem pouco, e não os que sabem muito, que afirmam positivamente que esse ou aquele problema nunca pode ser resolvido pela ciência”.

Charles Darwin*

* Darwin CA. Origem das espécies. São Paulo: Hemus; 2013.

Imbriani MJM. Nanoemulsão contendo hesperitina: Desenvolvimento, caracterização e avaliação de seus efeitos anti-inflamatório e pró-osteogênico [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2023.

RESUMO

Terapias alternativas associando produtos naturais e nanobiotecnologias abrem novas perspectivas para liberação controlada das drogas. Dentro deste contexto, nanoemulsões (NE) apresentam resultados promissores devido ao seu desenho estrutural e destacadas propriedades biológicas. A hesperitina (HT) é um metabolito da hesperidina, encontrado principalmente em frutas cítricas, com diversas propriedades biológicas tais como antioxidante, anti-inflamatória, anticancerígena, neuroprotetora, analgésica e com capacidade osteogênica. Em função dos efeitos terapêuticos da hesperitina, associado às caraterísticas promissoras das NE, o objetivo do presente estudo foi desenvolver uma nanoemulsão contendo hesperitina (NE-HT) com a finalidade de melhorar suas propriedades farmacodinâmicas e assim obter um material biocompatível e bioativo. Por outro lado, o estímulo elétrico (EE) tem mostrado efeitos promissores no comportamento celular, melhorando funções no metabolismo ósseo. Desta forma, consideramos que uma associação entre a NE-HT e EE pode sugerir uma melhora dos efeitos um do outro. Inicialmente, no **estudo 1**, NE-HT foi sintetizada e caracterizada, sua eficiência de encapsulação e morfologia foram avaliadas e seus efeitos sobre a osteogêneses foram determinados em linhagem celular de osteoblastos humanos através da proliferação celular, formação de nódulos mineralizados, expressão de genes reguladores do metabolismo ósseo, organização e maturação de colágeno e atividade da fosfatase alcalina (FAL). **No estudo 2**, foi avaliada a ação da HT em sua forma livre e da NE-HT associadas ou não com estímulo elétrico (EE) no comportamento de osteoblastos humanos MG63, através da análise da adesão celular, proliferação celular, atividade da fosfatase alcalina e migração celular. Finalmente, **no estudo 3**, a ação da NE-HT na expressão de mediadores inflamatórios e matirz metaloproteinases (MMPs), foi avaliada em monócitos/macrófagos humanos estimulados por *Fusobacterium nucleatum*. Os dados numéricos, obtidos pela aplicação dos protocolos laboratoriais, foram submetidos à análise estatística específica utilizando-se o software GraphPad Prism, e todos os testes desse estudo serão aplicados com nível de significância de 5 % (p<0.05). Os resultados mostraram que a nossa NE-HT apresenta índices de caracterização ideais e estáveis, além de uma eficiência de encapsulação de 74.07 ± 5,33%. Além disso o seu efeito benéfico na osteogênese foi comprovado pelo aumento dos parâmetros avaliados, ou aceleração dos mesmos. Já a associação da NE-HT com o EE mostra um melhor efeito do EE, ao permitir que uma alta voltagem de EE possa ser usada em osteoblastos não interferindo na adesão celular, aumentando a proliferação e melhorando os índices de atividade da FAL, além de mostrar um maior efeito da NE-HT na migração celular quando comparada a HT na forma livre. E por fim, a ação da NE-HT contra a inflamação pode ser comprovada pela redução das taxas de expressão de mediadores inflamatórios e MMPs. Portanto, podemos concluir que a NE-HT desenvolvida no presente estudo foi adequadamente caraterizada e sua aplicação direta ou associada a EE apresentam-se como potencial tratamento para doenças inflamatórias que envolvem o osso, porém, mais estudos *in vitro* e *in vivo* são necessários.

Palavras-chave: Doenças periodontais. Flavonoides. Nanopartículas. Regeneração Óssea. Inflamação.

Imbriani MJM. Hesperetin loaded nanoemulsion: development, characterization and evaluation of its anti-inflammatory and pro-osteogenic effects" [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2023.

ABSTRACT

Alternative therapies combining natural products and nanobiotechnologies open new perspectives to controlled drug release. In this context, nanoemulsions (NE) show promising results due to their structural design and outstanding biological properties. Hesperetin (HT) is a metabolite of hesperidin, found mainly in citrus fruits, with several biological properties such as antioxidant, anti-inflammatory, anticancer, neuroprotective, analgesic, and osteogenic capacity. Due to the therapeutic effects of hesperetin, associated with the promising characteristics of NE, the present study aimed to develop a hesperetin-loaded nanoemulsion (HT-NE) to improve its pharmacodynamic properties and thus obtain a biocompatible and bioactive drug. On the other hand, electrical stimulation (ES) has been shown promising effects on cell behavior, enhancing bone metabolism functions. In this way, we consider that an association between the HT-NE and ES provides the possibility that both can enhance the effects of each other. At first, in **study 1** the HT-NE was synthesized and characterized, its encapsulation efficiency and morphology were evaluated, and its effects on osteogenesis were determined, in human osteoblasts cell line, through cell proliferation, formation of mineralized nodules, expression of regulator genes to bone metabolism, collagen quantification, and alkaline phosphatase activity. In **study 2**, the effects of HT in its free form and HT-NE associated or not with ES were evaluated in human osteoblasts MG63 behavior, through the analysis of cell adhesion, cell proliferation, alkaline phosphatase (ALP) activity, and cell migration. Finally, in **study 3**, the action of HT-NE on the expression of inflammatory mediators and matrix metalloproteinases (MMPs) was evaluated in human monocytes/macrophages stimulated by *Fusobacterium nucleatum*. The numerical data, obtained by applying the laboratory protocols, was subjected to specific statistical analysis using the GraphPad Prism software, and all tests in this study was applied with a significance level of 5% *(p* <0.05). Our results shows ideal and stable indexes of charachterization, besides of an encapsulation efficiency of 74.07 ± 5,33%. Besides that, its benefic effect on osteogenesis was proved by the increase of evaluated assays, or the acceleration of them. The association of HT-NE with ES showed a better effect of ES, allowing high voltage to be used in human osteoblasts, not interfering the cells adhesion, increasing cell proliferation and enhancing ALP activity, besides of showing a higher effect of HT -NE in cell migration when compared with free HT. Finally, the HT-NE effect against inflammation can be proved by de reduction of inflammatory mediators and MMPs. For this reason, we can conclude that the HT-NE developed in this study was properly characterized and its direct application or its association with ES can be consider as a potential treatment to inflammatory diseases affecting bone, although *in vitro* and *in vivo* studies are necessary.

Keywords: Periodontal diseases. Flavonoids. Nanoparticles. Bone regeneration. Inflammation.

SUMÁRIO

1 INTRODUÇÃO 11

2 PROPOSIÇÃO 16

2.1 Objetivos Específicos 16

3 PUBLICAÇÃO 17

3.1 Publicação 1 17

3.2 Publicação 2 45

3.3 Publicação 3 67

4 CONCLUSÃO 84

REFERÊNCIAS 85

1 INTRODUÇÃO

A periodontite é uma doença inflamatória crônica multifatorial associada ao biofilme disbiótico, e caracterizada por causar a destruição progressiva dos tecidos de suporte dentário[1]. É identificada a partir da perda de inserção clínica, perda óssea radiograficamente visível, bolsa periodontal e sangramento gengival[1]. Os periodontopatógenos e seus componentes bacterianos tais como lipopolissacarídeos, proteases e toxinas encontradas no biofilme induzem a resposta imune do hospedeiro ativando vias de defesa para a prevenção do avanço da infecção nos tecidos periodontais[2]. Bactérias periodontopatogênicas Gram-negativas tais como *Porphyromona gingivalis, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* e *Tanerella forsythia*, estão fortemente envolvidas em diversas etapas da doença periodontal desde seu início[3-5].

Complexos eventos imunoinflamatórios estão envolvidos na patogênese da periodontite, como por exemplo, aumento na produção de citocinas inflamatórias, como as interleucinas IL-1β, IL-6 e fator de necrose tumoral (TNF) assim como, a perda da relação entre metaloproteases de matriz (MMP) e o tecido inibidor de metaloproteínase (TIMP), fazendo com que a MMP degrade colágeno, elastina, matriz glicoproteíca e proteoglicanos[5-7]. Além disso, o LPS das bactérias via receptores toll like (TLRs) e marcadores inflamatórios como TNF (fator de necrose tumoral), levam a ativação de receptor desencadeador expresso em células mielóides (sTREM-1) por células de defesa como neutrófilos e monócitos ou macrófagos, acarretando a expressão de citocinas pró inflamatórias, quimiocinas e receptores de superfície celular[8,9]. Além disso, o sTREM é detectado em altas concentrações no fluido gengival de indivíduos com doença periodontal[8,9]. Dessa forma, estes mediadores inflamatórios promovem, indiretamente, degradação dos tecidos periodontais pela indução da produção de metaloproteases de matriz, ativador do receptor nuclear Kappa-B ligante (RANKL) e atividade de osteoclastos, resultando na reabsorção óssea alveolar[10] que constitui a principal característica da periodontite e, eventualmente, resulta com a perda do dente[7,11].

O tratamento da periodontite tem como objetivo evitar a futura progressão da doença, minimizar os sintomas da mesma e muitas vezes possibilitando a restauração dos tecidos perdidos, evitando a perda do elemento dental e mantendo a saúde do

periodonto[12]. A terapia de redução bacteriana por meio do controle mecânico do biofilme dentário, assim como da terapia básica de raspagem e alisamento radicular manual, são tratamentos que apresentam bons índices de sucesso e ainda é a terapia mais indicada na prática clínica[12]. Contudo existem casos em que esta abordagem não é suficiente, exigindo complementações à terapia tradicional, podendo-se utilizar terapias coadjuvantes focada na tentativa de modular a resposta do hospedeiro e assim tentar suprimir a destruição dos tecidos periodontais pela reação inflamatória crônica[12].

Novas drogas bioativas e de origem natural têm se tornado atraentes devido aos seus efeitos benéficos na saúde humana, principalmente no que diz respeito às doenças infecciosas[13,14]. Os flavonoides são compostos bioativos do grupo dos polifenóis, apresentam mais de 5000 tipos e possuem diferentes estruturas[13,14]. Podem ser encontrados em diversos tipos de plantas, frutas e vegetais, apresentando um sistema de baixa citotoxicidade e propriedades biológicas como anti-oxidante, anti-inflamatória, cardioprotetiva, vasodilatadora, anti-cancerigena, antibacteriana e efeitos no metabolismo ósseo[14-18]. Devido às suas complexas estruturas os flavonoides podem ser divididos em flavonois, flavonas, flavononas, flavon-3-ol, isoflavononas e antocianins[19].

A hesperitina (HT) é um metabólito da hesperidina, pertencente ao subgrupo flavanona, encontrado principalmente em frutas cítricas como laranja e toranja[20]. Diversos estudos têm utilizado a HT como uma estratégia alternativa de novos usos terapêuticos, devido as suas diversas propriedades biológicas, incluindo efeitos analgésicos, anticancerígenas, anti-inflamatórias, antioxidantes, neuroprotetoras e efeitos benéficos ao metabolismo ósseo[14-18]. Estudos destacam sua propriedade anti-inflamatória, mostrando sua capacidade em reduzir a atividade de mieloperoxidase, produção de TNF-α, IL-6, IL-1β, MIP-2, ciclooxigenase-2 (COX-2), alanina aminotransferase (AST) e enzimas ativas de aspartate transferase (ALT), além de inibir a ativação da via de sinalização NF-κB[21-23]. Protocolos experimentais *in vivo* e *in vitro* puderam demonstrar a capacidade da HT em inibir fortemente as expressões proteicas de TNF-α, NF-kB, COX-2, iNOS, IL-6 e IL-1β[24-26]. Por meio da sua atuação na ligação MD2/TLR4, responsável pela identificação de LPS na superfície celular, a HT é capaz de atenuar lesões agudas de pulmão, diminuindo a concentração total de proteínas, número de neutrófilos e nível de citocinas inflamatórias IL-6 e TNF-α[27]. Chen et al.[28] (2017) mostraram em estudo *in vivo*, com indução de lesão hepática em ratos, e *in vitro*

com a indução de inflamação em macrófagos RAW 264.7 por LPS, que a HT apresenta efeito hepatoprotetivo e anti-inflamatório através da inibição das citocinas inflamatórias TNF-α, IL-1β e IL-6.

Nos últimos anos autores sugeriram que a HT está envolvida no metabolismo ósseo e mecanismos de ação na osteogênese[29,30]. Estudos mostraram que a HT alivia os efeitos inibitórios da alta glicose na diferenciação osteoblástica de células-tronco do ligamento periodontal[31,32]. Além disso, a HT promove a diferenciação osteogênica de células-tronco mesenquimais humanas através das vias de sinalização ERK e Smad1/5/8[34]. A ação benéfica da HT aos ossos, também se deve ao fato de a mesma aumentar a expressão de importantes genes, como ALP, RUNX 2, osteocalcina (OCN), Osterix, osteopontina (OPN), e COL1A1 em osteoblastos, células mesenquimais e células do ligamento periodontal[29,32,33]. Entretanto, os mecanismos de ação da HT sobre o metabolismo ósseo não estão completamente elucidados sendo de suma importância a busca de novas informações e constatações desse composto sobre estes postulados.

A nanotecnologia é o desenvolvimento de novas tecnologias em nível atômico, molecular e macromolecular para aplicações práticas, como dispositivos, moléculas e estruturas em escalas de nanômetros[34]. A mesma contribui para quase todos os campos da ciência, como por exemplo, física, ciência dos materiais, química, biologia, ciência da computação e engenharia, sendo recentemente aplicada na saúde humana com resultados altamente promissores[34]. Por outro lado, a biotecnologia se define como uma área multidisciplinar de aplicação tecnológica que utiliza sistemas biológicos e organismos vivos, para fabricar ou modificar produtos ou processos para utilização específica[35]. Estas novas tecnologias estão revolucionando o mundo da ciência e o resultado dessa união é a chamada “nanobiotecnologia”, ou seja, uma ciência avançada e altamente interdisciplinar que vem sendo frequentemente utilizada para várias aplicações[35]. Espera-se que a sociedade seja beneficiada pela fusão dessas duas tecnologias devido às potenciais aplicações em medicina, odontologia, agricultura, meio ambiente dentre outras áreas[35].

O interesse na nanotecnologia cresce cada vez mais como sistema de entrega de drogas com múltiplas vantagens: sua relação superfície/massa que é maior que de outras partículas, suas propriedades quânticas[36], conferindo numerosas vantagens para a entrega de medicamentos, especialmente controlando o comportamento físico-

químico do fármaco (solubilidade e liberação) e medicamentos visando o potencial do sito ativo, diminuindo os efeitos adversos[37-40]. Dentre as diferentes nanopartículas, sabe-se que as nanoemulsões são um tipo de carregador lipídico nanoestruturado[41] e podem ser formuladas de diversas formas, como líquidos, géis, cremes, aerossóis, dentre outras, e possibilitam diferentes formas de aplicação, além de poder apresentar administração de forma nasal, intravenosa, pulmonar e ocular[42]. Além disso, as gotículas de óleo presentes são capazes de solubilizar compostos farmacêuticos lipofílicos, além de protegê-los contra fatores externos, como pH, a oxidação e a hidrólise, sendo também mais resistentes a floculação e coalescência[43]. Portanto as nanoemulsões permitem a redução da variabilidade do composto encapsulado, destacando-se a sua capacidade de protegê-lo contra enzimas degenerativas e controlando sua liberação no sítio ativo de escolha[44]. Além disso a mesma pode gerar uma melhor biocompatibilidade do fármaco permitindo a utilização do mesmo em altas concentrações[44]. Por tanto, ao incorporar um composto natural com baixa toxicidade dentro de um sistema de liberação controlada como são as nanoemulsões, irá permitir de que a nova formulação se torne mais biocompatível, permita a redução de seus efeitos colaterais, aumente sua estabilidade e melhore os efeitos biológicos destes compostos naturais[45].

E por fim, a estimulação elétrica (EE) tem mostrado efeitos positivos nas atividades celulares, como por exemplo, no transporte de íons, na expressão gênica, produção de proteínas, espécies reativas de oxigênio intracelulares, secreção de citocinas e quimiocinas e migração celular[46]. Um estudo mostrou que a EE é capaz de gerar efeitos positivos na cicatrização através da regulação de citocinas pró inflamatórias e anti-inflamatórias, fatores de crescimento e migração celular em fibroblastos humanos de pacientes diabéticos[47]. Por outro lado, Meng et al.[48] (2011) mostraram que a EE melhora a proliferação e adesão de osteoblastos, aumenta a formação de nódulos de mineralização e a expressão de genes relevantes para o metabolismo ósseo. Estudos mais recentes tiveram como foco a melhora da aplicação do EE e, por esta razão, diferentes membranas foram usadas[47-50]. Dentre estas membranas, as compostas por Polypirrole (PPy), um polímero que possui uma boa compatibilidade, mostrou resultados promissores[51]. Apesar do PPy apresentar características positivas, o mesmo apresenta algumas limitações que levam a um prejuízo de sua função, como a fragilidade e insolubilidade. Assim, outro polímero o poly(l-lactidio) (PLLA) foi associado na síntese da membrana para torná-la mais forte e

resistente[49,51]. Além disso, foi testada a adição de heparina (HE) à formulação da membrana, obtendo-se resultados positivos como um aumento significativo da condutibilidade em meio aquoso e da adesão e crescimento celular[52]. Portanto com a utilização de uma membrana de PPy/HE/PLLA, a EE pode apresentar resultados promissores.

Contudo tem-se a hipótese de que a encapsulação da HT à uma nanoemulsão pode gerar uma melhora nos efeitos anti-inflamatório e pró-osteogênico assim como uma melhor estabilidade do flavonoide. Além disso, a associação entre a nanoemulsão contendo hesperitina (NE-HT) e EE podem apresentar perspectivas de aumento dos efeitos biológicos, uma da outra, sobre o metabolismo ósseo através da influência no comportamento dos osteoblastos.

2 PROPOSIÇÃO

Desenvolver e caracterizar uma nanoemulsão contendo hesperitina (NE-HT) com a finalidade de melhorar as propriedades farmacodinâmicas, a fim de obter um material biocompatível e bioativo, associando-a ou não ao estímulo elétrico, e avaliar *in vitro* o seu efeito anti-inflamatório e pró-osteogênico.l.

2.1 Objetivos Específicos

A. Sintetizar, caracterizar morfologicamente e estruturalmente a NE-HT, avaliando *in vitro*, sua biocompatibilidade e seu efeito sobre o metabolismo do tecido ósseo, verificando sua influência sobre a diferenciação e metabolismo de osteoblastos através da análise da proliferação celular, formação de nódulos de mineralização, expressão dos genes RUNX2, ALPL e TGF-β, produção de colágeno e atividade da fosfatase alcalina.
B. Avaliar *in vitro*, o efeito da HT livre e da NE-HT associadas à estímulo elétrico (EE) na adesão e proliferação de osteoblastos, além do seu efeito na atividade da fosfatase alcalina e migração celular.
C. Avaliar *in vitro*, o efeito da NE-HT na ativação mediada por *Fusobacterium nucleatum* na expressão de mediadores pró-inflamatórios TNF-α, lL-1β, IL-6, IL-8 e matirz metaloproteinases (MMP)-1, MMP-3 e MMP-9 por monócitos/macrófagos humanos.

3 PUBLICAÇÃO

Nas publicações a seguir serão apresentados os resultados deste estudo. Na Publicação 1 estão os resultados sobre o desenvolvimento e caracterização da NE-HT, e seu efeito na osteogênese. A Publicação 2, mostra os resultados obtidos no intercâmbio BEPE-FAPESP realizado na Faculté de médecine dentaire da Université Laval em Quebec no Canada sob supervisão do Prof. Dr Mahmoud Rouabhia. Nesta, está descrito o efeito da NE-HT e HT associadas ou não ao estímulo elétrico no comportamento de osteoblastos. E, por fim, a Publicação 3 traz os resultados da ação NE-HT sobre a inflamação.

3.1 Publicação 1*

Formulation of a novel hesperetin-loaded nanoemulsion and its promising effect on osteogenesis

Maria Júlia Mancim-Imbriani[1,2], Jonatas Lobato Duarte[3], Leonardo Delello Di Filippo[3], Leticia Pereira Lima Durão[2], Marlus Chorilli[3], Denise Madalena Palomari Spolidorio[2], Patricia Milagros Maquera-Huacho[1,2,*]

[1]Department of Diagnosis and Surgery, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

[2]Department of Physiology and Pathology, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

[3]Department of Drugs and Medicines, School of Pharmaceutical Sciences, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

*Correspondence:

Patricia Milagros Maquera-Huacho, Department of Physiology and Pathology, School of Dentistry, São Paulo State University (Unesp), Araraquara – SP, Humaita St, 1680, CEP 14801-903 Araraquara, São Paulo, Brazil.

Email: patricia.maquera@unesp.br

* Artigo seguiu as normas da revista *Pharmaceuticals* para a qual será submetido.

Abstract: Alternative therapies associating natural products and nanobiotechnology show new perspectives to controlled drug release. In this context, nanoemulsions (NE) present promising results for their structural design and properties. Hesperetin (HT), a flavonoid mainly found in citrus fruits, presents highlighted bone benefits. In this context, we developed a hesperetin-loaded nanoemulsion (HT-NE) by sonication method and characterized it by dynamic light scattering, analyzing its encapsulation efficiency, and registering its particle size by transmission electron microscopy. The biocompatibility in human osteoblasts Saos-2-like was evaluated by the cytotoxicity assay and IC_{50}. Then, the effects of the HT-NE on osteogenesis were evaluated by the cellular proliferation, calcium nodule formation, bone regulators gene expression, collagen quantification and alkaline phosphatase activity. The results showed that the formulation presented ideal values of droplet size, polydispersity index and zeta potential, and the encapsulation efficiency was 74.07 ± 5,33%, showing good morphology. Finally, HT-NE showed to be biocompatible and increased cellular proliferation, calcium nodule formation, regulated the expression of Runx2, ALPL and TGF-β genes, and increased the collagen formation and alkaline phosphatase activity. Therefore, the formulation of this NE encapsulated the HT appropriately, allowing the increasing of its effects on mechanisms to improve or accelerate the osteogenesis process.

Keywords: Nanoparticles. Flavonoids. Materials Testing. Cytotoxicity Tests. Bone Regeneration

1. Introduction

Nowadays, we have a wide array of conditions presenting bone loss, including periodontal diseases, osteoporosis, and fractures in the bone [1], so the search for treatments to these diseases is growing. To the treatment of these diseases, several treatments, medicines and biomaterials are proposed but in some cases is not enough to restore bone formation [2]. On the other hand, several disadvantages and side effects have been observed, for example, bisphosphonate is used to the treatment of osteoporosis, but its use can cause bone necrosis [3],

In recent years, drug discovery and development that presents a drug delivery system is increasing. Among them, nanoparticles are formulations with benefits as its relation surface mass/bigger and its diverse quantic properties [4,5]. Besides the drug release, they can present others positive characteristics as increasing the solubility and more selective distribution, and therefore decreasing its adverse effects [6-10]. Among the nanoparticles, nanoemulsions (NE) are a mix of oil phase (non-polar) and aqueous phase (polar) with the presence of an emulsifier. They have a good potential as drug delivery systems, controlling the release and directing it to its active site [11]. Furthermore, NE are capable to carry lipophilic pharmaceutical compounds, protecting them against external factors, like pH, oxidation, and hydrolysis, and it has also been more resistant to flocculation and coalescence when compared to emulsions [12].

Flavonoids are bioactive compounds that belong to the polyphenol group, they can be classified in flavonols, flavones, flavonones, flavon-3-ol, isoflavonones and anthocyanins [13]. Hesperetin (HT) is a metabolite of hesperidin, belonging to flavonone subgroup [14]. It can be found mainly in citric fruits, like oranges and grapefruit [14]. Several studies suggested the biological properties of hesperetin like analgesic, anti-cancer, anti-inflammatory, antioxidant, and neuroprotector effects [15-18]. Among these properties, benefic effects on bone metabolism are highlighted [15-19].

HT presents characteristics that can improve bone mineral density through different mechanisms [20]. Trzeciakiewicz et al. [21], 2010 suggested that this flavonoid can stimulate the differentiation of primary osteoblasts in rats, by Smad-dependent BMP (bone morphogenetic protein) and MAPK signaling pathways. Hesperetin can enhance the expression of bone metabolism genes, like ALP, RUNX 2, OCN, Osterix, osteopontin (OPN) and COL1A1 in osteoblasts, mesenchymal and

periodontal ligament stem cells [21-23]. Also, in high glucose conditions, hesperetin can promote osteoblastic differentiation [24, 25]. In this context, HT suppress the osteogenesis inhibitor factors through activation of PI3K/Akt pathway and suppression of ROS [23]. Additionally, Liu et al [26] suggest that hesperetin is able to suppress glucocorticoid effects through regulation of ERK signaling pathway.

Given that HT is a flavonoid with benefic and promising properties specifically on bone metabolism, the aim of this study was to formulate a new hesperetin-loaded nanoemulsion (HT-NE), evaluate its colloidal stability and the *in vitro* effect on osteogenesis. To the best of our knowledge, this is the first manuscript on incorporating HT in NE as a drug delivery system and investigating its effects for biomedical applications including bone tissue regeneration.

2. Results

Droplet Size, Polydispersity Index, and Zeta Potential

The HT-NE showed a translucence or slight turbidity of its surface during all periods under analysis (Figure 1), a common characteristic of this type of nanoparticle due to the small size of its droplets.

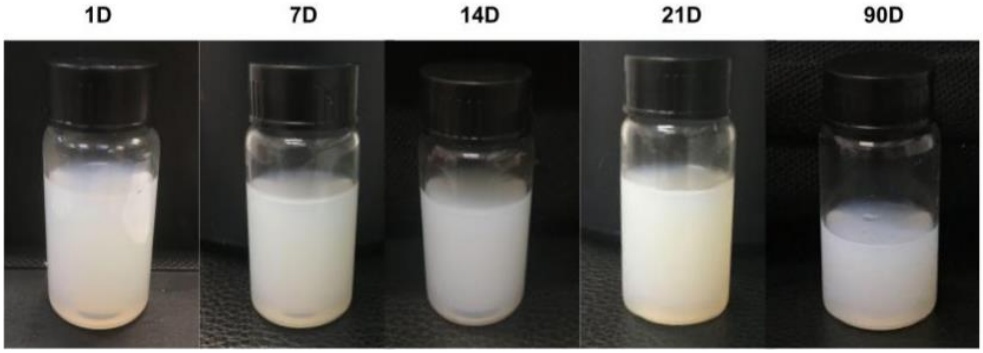


Figure 1. Representative images of HT-NE in different analyzed period (1, 7, 14, 21 and 90 days).

Table 1 shows the results of HT-NE characterization by dynamic light scattering (DLS). It is possible to observe that the droplet size was kept stable during the evaluated periods, 1 to 90 days, presenting values around 89.41 ± 5.81 nm. The polydispersity index (PDI) presented a stable value around of 0.267 ± 0.008 for the periods until 21 days, and a value of 0.584 ± 0.035 was obtained after 90 days. HT-NE

zeta potential presented values higher than -20 mV and less than 20 mV for all the periods.

Table 1. Droplet size, polydispersity index and zeta potential of HT-NE.

Time (days)	Droplet size (nm)	Polydispersity Index	Zeta Potential (mV)
1	88.79 ± 0.09	0.276 ± 0.003	-13.27 ± 2.7
7	88.26 ± 0.75	0.273 ± 0.006	-17.37 ± 1.7
21	89.01 ± 1.15	0.266 ± 0.003	-14.97 ± 0.6
90	82.11 ± 3.89	0.584 ± 0.035	-4.75 ± 0.1

Table 2 showed values of droplet size, polydispersity index, and zeta potential of the Blank NE analyzed for 1 day. The results showed that the Blank NE presents droplet size value of 97.86 ± 0.50 nm and polydispersity index of 0.278 ± 0.003, similar to HT-NE (results shown in Table 1). On the other hand, the Blank NE showed a zeta potential value of -26.9 ± 2.4 mV.

Table 2. Droplet size, polydispersity index, and zeta potential of the Blank NE.

Time (days)	Droplet Size (nm)	Polydispersity Index	Zeta Potential (mV)
1	97.86 ± 0.50	0.278 ± 0.003	-26.9 ± 2.4

Encapsulation efficiency

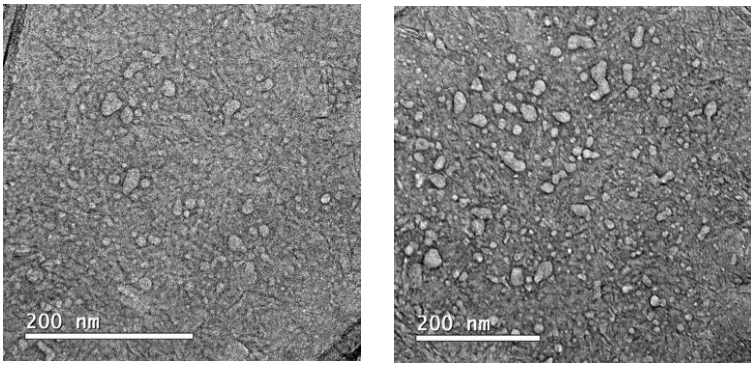
Table 3 shows the results of the encapsulation efficiency in percentage. It is possible to observe that the average obtained was 74.07% with a standard deviation of 5.33%.

Table 3. Encapsulation efficiency (%) from values of 3 replications, based on the analytic curve.

Formulation	Encapsulation efficiency (%) ± SD
HT-NE	74.07 ± 5.33

Transmission Electron Microscopy (TEM)

Figure 2 was obtained by transmission electronic microscopy. The images showed that the droplets of HT-NE and droplets of Blank NE present similar characteristics, with a circular format and sizes smaller than 100 nm.



a *b*

Figure 2. Images obtained by transmission electron microscope (JEOL JEM-2100) at 250.000x magnification and scale bar of de 200 nm. a) Image of HT-NE and b) Image of blank NE.

Cytotoxicity

Figure 3 shows the results of the cytotoxicity assay. The treatment with Free HT and HT-NE in concentrations greater than 512 µM showed a statistically significant decrease in osteoblasts cells after exposure for 7 days in comparison to the negative control group (p<0.001). On the other hand, Blank NE showed a cytotoxic effect in concentrations greater than 1025 µM in comparison to the negative control group (p<0.001).

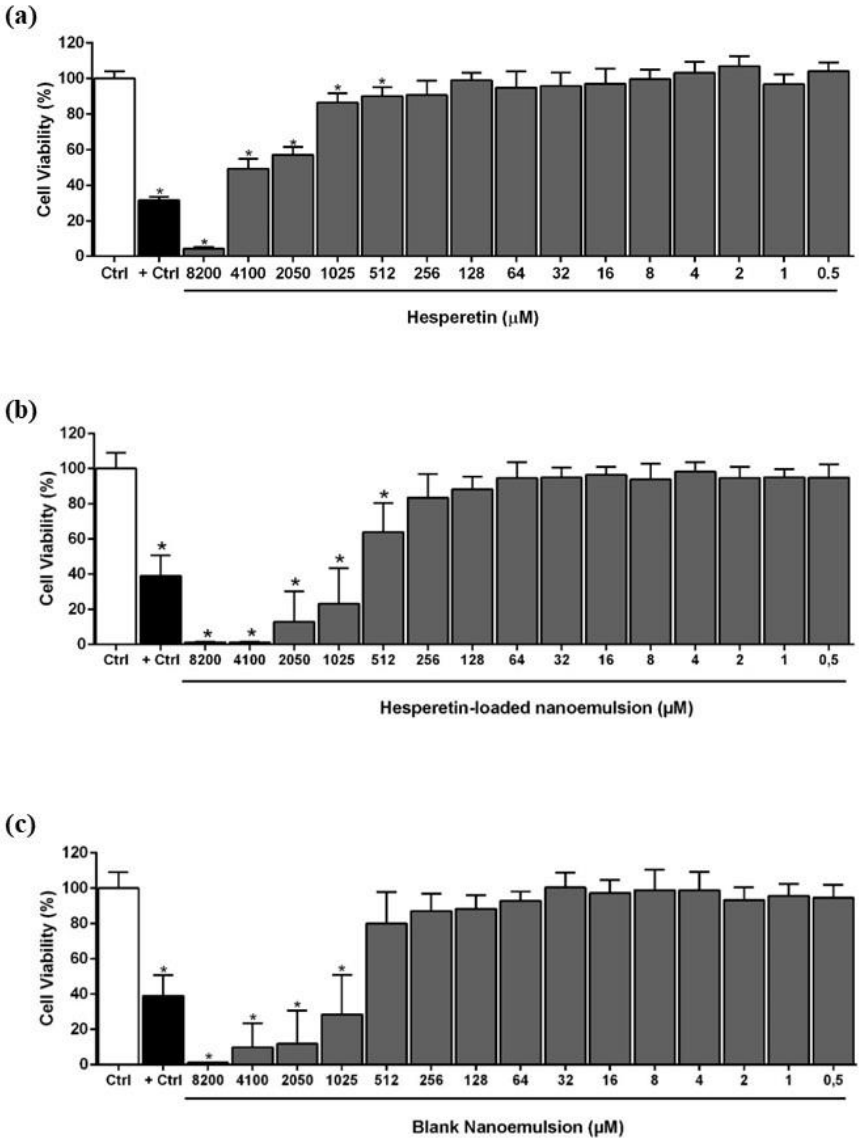


Figure 3. Cell viability percentage of osteoblasts Saos-2 after 7 days of treatment with different concentrations of Free HT (a), HT-NE (b), and Blank NE (c). *Difference statistically significant compared with the negative control group (ANOVA, p<0.001).

IC_{50} value

Figure 4 shows the IC_{50} values of the treatments: Free HT and nanoemulsions (HT-NE and Blank NE). The IC_{50} values were 3.46 log, 2.82 log and 2.87 log (concentrations) to Free HT, HT-NE and Blank NE, respectively.

Taking this into account, it is possible to note that Free HT presented an IC_{50} value higher than the NE tested.

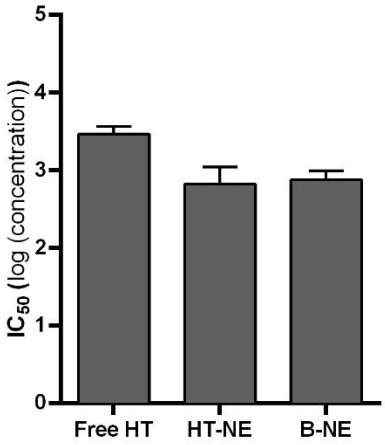


Figure 4. The IC_{50} value of the Saos-2-like cell line treated with Free HT, HT-NE and Blank NE (ANOVA, $p<0.05$).

Cell Proliferation Analysis

Figure 5 shows the cell proliferation results. It is possible to observe an increase in all groups when they are compared between different periods, showing a statistically significant difference ($p<0.05$). On the first day of evaluation the groups did not present a statistical difference between them ($p>0.05$), however, in 3, 5, and 7 days of evaluation the groups treated with HT-NE showed a statistically significant increase in cell proliferation when compared to the control group ($p<0,05$). An interesting point was that at 5 and 7 days, the HT-NE treated groups with 128 µM and 64 µM concentrations, showed an increase in cell proliferation and showed a statistical difference compared to the control and HT-NE 32 µM groups ($p<0.05$).

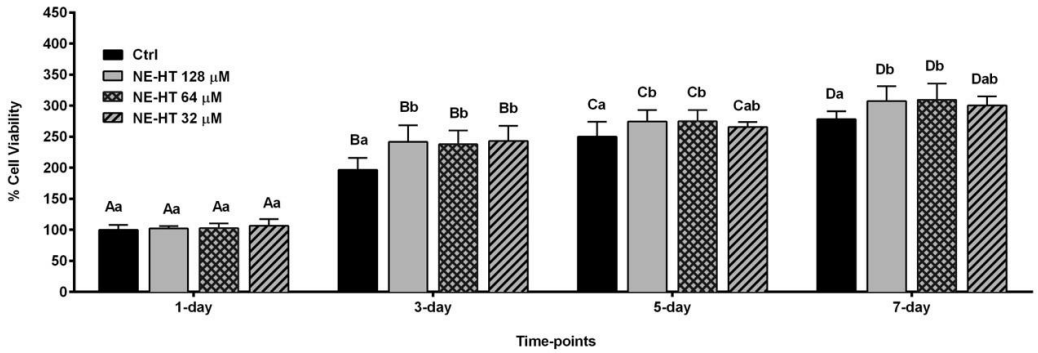


Figure 5. HT-NE effect on osteoblasts Saos-2 proliferation after treatment with non-cytotoxic concentrations (32 µM, 64 µM, and 128 µM) for 1, 3, 5, and 7 days. The same

letters represent no statistical difference, different letters indicate statistical difference. The upper case indicates a comparison between periods and the lower case indicates a comparison between groups in the same period. (Two-way ANOVA, $p<0.05$).

Mineralized Nodules Formation

Figure 6 described the results of mineralized nodules formation. HT-NE treated groups at 32 µM and 64 µM showed a statistically significant increase in mineralized nodule formation when compared with group treated with osteogenic medium only ($p<0.05$, $p<0.01$). However, the HT-NE 128 µM did not present a statistically significant difference ($p>0,05$). Although, we can observe that all groups treated with HT-NE, showed an increase in mineralized nodule formation compared to the group that just received a normal medium ($p<0.0001$).

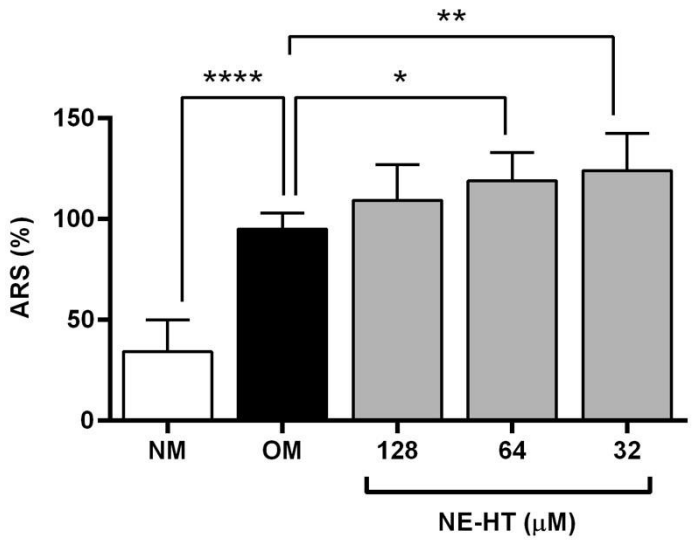


Figure 6. Calcium nodule formation quantification in human osteoblasts Saos-2 like after treatment with non-cytotoxic HT-NE (128 µM, 64 µM, and 32 µM) concentrations for 7 days. Measurement in the percentage of Alizarin red absorbance. (ANOVA, α=0,05). ⌐¬ represents statistically significant difference compared to osteogenic group (*$p<0.05$, **$p<0.01$, ****$p<0.0001$). NM = normal medium, OM = osteogenic medium.

Bone Metabolism Gene Expression Quantification

Figure 7 represents the gene expression quantification of TGF-β, Runx2 and ALPL by human osteoblasts Saos-2 treated with non-cytotoxic HT-NE concentrations after 1, 3, and 7 days. The TGF-β expression was significant increased after the treatment with HT-NE 64 µM at 3 and 7 days (p<0.05, p<0.001). Runx2 gene expression showed a statistically significant increase after all tested periods when treated with HT-NE 32 µM (p<0.05, p<0.001, p<0.0001). At 64 µM concentration of HT-NE, the increase in its expression was observed at 3 and 7 days (p<0.05). The concentration of HT-NE 128 µM showed an increase effect of Runx2 gene expression in just after 7 days (p<0.01). Finally, ALPL gene showed a statistically significant increase after the treatment with HT-NE 32 µM at 1 and 7 days (p<0.01, p<0.0001). Additionally, after 7 days HT-NE 64 µM and 128 µM showed a statistically significant increase in the expression of this gene (p<0.05, p<0.0001).

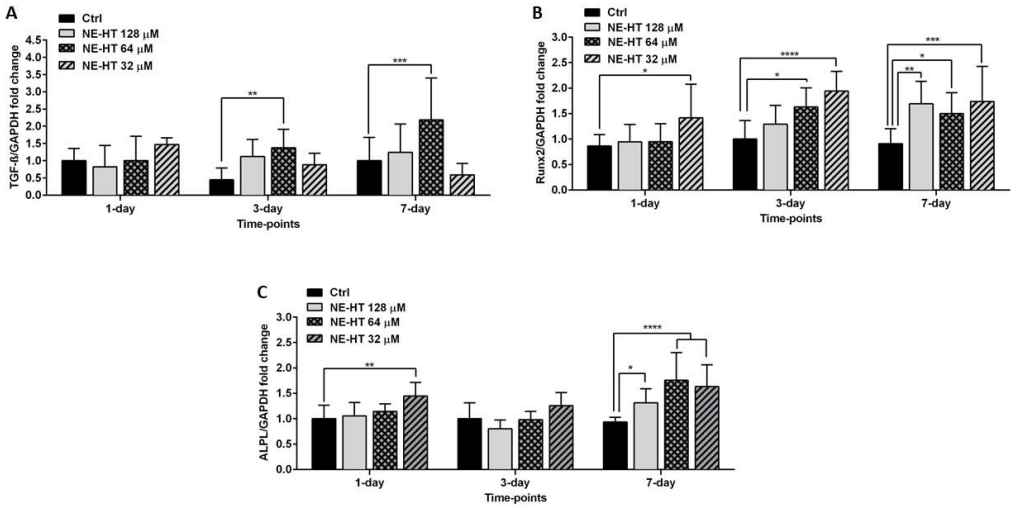


Figure 7. Gene expression levels of TGF-β (A), Runx2 (B) e ALPL (C) in human osteoblasts Saos-2 treated with non-cytotoxic HT-NE (128 µM, 64 µM and 32 µM) after 1, 3 and 7 days. *Represents a statistically significant difference compared to the control group (One-way ANOVA, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001).

Collagen Production by Picrosirius Red

Figure 8 shows the collagen quantification after treatment with different HT-NE concentrations (128 µM, 64 µM and 32 µM) for 3 and 7 days. The results showed that at 3 days of treatment, HT-NE treated groups did not show statistical difference compared to the control group (p>0.05). However, at 7 days of treatment the HT-NE at 128 µM and 32 µM showed an increase in collagen production statistically significant compared to the control group (p<0.05).

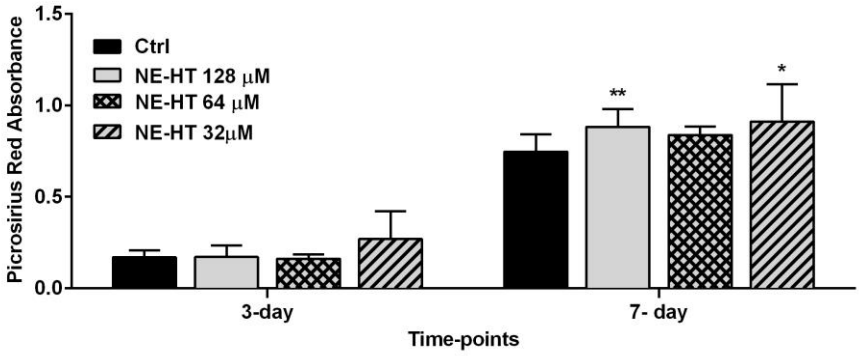


Figure 8. Collagen production quantification after treatment with non-cytotoxic HT-NE concentrations (128 µM, 64 µM and 32 µM) for 3 and 7 days. *Represents a statistically significant difference compared to the control group (One-way ANOVA, *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001).

Alkaline Phosphatase Activity

Figure 9 shows the results of ALP activity analysis. It is possible to note that after the treatment with all HT-NE concentrations, no statistical difference between groups was observed at 3 days of treatment (p>0.0001). On the other hand, we can observe that at 7-days the treatment with 128 µM of HT-NE showed a statistically significant increase in ALP activity compared to the control group (p<0.0001).

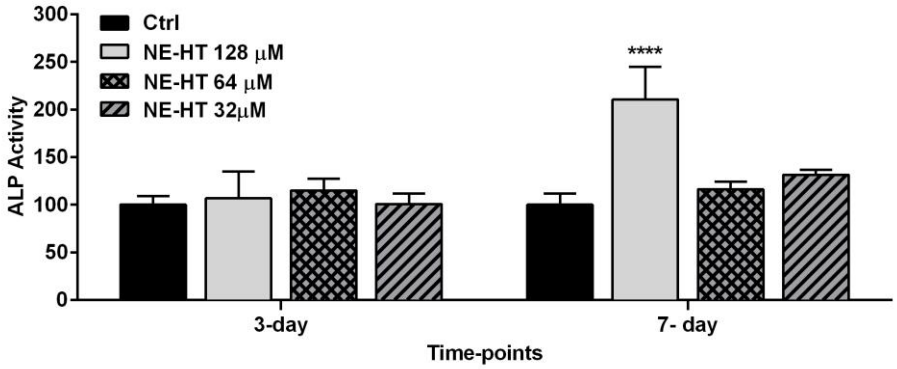


Figure 9. Alkaline Phosphatase activity at the periods of 3 and 7 days after the treatment of osteoblasts Saos-2-like with HT-NE 128 µM, 64 µM and 32 µM. * represents statistically significant difference compared to the control group (ANOVA, ****p<0.0001).

3. Discussion

Bone diseases are a recurring problem in the general population, being presented in diverse ways leading to mass bone loss [1]. New treatments are studied to solve this problem with less collateral effect and good efficacy [27]. In this way, we hypothesize that a new formulation of HT-NE may permit a controlled release of hesperitin and provide safe treatment techniques, besides of enhance its biocompatibility and biological activities. The focus of the present study was to develop a new HT-NE and characterized through the evaluation of droplet size, polydispersity index, and zeta potential to guarantee its colloidal stability and its correct function. Additionally, we investigated its effects on bone metabolism through different analyses in human osteoblasts Saos-2-like, such as cell proliferation, calcium nodule formation, genes expression, collagen production and ALP activity.

A droplet size smaller than 100 nm can reduce the interfacial tension between the oil phase and water, preventing the droplets' coalescence and maintaining their disposition and distribution [26]. Additionally, nanoemulsions with small droplet size present trans-lucence or slight turbidity [28] as observed in our formulations. Regarding the PDI, values less than 0.5 are ideal to obtain a good dispersion [29]. Taking this into account, the new HT-NE development in the present study showed excellent parameters with mean values of PDI of 0.267 +- 0.008 during 21 days. In a previous study by Bosly [30], in 2022, it was prepared a nanoemulsion using a high sonication method to obtain nanoparticles, similar to our study. The authors confirmed the

homogeneity, stability, and adequate particle distribution of the nanoemulsion with a PDI of 0.342, that was optimized, acquiring a PDI of 0.263 [29]. Our result of 90 days of characterization shows a PDI of 0.584, which did not mean an ideal index, but is closer to the expected, besides that it can show the stability of the formulation in smaller periods.

The zeta potential is another important characteristic to the development of a new nanoemulsion because it will generate the necessary repulsive potential to prevent the coalescence of the droplets [31]. Besides that, the zeta potential will affect directly the nanoemulsion capacity of permeability in cell membranes, since these ones are negatively charged, and request a specific cationic and anionic balance to allow biocompatibility, in order to reach this biocompatibility as better as possible, the ideal values of zeta potential should be kept between -30 mV and 30 mV [32]. Our HT-NE presented values of zeta potential between 20 mV and -20 mV, showing this way an interesting potential to stability of the formulation and promotion of the biocompatibility.

To evaluate the amount of the main compound present into the HT-NE, the encapsulation efficiency was performed. Our results showed an encapsulation efficiency of 74 ± 5.33% demonstrating the great capacity of the nanoemulsion to carry the HT. Similar results of encapsulation efficiency were shown by Duranoğlu et al., 2018 [33], who developed a nanoparticle system using HT and obtained an encapsulation efficiency of 80 ± 4.9%. Although, they obtained a minimum particle size of 260.2 ± 16.5 nm, showing that our HT-NE allowed optimization nanoparticle system, maintaining a good encapsulation and droplet size. In addition, it was demonstrated that the use of polysorbate 80 as a surfactant, enables an encapsulation efficiency of 80.7 ± 7.15% and satisfactory results of characterization [34].

Transmission Electron Microscopy (TEM) is commonly used in nanoparticles formulations to show the surface morphology of droplets, being able the observation of droplet size [35]. The images obtained by TEM, show the morphology of HT-NE and Blank NE droplets, making possible the comparison between them. The small size of the droplets is an important parameter that can avoid their junction [35]. Opposite to this, when they are bigger than they should be, it will prejudice the capacity of the nanoemulsion to exercise its functions, losing its stability [35]. Therefore, it is possible to observe in TEM images that our formulation kept a size around 100 nm, maintaining its dispersion. Then, this can contribute to the preservation of its functions.

In terms of possible clinical use, the cytotoxicity test is extremely important for the evaluation of new therapeutic drug delivery systems. Thus, the cytotoxicity of our new HT-NE was evaluated on osteoblasts Saos-2, which are important cells present in bone metabolism. The cells exposed for 7 days to the HT-NE and Free HT, showed a concentration-dependent effect. Our HT-NE has a similar biocompatibility compared with Free HT, which is an important observation since the nanoemulsion kept the biocompatibility having a more complex composition, responsible for bringing more benefits as a differential delivery of drugs and a controlled release, avoiding compound oxidation and other destructive processes [35,36].

Our formulation did not present cytotoxic effects in concentrations less than 256 µM, showing interesting enhancement of the biocompatibility when compared with Xue et al. [37] in 2017, which showed an inhibition of pre-osteoblastic cell proliferation with HT treatment at 100 µM. Also, Blank NE presents a non-cytotoxic effect in concentrations less than 512 µM. In contrast, the Blank NE developed by Vaz et al. [38], was formulated similarly to ours, using a high-energy method, but using castor oil instead of capric/caprylic acid. The authors showed a reduction of nasal cavity cell viability (around 80%) with the Blank NE at 131 µM [38]. Since there are not many studies showing osteoblasts and nanoemulsions biocompatibility, we can suggest that the new formulation and its chemicals' combination developed in the present study showed excellent parameters of biocompatibility.

Osteoblasts cells have a very important role in bone formation and differentiation [39]. The evaluation of cell proliferation is an important marker to show if our formulation has a good effect on the cells that are being treated and confirm its biocompatibility [39]. An enhancement in osteoblast cell metabolism can offer the possibility of the positive effect of HT-NE on bone metabolism. There are few studies regarding encapsulating in a nanosystem and evaluation of the biological properties of the HT using osteoblasts test models, and none of them evaluated the cell proliferation. On the other hand, Trzeciakiewicz et al., in 2009 [21] showed in their study that the Free HT did not affect the primary rat osteoblasts proliferation in the highest non-cytotoxic concentration (10 µM). This study corroborates with the hypothesis that the encapsulation of HT in a nanoemulsion, can enhance its properties.

In osteogenesis, many processes are involved from the beginning of formation until the maturation of bone. Between them, an important step is the calcium nodule mineralization by osteoblasts, which generates the production of hydroxyapatite, the

inorganic compound responsible for bone formation [40]. Our formulation showed an increase of the mineralization in all concentrations evaluated, which contrast with a previous study that tested the HT in its free form and did not find this effect [21]. A previous study, evaluated the influence of HT and a HT nanocrystal in calcium nodule formation after 3 weeks of treatment [41]. The authors showed an increase on nodule formation after the treatment with HT and a better effect by HT nanocrystals [41]. These previous results are in according with ours, because we demonstrated that HT-NE was also able to promote this increase, but in a shorter period (14 days), giving the hypothesis of the acceleration of this function.

On the other hand, the alkaline phosphatase (ALP) activity had correlation with nodules mineralization [42], because the increase of alkaline phosphatase is essential to the beginning of nodule formation. In the present study, at the period of 7 days, the HT-NE presents a great effect in the increase of ALP activity and shows the nodule formation increase. Trzeciakiewicz et al. [24] showed that the ALP activity of Free HT was highly expressed at 14 days of treatment in primary rat osteoblasts. Our results are in according with these authors, because Free HT and HT encapsulated in a NE can express high levels of ALP in early periods of mineralization.

Bone-specific gene expression patterns are highly involved in bone formation process, preceding the osteoblasts differentiation, or during the maturation and proliferation of these cells. [43-46]. Runx2 is one of the first transcription factors expressed in mesenchymal cell, leading to osteoblast formation and maturation [43,44]. The ALPL (alkaline phosphatase) gene is responsible for bone maturation keeping its density and avoiding dysplasia [45] and TGF-β, is a gene responsible for cell survivor, proliferation and differentiation, leading to bone growth through BMP9 signaling pathway [47]. Their roles in bone metabolism elucidate the understanding about our formulation action. Similar to our results, Trzeciakiewicz et al. [21], showed a time-dependent effect of HT in the expression of genes Runx2 and ALPL using primary rat osteoblasts. On the other hand, it was shown that the deficiency of TGF-β in mouses led to a reduction of bone growth and mineralization [48]. Regarding the effect of HT or a new nanoemulsion associated to HT on TGF-β expression, we demonstrate for the first time the positive effect of this flavonoid on this gene expression.

Finally, collagen is an essential protein to bone composition. The collagen fiber associated with the hydroxyapatite are responsible for bone formation, being capable

of elevating bone mass [49]. For this reason, many biocompatible materials are being developed aiming to enhance and increase collagen production [50]. As showed in the results section, at 7 days our formulation showed an increase in collagen production, corroborating the hypothesis that the HT-NE has a promising effect on osteogenesis, mainly in the beginning of osteoblasts maturation, allowing the enhance of bone density. Besides that, a study using the HT in its free form did not find an influence in collagen production [24]. Taking these results into account, the encapsulation of HT in our nanoemulsion improved its biological effect, specifically on collagen production.

Despite the limitations of the present study, new *in vitro* and *in vivo* models can provide other answers and complement our results to future medical applications. Besides that, we note that the HT-NE developed in the present study did not present a dose-dependent effect in our results. Then, there are still numerous questions about HT-NE and new studies are necessary to corroborate our findings.

4. Materials and Methods

Nanoemulsion (NE) Preparation

The formulations were prepared according to Bouchemal et al. [51] with modifications. The oil phase was prepared with soy phosphatidylcholine (Lipoid, Germany) (0.4% w/v), capric/caprylic acid triglycerides (KLK, Brazil) (1.8% w/v), polysorbate 80 (Deg, Brazil) (2.1% w/v), and 100 mM of hesperetin (HT) (Sigma-Aldrich, Brazil). The aqueous phase was composed of ultra-pure water that was added dropwise to the oil phase, resulting in a pre-emulsion. In sequence, this pre-emulsion was sonicated for 2 minutes in a sonicator (Qsonica, USA), with a pulse on of 1 min and pulse off for 30 seconds, and an amplitude of 35. The blank formulation was prepared as described, without the HT addition (Blank NE).

Droplet Size, Polydispersity Index, and Zeta Potential

The droplet size, polydispersity index, and zeta potential were obtained using the dynamic light scattering (DLS) technique, which was performed on Zetasizer equipment (Nano ZS, UK). The formulations were diluted in deionized water (1: 25), and the analysis of stability was performed in triplicate for 1, 7, 14, 21, and 90 days to

evaluate the hesperetin-loaded nanoemulsion (HT-NE). Additionally, an evaluation of 1 day was performed to the Blank NE.

Analytic Methodology for Hesperetin Quantification

The High-performance liquid chromatography (HPLC) analysis was performed in an HPLC Agilent 1220 infinity system (Agilent, USA) equipped with a column Synergy (150 x 4.6 mm, 4 µm). The volume of injection was 20 µL with a 1.0 mL/min flow. The mobile phase was constituted in acetonitrile/acidified water with acetic acid 0.1% (50:50, v/v). The detector was fixed at 290 nm for chromatogram acquisition.

To obtention of the analytical curve, a standard solution (2000 µg/mL) of HT in acetonitrile (J.T. Baker, USA) was prepared. The analytic curve was performed in triplicate at the following concentrations: 25, 75, 125, 250, 375, 500, and 750 µg/mL of HT in acetonitrile. Three independent analytic curves were created, and the linearity was obtained by linear regression using the minimum squares method.

Encapsulation Efficiency

The encapsulation efficiency of HT on the nanoemulsion was calculated by the difference between the mass of encapsulated HT and total HT mass. Briefly, 1 mL of the formulation was submitted to centrifugation of 14000 rpm for 30 min. A supernatant aliquot of 100 µL was added to a flask containing 900 µL of acetonitrile and sonicated (amplitude 35, during 1 min). The HT quantification was performed by HPLC as described previously.

Transmission Electron Microscopy (TEM)

The HT-NE was suspended in an aqueous solution and 3 µL were added to the copper grids. After 30 sec, the excess amount was dried with paper and colored with aqueous uranyl acetate (m/v) for 2 min. After that, the exceed of acetate was dried with paper, and the grids were placed at room temperature to dry. Finally, the grids were submitted to Transmission Electron Microscopy (JEOL JEM-2100, Japan) analysis (emission equipment LaB6) at 100 kV.

Cell Line Culture

Human Osteoblasts cell line Saos-2-like, purchased from American Type Culture Collection (ATCC hTB-85), were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 IU mL^{-1} penicillin, and 100 µg/mL^{-1} streptomycin (Gibco, USA). The cell culture was incubated at 37 °C in a humidified incubator containing 5% of CO_2.

Cytotoxicity Assay

Previously, HT ≥ 98% (Sigma-Aldrich, Brazil) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 M and stored at -20 °C to the obtention of desired concentrations. The final concentration of DMSO in the assay was less than 0,1% [52].

Human Osteoblasts cell line Saos-2-like were seeded in a 96-well plate ($5x10^4$ cell/well) and incubated for 24 h. Thereafter, the culture medium of the confluent cell monolayer was replaced by Free HT, HT-NE, Blank NE concentrations (8200 µM – 0.5 µM v/v), Camptothecin 10 µM (positive control group) and culture medium (negative control group). The plate was incubated for 7 days at 37 °C in a humidified atmosphere containing 5% of CO_2 and the spent medium was refreshed every 48 h. After 7 days, the cytotoxic effect was evaluated by methyl tetrazolium (MTT) (Sigma-Aldrich, EUA) assay.

The colorimetric analysis with MTT evaluates the cell metabolism through the cytochemical activity of the succinic dehydrogenase (SDH) enzyme, which represents the mitochondrial breath rate of viable cells [53]. Each well of the experimental and control groups received a solution containing 90 µL of culture medium and 10 µL of MTT solution, which was prepared with 5 mg of MTT powder and 1 mL of sterile Phosphate-buffered saline (PBS). The cells were incubated for 4 hours at 37 °C and 5% CO_2. Then, the culture medium with MTT was aspirated, and 100 µL of acidified isopropanol (HCl 0.04 N) was applied in each well. The cell viability was evaluated considering that cells with normal mitochondrial activity were colored with an intense violet. Optical density was read at 570 nm wavelength in a spectrophotometer (Synergy H1 Multi-Mode Reader-BioTek, EUA).

Finally, IC_{50} (half-maximum inhibitory concentration) is the concentration of the treatments at an inhibition ratio of 50%. Considering this, the IC_{50} values were

measured using the software GraphPad Prism, where the X values are the logarithm (log) of concentration and Y values are the log of C/C0 x 100%, where C are each concentrations' optical densities and C0 is the control group's optical density obtained by MTT assay.

Cell Proliferation Analysis

To analyze cell proliferation, osteoblasts Saos-2-like were seeded in 48 wells plates at $1x10^4$ cell/well. After 24 h the medium was refreshed with non-cytotoxic concentrations of HT-NE. The plate was incubated at 37 ºC and 5% CO_2 and the proliferative effect was evaluated after 1, 3, 5 and 7 days by Alamar Blue assay®, which is based on the capacity of metabolic active cells to convert the reagent in a fluorescent and colorimetric indicator. So, at the end of each period evaluated, the wells received the Alamar Blue® solution 10% diluted in culture medium and the plates were incubated for 4 h in dark conditions. After this time, an aliquot of 100 µL of each well was transferred to a new 96-well plate, to be read in a spectrophotometer (Synergy H1 Multi-Mode Reader-BioTek, EUA) with wavelengths of 570 nm and 600 nm.

Mineralized Nodules Formation

To evaluate the mineralized nodules deposited by the cells it was performed the alizarin red staining after 7 days of treatment. This way, the cells were seeded in 96-well plates, at 1×10^4 cell/well. After 24 h the culture medium was refreshed for a new one containing non-cytotoxic concentrations of HT-NE, previously prepared in osteogenic medium. While the control group just received culture medium. The medium was refreshed each 48 h. After 7 days, the attached cells were washed two times with Phosphate Buffer Solution (PBS) with a pH 7.2 and fixed with 100 µL ethanol 70% for 30 min. Then, each well was washed with distilled water two times and stained with 150 µL of alizarin red stain solution (40 mM, pH 4,2 - Sigma-Aldrich, Brazil). The plates were kept in contact with the stain solution for 20 min at room temperature and slow agitation (300 rpm) (VDRL Shaker, Biomixer, Brazil). Then, the wells were washed with distilled water until remove the excess of stain solution. The mineralized nodules were dissolved with 200 µL de cetylpyridine 10 % (Sigma-Aldrich, Brazil) for 15 min under slow agitation (300 rpm) and room temperature conditions. Then, the

absorbance was measured in a microplate reader (Synergy H1 Multi-Mode Reader-BioTek, EUA) at 562 nm.

Bone Metabolism Gene Expression Quantification

Cells were seeded in a 12-well plate, at 9 x 10^4 cells/well and treated with non-cytotoxic concentrations of HT-NE. After 1, 3, and 7 days, the cellular lysis and purification were performed using the RNeasy mini kit (Qiagen GmbH, Germany) following the manufacturer protocol. The RNAm were quantified using the Synergy H1 equipment (Biotek, USA) and RNAm of each sample (800 ng/mL) was transcript in cDNA using a thermocycler and the High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, USA), following the manufacturer's protocol.

The cDNAs obtained were used in real-time PCR reactions to determine the relative amount of Runx2 (Hs00231692_m1), ALPL (Hs01029144_m1), and TGF-β (Hs00248373_m1) genes. The relative abundance was measured by reverse transcription in real-time PCR (RT-qPCR) using Taqman, primer and specific probe (TaqMan Gene Expression Assays, Applied Biosystems, USA) in a thermocycler StepOne Plus Real–Time PCR System (Applied Biosystems, USA). The reactions were performed in 96-well plates with a 20 µL final volume, including Taqman Universal PCR Master Mix (Applied Biosystems, USA), Taqman Gene Expression Assay (Applied Biosystems, USA) to gene and model cDNA. The optimized thermocycler conditions were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The normalization was accomplished by the GAPDH (Hs02786624_g1) expression, which was not modified by experimental conditions. To compare the expression levels between the samples, the relative gene expression level was calculated using ΔΔCT comparative method with the thermocycler software.

Collagen Production by Picrosirius Red

After 3 and 7 days of treatment with non-cytotoxic concentrations of HT-NE, the supernatants were collected to evaluate the collagen quantification. Picrosirius Red was added to the samples in a 1:1 proportion, then they were kept in an Eppendorf ThermoMixer equipment at 400 rpm and 25 ºC for 1 h. After this period the samples were centrifuged at 12000 rpm and 25 ºC for 10 min. The supernatants were thrown

away and HCl 0,01 M was added to each sample, a new centrifugation was performed at 12000 rpm and 25 ºC for 10 min. Finally, the supernatants were thrown away, and the samples dissolved in NaOH 0,5 M. The absorbance was read in a spectrophotometer (Synergy H1 Multi-Mode Reader-BioTek, EUA) at 555 nm wavelength.

Alkaline Phosphatase Activity

After the periods of osteogenic induction, at 3 days and 7 days, the Alkaline Phosphatase (ALP) activity was measured using the Alkaline Phosphatase kit (ABCAM, USA) following the manufacturer's instructions. Briefly, the samples and the substrate of pNPP (p-nitrophenyl phosphate) were mixed and reacted for 60 min, then the reaction was interrupted with the Stop solution. The absorbance was read in a spectrophotometer (Synergy H1 Multi-Mode Reader-BioTek, EUA) with a wavelength of 405 nm and the ALP activity was calculated based on the standard curve graph.

5. Conclusions

The results obtained indicate excellent parameters of characterization and promisor biological performance of the novel HT-NE. The new biocompatible formulation allowed the incorporation of HT without a cytotoxic effect. Besides that, the present results shows that the HT-NE can enhancing the HT properties, allowing an increase or acceleration of its effects on bone metabolism, through the increase of cell proliferation, calcium nodule formation, regulation of important genes to bone metabolism, ameliorate the collagen production and ALP activity. This way, we consider that this new biomaterial can be used as a promising treatment for bone diseases.

Supplementary Materials: All materials are included in the main text.

Author Contributions: Conceptualization, Maria Júlia Mancim-Imbriani, Marlus Chorilli, Denise Spolidorio and Patricia Milagros Maquera-Huacho; Funding acquisition, Maria Júlia Mancim-Imbriani, Letícia Durão, Denise Spolidorio and Patricia Milagros Maquera-Huacho; Investigation, Maria Júlia Mancim-Imbriani, Jonatas

Duarte, Letícia Durão and Patricia Milagros Maquera-Huacho; Methodology, Maria Júlia Mancim-Imbriani, Jonatas Duarte, Leonardo Di Filippo, Letícia Durão and Patricia Milagros Maquera-Huacho; Resources, Marlus Chorilli and Denise Spolidorio; Supervision, Denise Spolidorio and Patricia Milagros Maquera-Huacho; Validation, Maria Júlia Mancim-Imbriani, Jonatas Duarte, Leonardo Di Filippo, Letícia Durão and Patricia Milagros Maquera-Huacho; Writing – original draft, Maria Júlia Mancim-Imbriani, Jonatas Duarte, Leonardo Di Filippo and Patricia Milagros Maquera-Huacho; Writing – review & editing, Maria Júlia Mancim-Imbriani, Marlus Chorilli, Denise Spolidorio and Patricia Milagros Maquera-Huacho.

Funding: This research was funded by São Paulo Research Foundation, grant numbers 2020/14155-0, 2021/05881-1, 2018/16540-8 and 2021/15178-6.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors would like to thank São Paulo Research Foundation for financial support to the research. Master's degree scholarship for Maria Júlia Mancim Imbriani (grant #2020/14155-0, São Paulo Research Foundation (FAPESP)). Scientific initiation scholarship for Letícia Pereira Lima Durão (grant #2021/15178-6, São Paulo Research Foundation (FAPESP)). Post-Doctoral fellowship for Patricia Milagros Maquera Huacho (grant #2018/16540-8, São Paulo Research Foundation (FAPESP)). Additionally, a research scholarship for the Laboratory of Microbiology and Molecular Biology, Department of Physiology and Pathology, School of Dentistry of Araraquara – UNESP (grant #2021/05881-1, São Paulo Research Foundation (FAPESP)).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Yu B, Wang CY. Osteoporosis and periodontal diseases - An update on their association and mechanistic links. Periodontol 2000. 2022;89(1):99-113. doi: 10.1111/prd.12422.
2. Karaguzel G, Holick MF. Diagnosis and treatment of osteopenia. Rev Endocr Metab Disord. 2010;11(4):237-51. doi: 10.1007/s11154-010-9154-0.
3. Izzotti A, Menini M, Pulliero A, et al. Biphosphonates-associated osteonecrosis of the jaw: the role of gene-environment interaction. J Prev Med Hyg. 2013;54(3):138-45.
4. De Jong WH, Borm PJ. Drug delivery and nanoparticles: applications and hazards. Int J Nanomedicine. 2008;3(2):133-49. doi: 10.2147/ijn.s596.
5. Dos Santos Ramos MA, Dos Santos KC, da Silva PB, et al. Nanotechnological strategies for systemic microbial infections treatment: A review. Int J Pharm. 2020;589:11978. doi: 10.1016/j.ijpharm.2020.119780.
6. Lotfipour F, Abdollahi S, Jelvehgari M, Valizadeh H, Hassan M, Milani M. Study of antimicrobial effects of vancomycin loaded PLGA nanoparticles against enterococcus clinical isolates. Drug Res (Stuttg). 2014;64(7):348-52. doi: 10.1055/s-0033-1358747.
7. Bonifácio BV, Silva PB, Ramos MA, Negri KM, Bauab TM, Chorilli M. Nanotechnology-based drug delivery systems and herbal medicines: a review. Int J Nanomedicine. 2014;9:1-15. doi: 10.2147/IJN.S52634.
8. Souza ALR, Kiill CP, Santos FK, da Luz GM, Silva HR, Chorilli M, Gremiao MPD. Nanotechnology-based drug delivery systems for dermato mycosis treatment. Curr. Nanosci. 2012;8:512–519. doi: 10.2174/157341312801784311.
9. Sato MR, da Silva PB, de Souza RA, dos Santos KC, Chorilli M. Recent advances in nanoparticle carriers for coordination complexes. Curr Top Med Chem. 2015;15(4):287-97. doi: 10.2174/1568026615666150108145614.
10. Duarte JL, Maciel de Faria Motta Oliveira AE, Pinto MC, Chorilli M. Botanical insecticide–based nanosystems for the control of Aedes (Stegomyia) aegypti larvae. Environ Sci Pollut Res. 2020;28737–28748. https://doi.org/10.1007/s11356-020-09278-y.

11. Pandey P, Gulati N, Makhija M, Purohit D, Dureja H. Nanoemulsion: A novel drug delivery approach for enhancement of bioavailability. Recent Pat Nanotechnol. 2020;14(4):276-293. doi: 10.2174/1872210514666200604145755.
12. Chen LH, Cheng LC, Doyle PS. Nanoemulsion-loaded capsules for controlled delivery of lipophilic active ingredients. Adv Sci (Weinh). 2020;7(20):2001677. doi: 10.1002/advs.202001677.
13. Cassidy A, Minihane AM. The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. Am J Clin Nutr. 2017;105(1):10–22. doi: 10.3945/ajcn.116.136051.
14. Muhammad T, Ikram M, Ullah R, Rehman SU, Kim MO. Hesperetin, a citrus flavonoid, attenuates LPS-induced neuroinflammation, apoptosis and memory impairments by modulating TLR4/NF-κB signaling. Nutrients. 2019;11(3):648. doi 10.3390/nu11030648.
15. Nones J, E-Spohr TC, Gomes FC. Hesperidin, a flavone glycoside, as mediator of neuronal survival. Neurochem Res. 2011;36(10):1776-84. doi: 10.1007/s11064-011-0493-3.
16. Ahmad S, Alam K, Hossain MM, et al. Anti-arthritogenic and cardioprotective action of hesperidin and daidzein in collagen-induced rheumatoid arthritis. Mol Cell Biochem. 2016;423(1-2):115-127. doi: 10.1007/s11010-016-2830-y.
17. Mamun MA, Hosen MJ, Khatun A, Alam MM, Al-Bari MAA. Tridax procumbens flavonoids: a prospective bioactive compound increased osteoblast differentiation and trabecular bone formation. Biol Res. 2017;50(1):28. doi: 10.1186/s40659-017-0134-7.
18. Nobakht M, Trueman SJ, Wallace HM, Brooks PR, Streeter KJ, Katouli M. Antibacterial properties of flavonoids from kino of the eucalypt tree, Corymbia torelliana. Plants (Basel). 2017;6(3):39. doi: 10.3390/plants6030039.
19. Rothwell JA, Knaze V, Zamora-Ros R. Polyphenols: dietary assessment and role in the prevention of cancers. Curr Opin Clin Nutr Metab Care. 2017;20(6):512-21. doi: 10.1097/MCO.0000000000000424.
20. Ortiz AC, Fideles SOM, Reis CHB, et al. Therapeutic effects of citrus flavonoids neohesperidin, hesperidin and its aglycone, hesperetin on bone health. Biomolecules. 2022;12(5):626. doi: 10.3390/biom12050626.

21. Trzeciakiewicz A, Habauzit V, Mercier S, et al. Hesperetin stimulates differentiation of primary rat osteoblasts involving the BMP signalingng pathway. J Nutr Biochem. 2010;21(5):424-31. doi: 10.1016/j.jnutbio.2009.01.017.
22. Xue D, Chen E, Zhang W, Gao X, Wang S, Zheng Q. The role of hesperetin osteogenesis of human mesenchymal stem cells and its function in bone regeneration.Oncotarget. 2017;8(13):21031-21043. doi: 10.18632/oncotarget.15473.
23. Kim SY, Lee JY, Park YD, Kang KL, Lee JC, Heo JS. Hesperetin alleviates the inhibitory effects of high glucose on the osteoblastic differentiation of periodontal ligament stem cells. PLoS One. 2013;8(6):e67504. doi: 10.1371/journal.pone.0067504.
24. Trzeciakiewicz A., Habauzit V., Mercier S., et al. Molecular mechanism of hesperetin-7-O-glucuronide, the main circulating metabolite of hesperidin, involved in osteoblast differentiation. J. Agric. Food Chem. 2009;58:668–675. doi: 10.1021/jf902680n.
25. Liu L, Zheng J, Yang Y, Ni L, Chen H, Yu D. Hesperetin alleviated glucocorticoid-induced inhibition of osteogenic differentiation of BMSCs through regulating the ERK signaling pathway. Med Mol Morphol. 2021;54(1):1-7. doi: 10.1007/s00795-020-00251-9.
26. Ali M, Khan NR, Subhan Z, et al. Novel curcumin-encapsulated α-tocopherol nanoemulsion system and its potential application for wound healing in diabetic animals. Biomed Res Int. 2022;2022:7669255. doi: 10.1155/2022/7669255.
27. Lin Z, Zheng K, Zhong J, Zheng X. Advances in microneedle-based therapy for bone disorders. Biomed Pharmacother. 2023;165:115013. doi: 10.1016/j.biopha.2023.115013.
28. McClements DJ. Advances in edible nanoemulsions: Digestion, bioavailability, and potential toxicity. Prog Lipid Res. 2021;81:101081. doi: 10.1016/j.plipres.2020.101081.
29. Luo W, Ju M, Liu J, Algharib SA, Dawood AS, Xie S. Intelligent-responsive enrofloxacin-loaded chitosan oligosaccharide-sodium alginate composite core-shell nanogels for on-demand release in the intestine. Animals (Basel). 2022; 12(19):2701. doi: 10.3390/ani12192701.

30. Bosly HAE. Evaluation of larvicidal enhanced activity of sandalwood oil via nano-emulsion against Culex pipiens and Ades aegypti. Saudi J Biol Sci. 2022; 29(12):103455. doi: 10.1016/j.sjbs.2022.103455.
31. Kelmann RG, Kuminek G, Teixeira HF, Koester LS. Carbamazepine parenteral nanoemulsions prepared by spontaneous emulsification process. Int J Pharm. 2007; 342(1-2):231-9. doi: 10.1016/j.ijpharm.2007.05.004.
32. Clogston JD, Patri AK. Zeta potential measurement. Methods Mol Biol. 2011; 697:63-70. doi: 10.1007/978-1-60327-198-1_6.
33. Duranoğlu D, Uzunoglu D, Mansuroglu B, Arasoglu T, Derman S. Synthesis of hesperetin-loaded PLGA nanoparticles by two different experimental design methods and biological evaluation of optimized nanoparticles. Nanotechnology. 2018; 29(39):395603. doi: 10.1088/1361-6528/aad111.
34. García-Melero J, López-Mitjavila JJ, García-Celma MJ, Rodriguez-Abreu C, Grijalvo S. Rosmarinic acid-loaded polymeric nanoparticles prepared by low-energy nano-emulsion templating: formulation, biophysical characterization, and *in vitro* studies. Materials (Basel). 2022; 15(13):4572. doi: 10.3390/ma15134572.
35. Gupta A, Eral HB, Hatton TA, Doyle PS. Nanoemulsions: formation, properties and applications. Soft Matter. 2016; 12(11):2826-41. doi: 10.1039/c5sm02958a.
36. Rashed MMA, You L, Ghaleb ADS, Du Y. Two-phase extraction processes, physicochemical characteristics, and autoxidation inhibition of the essential oil nanoemulsion of citrus Reticulata blanco (tangerine) leaves. Foods. 2022; 12(1):57. doi: 10.3390/foods12010057.
37. Xue D, Chen E, Zhang W, et al. The role of hesperetin osteogenesis of human mesenchymal stem cells and its function in bone regeneration. Oncotarget. 2017;8(13):21031-21043. doi: 10.18632/oncotarget.15473.
38. Vaz G, Clementino A, Mitsou E, et al. *In vitro* Evaluation of Curcumin- and Quercetin-Loaded Nanoemulsions for Intranasal Administration: Effect of Surface Charge and Viscosity. Pharmaceutics. 2022;14(1):194. doi: 10.3390/pharmaceutics14010194.
39. Bagherpour I, Yaghtin A, Naghib SM, Molaabasi F. Synthesis and investigation on microstructural, mechanical features of mesoporous hardystonite/reduced graphene oxide nanocomposite for medical applications. Front Bioeng Biotechnol. 2023;11: 1073435. doi: 10.3389/fbioe.2023.1073435.

40. Meng S, Zhang Z, Rouabhia M. Accelerated osteoblast mineralization on a conductive substrate by multiple electrical stimulation. J Bone Miner Metab. 2011;29(5):535-44. doi: 10.1007/s00774-010-0257-1.
41. Alipour M, Sharifi S, Samiei M, Shahi S, Aghazadeh M, Dizaj SM. Synthesis, characterization, and evaluation of Hesperetin nanocrystals for regenerative dentistry. Sci Rep. 2023;13(1):2076. doi: 10.1038/s41598-023-28267-y.
42. Bellows CG, Aubin JE, Heersche JN. Initiation, and progression of mineralization of bone nodules formed *in vitro*: the role of alkaline phosphatase and organic phosphate. Bone Miner. 1991;14(1):27-40. doi: 10.1016/0169-6009(91)90100-e.
43. Kushwaha AC, Mohanbhai SJ, Sardoiwala MN, Jaganathan M, Karmakar S, Roy Choudhury S. Nanoemulsified Genistein and Vitamin D Mediated Epigenetic Regulation to Inhibit Osteoporosis. ACS Biomater Sci Eng. 2022;8(9):3810-3818. doi: 10.1021/acsbiomaterials.2c00165.
44. Komori T. Runx2, an inducer of osteoblast and chondrocyte differentiation. Histochem Cell Biol. 2018;149(4):313-323. doi: 10.1007/s00418-018-1640-6.
45. Meyer MB, Benkusky NA, Pike JW. The RUNX2 cistrome in osteoblasts: characterization, down-regulation following differentiation, and relationship to gene expression. J Biol Chem. 2014;289(23):16016-31. doi: 10.1074/jbc.M114.552216.
46. Liu W, Zhang L, Xuan K, et al. Alpl prevents bone ageing sensitivity by specifically regulating senescence and differentiation in mesenchymal stem cells. Bone Res. 2018;6:27. doi: 10.1038/s41413-018-0029-4.
47. Yao H, Zou Y, Yang K, Yin L, Liu Y, Li R. TGFβ1 induces bone formation from BMP9-activated Bone Mesenchymal Stem Cells, with possible involvement of non-canonical pathways. Int J Med Sci. 2020;17(12):1692-1703. doi: 10.7150/ijms.45786.
48. Geiser AG, Hummel CW, Draper MW, et al. A new selective estrogen receptor modulator with potent uterine antagonist activity, agonist activity in bone, and minimal ovarian stimulation. Endocrinology. 2005;146(10):4524-35. doi: 10.1210/en.2005-0024.
49. Ambroszkiewicz J, Gajewska J, Mazur J, et al. Dietary Intake and Circulating Amino Acid Concentrations in Relation with Bone Metabolism Markers in Children Following Vegetarian and Omnivorous Diets. Nutrients. 2023;15(6):1376. doi: 10.3390/nu15061376.

50. Huang Y, Wang J, Yang F, Shao Y, Zhang X, Dai K. Modification and evaluation of micro-nano structured porous bacterial cellulose scaffold for bone tissue engineering. Mater Sci Eng C Mater Biol Appl. 2017;75:1034-1041. doi: 10.1016/j.msec.2017.02.174
51. Bouchemal K, Briançon S, Perrier E, Fessi H. Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimization. Int J Pharm. 2004;280(1-2):241-51. doi: 10.1016/j.ijpharm.2004.05.016.
52. Kara S, Gencer B, Karaca T, et al. Protective effect of hesperetin and naringenin against apoptosis in ischemia/reperfusion-induced retinal injury in rats. ScientificWorldJournal. 2014;2014:797824. doi: 10.1155/2014/797824.
53. Huacho PMM, Nogueira MNM, Basso FG, Jafelicci Junior M, Francisconi RS, Spolidorio DMP. Analyses of Biofilm on Implant Abutment Surfaces Coating with Diamond-Like Carbon and Biocompatibility. Braz Dent J. 2017;28(3):317-323. doi: 10.1590/0103-6440201601136.

3.2 Publicação 2*

Effects of hesperetin and hesperetin-loaded nanoemulsion on osteoblast behavior associated with electrical stimulation

Maria Júlia Mancim Imbriani[1,2,3], Atieh Abedin-Doo[1,4], Ze Zhang[4], Denise Madalena P. Spolidorio[2], Patricia Milagros Maquera-Huacho[1,2], Mahmoud Rouabhia[1*]

[1]Groupe de Recherche en Écologie Buccale, Faculté de Médecine Dentaire Université Laval, Quebec, QC, Canada.

[2]Department of Physiology and Pathology, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

[3]Department of Diagnosis and Surgery, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

[4]Axe Médecine Régénératrice Centre de Recherche du CHU de Québec Département de Chirurgie Faculté de Médecine, Université Laval, Quebec, QC, Canada.

Running title: Hesperetin and osteoblasts behavior

*Corresponding author: Dr Mahmoud Rouabhia, Faculté de médecine dentaire, Université Laval
2420 de la Terrasse, Québec (Québec), Canada G1V 0A6
Email: mahmoud.rouabhia@fmd.ulaval.ca

* Artigo seguiu as normas da revista *Tissue Engeneering and Regenerative Medicine* para a qual pretende-se submeter.

ABSTRACT

The benefic effects of Hesperetin (HT) and the advantages of nanoemulsions proved that a hesperetin-loaded nanoemulsion (HT-NE) could enhance the HT effects on bone metabolism. The electrical stimulation (ES) showed positive effects on cellular behavior. This way, an association between HT and HT-NE with ES can bring good perspectives that can enhance the effects of each other. Then, the objective of this study was to evaluate the effects of HT and HT-NE at concentrations of 32 µM, 64 µM and 128 µM associated or not with ES at 50 mV/mm² on osteoblasts behavior, measuring cell proliferation, cell adhesion, alkaline phosphatase (ALP) activity, and cell migration. The results showed that the HT and HT-NE presented the enhancement of all analyzed parameters, being better the results provided from HT-NE, and bolt of them didn't avoid cell adhesion on the membranes, generating a protective effect on high levels of ES, and enhancing osteoblasts behavior. Our results showed positive effects of this unprecedented association, being able new approaches to their synergistic effect on bone, leading to the opportunity for the execution of new studies *in vitro*, becoming deeper the investigations about the effect of HT and HT-NE with ES in bone formation.

Keywords: Flavonoids. Nanoparticles. Electrical Stimulation. Osteoblasts.

INTRODUCTION

Hesperetin (HT) is a metabolite of hesperidin, belonging to the flavanone subgroup, found mainly in citric fruits like orange and grapefruit (Muhammad et al., 2019). Several studies suggested different biological properties of HT, including analgesic, anti-cancer, anti-inflammatory, antioxidant, and neuroprotector effects (Nones et al., 2011; Ahmad et al., 2016; Mamun et al., 2017; Nobakht et al., 2017; Rothwell et al., 2017). Furthermore, available studies showed the benefic effects of hesperetin on osteogenesis by regulating and increasing the expression of genes that regulate bone metabolism, like runt-related transcription factor 2 (RUNX2), OCN, COL1A1, and ALP activity (Cassidy et al., 2017; Xue et al., 2017). Although studies support the hypothesis that hesperetin has positive effects on bone metabolism, its mechanism of action is still poorly understood, and its effect on osteoblasts needs more evidence. Indeed, gene activation may not lead to biological functions such as cell growth, phosphatase alkaline secretion, etc. Thus, additional studies on the interaction between HT and osteoblasts are needed. These studies can be performed by using HT directly or through nanoemulsion.

Nanoemulsions are oil in water or water in oil dispersions of two immiscible liquids stabilized through surfactants to form a single phase (Carvalho et al., 2019; Sabjan et al., 2020; Singh et al., 2017). They can be presented in different forms, like liquids, gel, creams, and aerosols, among others, enabling different application forms, besides they can present nasal, intravenous, pulmonary, and ocular administration (Singh et al., 2017). Nanoemulsion presents a good drug delivery system, controlling the release and directing it to its active site (Pandey et al., 2020). Furthermore, oil droplets can solubilize lipophilic pharmaceutical compounds, protecting them against external factors like pH, oxidation, and hydrolysis. Nano-emulsion is resistant to flocculation and coalescence (Chen et al., 2017). These factors and the biggest area of active surface for droplets volume have allowed a longer life cycle and better availability of the active ingredients incorporated (Chen et al., 2017).

A hesperetin-loaded nanoemulsion (HT-NE) showed colloidal stability and excellent characteristics that allow good encapsulation efficiency and avoid its coalescence and flocculation, besides present biocompatibility with osteoblasts (Mancim-Imbriani et al., 2023 –). Our previous study showed that the HT-NE could enhance the activity of hesperetin in bone metabolism, enhancing or accelerating its

effects on osteoblasts proliferation, calcium nodule formation, the expression of important genes to bone metabolism, collagen production, and alkaline phosphatase activity (Mancim-Imbriani et al., 2023).

Electrical stimulation (ES) has been shown to have good effects on cellular activities such as ion transportation, gene expression, production of proteins, intracellular reactive oxygen species, secretion of chemokines and cytokines, neurite growth, and cell migration (Meng et al., 2021). A previous study showed that ES could positively affect wound healing, regulating pro-inflammatory and anti-inflammatory cytokines and growth factors secretion in diabetics' human skin fibroblasts (Meng et al, 2011). Besides that, another study published by Rouabhia et al. (2020) showed that ES could promote skin keratinocyte viability and proliferation, increase growth factors secretion, and decrease pro-inflammatory cytokines secretion, like interleukin IL-6. Furthermore, ES exposure enhanced osteoblast adhesion and proliferation, and increased the mineral nodule formation and the expression of relevant genes in bone metabolism (Meng et al., 2011).

To became possible ES conduction through the cells, different membranes have been used (Rouabhia et al., 2020; Abedin-Do et al, 2021; Cui et al, 2021). Between them, the membranes of Polypyrrole (PPy) are usually used due to their biocompatibility (Cui et al., 2021). Using the PPy membrane another polymer can be combined, for example the poly(l-lactic) (PLLA), which provides flexibility to the membrane, becoming more resistant (Cui et al., 2021; Cui et al., 2021). Also, the use and combination of heparin into these models can increase the conductivity in aqueous environment as well as the cells adhesion (Meng et al., 2008).

Therefore, we have the hypothesis that the HT-NE associated with the ES can enhance the effects of each other, being a promising treatment for bone disorders.

METHODS

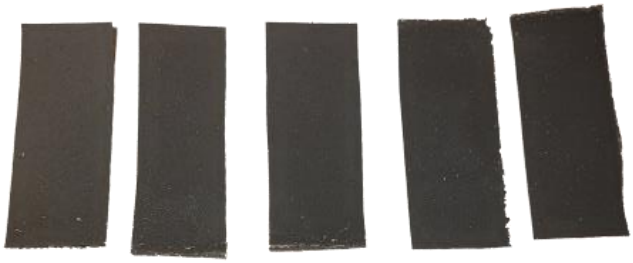
1. Cell Culture

MG63 (osteoblast-like cell line from human osteosarcoma - American Type Culture Collection, Manassas, VA, USA) were subcultured in 75 cm^2 culture flasks in Dulbecco's Modified Eagle's Medium with Ham's F-12 (Invitrogen, Burlington, ON, Canada) supplemented with 100 U/mL of penicillin and 100 U/mL of streptomycin (Schering, Pointe-Claire, QC, Canada), 250 mg/mL of amphotericin B (Sigma–Aldrich

Canada Ltd., Oakville, ON, Canada) and 10% fetal bovine serum (FBS) (Gibco, Burlington, ON, Canada). The medium was changed three times a week. When the cultures reached 90% confluence, the cells were detached from the flasks using a 0.25% trypsin solution, resuspended in 10% FBS-supplemented DMEM medium, and used for subsequent experiments.

2. Preparation of PPy/PLLA/HE Conductive Membranes

Polypyrrole (PPy) particles were formed in a water-in-oil emulsion containing pyrrole, $FeCl_3$, H_2O_2 and heparin (HE). This emulsion was maintained under vigorous stirring overnight at room temperature. The PPy/HE particles were collected, washed with methanol to remove the emulsifier and impurities, and mixed with a PLLA solution in chloroform at a 1:9 weight ratio (PPy:PLLA). The mixture was transferred into a glass petri dish and kept at room temperature (RT) in a fume hood to evaporate the chloroform, forming the PPy/HE/PLLA membranes. The membranes were then washed 5 × 2 h with deionized water and dried overnight at RT. The conductivity of the PPy/HE/PLLA membranes was assessed with a four-point probe, while the thickness was measured with a digital thickness gauge model MTG-DX2 (Rex Gauge, Buffalo Grove, IL, USA). The membranes were then cut into the appropriate size, mounted onto a homemade electrical tissue culture device (Figure. 1), and sterilized with 75% ethanol, followed by extensive washes with sterile PBS and culture medium before cell culture. The device's PPy/HE/PLLA membranes were assessed for conductivity using a digital multimeter (Keithley, Cleveland, OH, USA) (Abedin-Do et al., 2021).



Membranes cut into appropriate sizes.

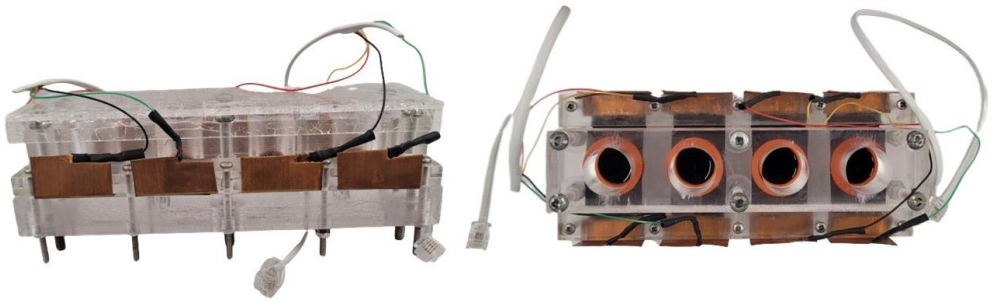


Figure 1. Electrical Stimulation homemade device.

3. Treatment and conditions

MG63 were seeded onto PPy/HE/PLLA membranes at $2x10^5$ cells per membrane and immediately treated and/or stimulated with 1) HT (32 µM, 64 µM, and 128 µM), 2) HT (32 µM, 64 µM, and 128 µM) and ES at 50 mV/mm, 3) HT-NE (32 µM, 64 µM, and 128 µM), 4) HT-NE (32 µM, 64 µM, and 128 µM) and ES at 50 mV/mm² , for 24 h to the evaluation of adhesion and the cell nucleus morphology in this period.

To cell proliferation and cell nucleus integrity evaluation the cells were seeded onto PPy/HE/PLLA membranes at $2x10^5$ cells per membrane for 24h, then they were treated and/or stimulated in the same conditions cited above for 24h.

4. Cells Adhesion Quantification and Nucleus Morphology

After 24 h, the cells adhesion was quantified using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Sigma, St. Louis, MO, USA) staining assay. This assay measures viable cells as a function of mitochondrial activity (Kabakov et al., 2018). The culture medium of each membrane was supplemented with 10% (v/v) of an MTS solution and incubated for 3 h at 37 °C in dark conditions. Then aliquots of 125 µL of each well were transferred from a new 96-well plate to be read in a spectrophotometer (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA) with wavelengths of 490 nm.

To evaluate the nucleus morphology, the cells were fixed with 4% paraformaldehyde, then washed 3 times with Phosphate Buffer Saline (PBS), and finally stained with Hoechst 33342 (Sigma-Aldrich, Oakville, ON, Canada) in distilled

water for 15 min at RT. After rinsing 3 times with PBS, the samples were observed under an epifluorescence light microscope (Axiophot, Zeiss, Oberkochen, Germany) and photographed.

5. Cell Proliferation Quantification

The proliferation of osteoblast cells was assessed using the MTS (Sigma, St. Louis, MO, USA) staining assay. The culture medium of each membrane was supplemented with 10% (v/v) of an MTS solution and incubated for 3 h at 37 °C in dark conditions. Then aliquots of 125 µL of each well were transferred from a new 96-well plate to be read in a spectrophotometer (Model 680, Bio-Rad Laboratories Inc., Hercules, CA, USA) with wavelengths of 490 nm.

6. Cell Nucleus Integrity Evaluation

After the proliferation assay by MTS, the osteoblasts were fixed with a 4% paraformaldehyde, then washed 3 times with Phosphate Buffer Saline (PBS), and finally stained with Hoechst 33342 (Sigma-Aldrich, Oakville, ON, Canada). in distilled water for 15 min at RT. After rinsing 3 times with PBS, the samples were observed under an epifluorescence light microscope (Axiophot, Zeiss, Oberkochen, Germany) and photographed.

7. Cell Migration

To evaluate cell migration, osteoblasts were seeded at $3x10^5$ in 6-well cell culture plates. When the cells became confluent after 4 days, a sterile 20–200 µL pipette tip was used vertically to scratch across each well, and the cells were treated with different concentrations of HT (32 µM, 64 µM, and 128 µM) or HT-NE (32 µM, 64 µM, and 128 µM) for 48 h. The images were acquired at 0, 6, 12, 18, 24, and 48 h. A camera performed the pictures of each well, and analysis of the open wound area was measured with the calculation of wound area (percentage) before and after 48h of treatment. Additionally, the cell activity during cell migration was evaluated as described above.

8. Alkaline Phosphatase (ALP) Activity

The supernatants of each well were collected from cell migration assay, and the ALP activity was analyzed by an Alkaline Phosphatase kit (Abcam, ab83369, Cambridge, UK) following the manufacturer's protocol. Briefly, the samples and pNPP

(p-nitrophenol Phosphate) substrate were mixed and reacted for 60 min; then, the reaction was interrupted with a stop solution. The plate was read at 405 nm of absorbance, and ALP was calculated based on the standard curve graphic.

RESULTS

1. Cells Adhesion Quantification and Nucleous Morphology

Figure 2 shows the quantitative analysis of cell adhesion after the treatment of the osteoblastic cells with HT or HT-NE (at 32 µM, 64 µM and 128 µM) and stimulated or not by ES at 50 mV/mm². The ES showed similar results to the control group, with a slight increase in the viable cell percentage (5%). HT did not affect the cell adhesion, enhancing this condition at 32 µM and 64 µM (increasing 7% the cell viability, $p<0.0001$). All concentrations of HT-NE (32 µM, 64 µM, and 128 µM) enhanced the adhesion of the cells to the PPy/HE/PLLA membranes, increasing the cell viability in 7%, 11% and 4%, respectively ($p<0.0001$ and $p<0.01$). The concentration of HT 32 µM associated with ES at 50 mV/mm² represented a great significant enhancement in cell adhesion, increasing the cell viability in 28 % ($p<0.0001$), while the interaction between the HT at 128 µM and ES at 50 mV/mm² presented a decrease of 5% ($p<0.01$) in cell viability and the HT at 64 µM kept the adhesion similar to the control group that only received ES. Finally, the interaction between ES and HT-NE at 32 µM and 64 µM ameliorated the adhesion by 16% ($p<0.0001$) and 4% ($p<0.001$), respectively, while the interaction with HT-NE at 128 µM, decrease this adhesion by 8% ($p<0.0001$).

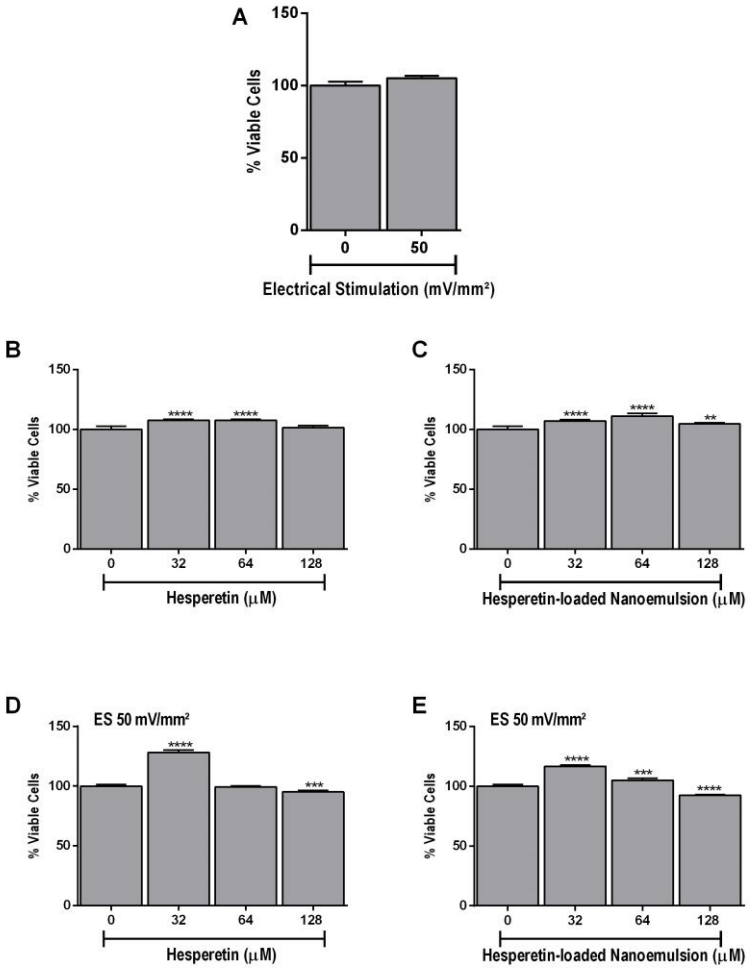


Figure 2. Quantitative analysis of adhesion by osteoblastic cells after the HT and HT-NE treatment with different concentrations (32 µM, 64 µM, and 128 µM) and ES at 50 mV/mm² after 24h of stimulus and treatment. (A) represents the stimulus with ES at 50 mV/mm², (B) represents the treatment with HT only, (C) represents the treatment with HT-NE only, (D) represents the treatment with HT and ES at 50 mV/mm², (E) represents the treatment with HT-NE and ES at 50 mV/mm. * Differences statistically significant compared with the control group (ANOVA, ***p*< 0.01 ****p* < 0.001 *****p*< 0.0001).

Figure 3 confirms the cell adhesion after treatment of the osteoblastic cells with HT or HT-NE (at 32 µM, 64 µM and 128 µM) and stimulated or not by ES at 50 mV/mm². Proving that the association of HT or HT-NE with ES still allows the adhesion of the cells to the membrane.

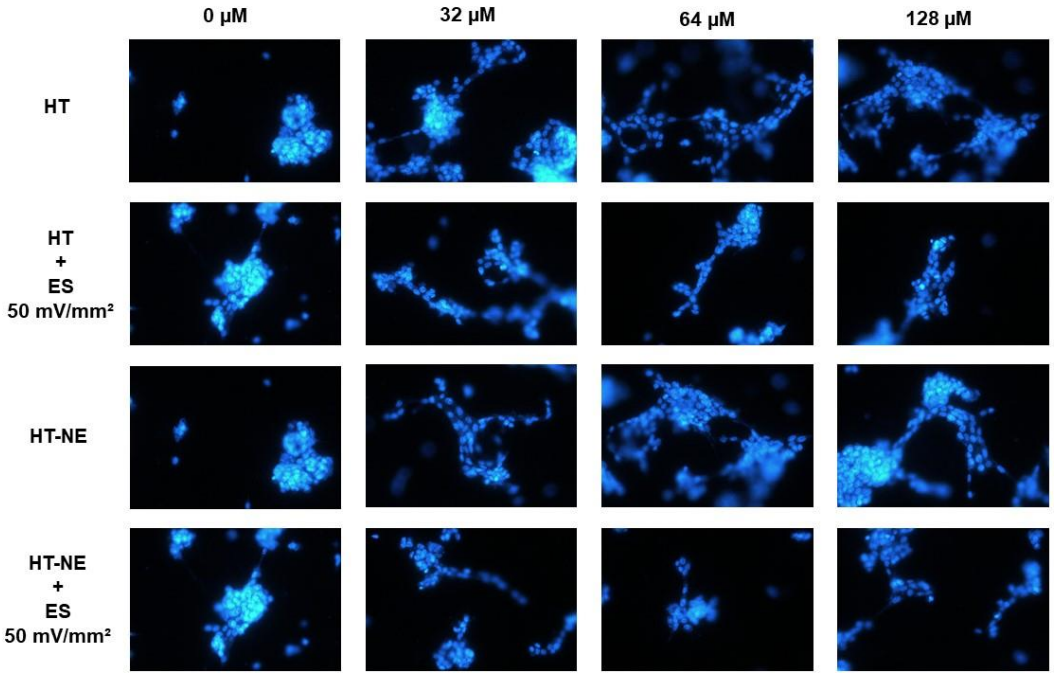


Figure 3. Adhesion of osteoblastic cells after the HT and HT-NE treatment with different concentrations (32 µM, 64 µM, and 128 µM) and ES at 50 mV/mm², representation of the qualitative analysis by images obtained on an epifluorescence light microscope with 10x magnificence.

2. Cell Proliferation Quantification

In Figure 4 the effect of HT or HT-NE associated or not with ES in the proliferation of osteoblastic cells seeded on PPy/HE/PLLA membranes is shown. The exposition of osteoblastic cells to ES at 50mV/mm² presented the enhancement on the cell proliferation, with percentages of increase 30% to ES at 50 mV/mm² (p<0.0001). The HT in all concentrations (32 µM, 64 µM, and 128 µM) increased cell proliferation in 28%, 32%, and 51%, respectively (p<0.0001). The effect of HT-NE in the proliferation of the osteoblastic cells is represented a significant enhancement in all concentrations, being them 64% to HT-NE at 32 µM, 45% to HT-NE at 64 µM and 74% to HT-NE at 128 µM (p<0.0001). Presenting this way, a statistically significant increase compared with the group that did not receive any treatment. The interaction between HT and ES generated an increase of cell proliferation in the medium (64 µM) and highest (128 µM) concentrations, the medium concentration led to an increase of 22% in viable cells, while the highest concentration increased the cell viability by 28% (p<0.05 and p<0.01). Finally, the interaction between HT-NE and ES also presented an enhancement in ploriferation. When exposed to 50mV/mm² of ES we noticed that

the lowest and medium concentrations of HT-NE (32 µM and 64 µM) increased 50% and 40%. In contrast, the highest concentration did not differ from the control group (p<0.0001).

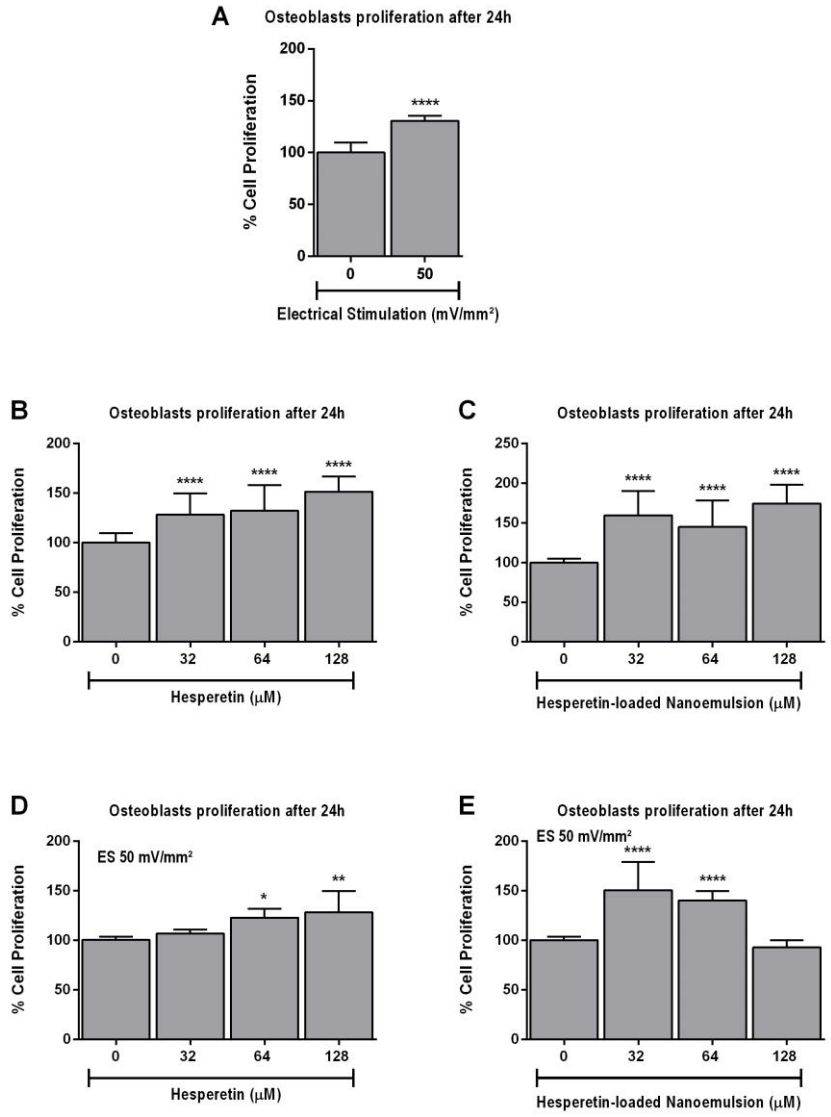


Figure 4. Quantitative analysis of osteoblasts MG63 proliferation after 24h with treatments of HT or HT-NE (32 µM, 64 µM, or 128 µM) and ES at 50 mV/mm². (A) The proliferation of osteoblasts stimulated with ES at 50 mV/mm², (B) Proliferation of osteoblasts treated with HT at 32 µM, 64 µM, or 128 µM, (C) Proliferation of osteoblasts treated with HT-NE at 32 µM, 64 µM, or 128 µM, (D) Proliferation of osteoblasts treated with HT at 32 µM, 64 µM, or 128 µM and ES at 50 mV/mm², (E) Proliferation of osteoblasts treated with HT-NE at 32 µM, 64 µM, or 128 µM and ES at 50 mV/mm², *Differences statistically significant compared to the control group. (ANOVA, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001).

In Figure 5 is the comparison of the proliferation between the groups. In Figures 5A and 5B, we can observe that the use of HT and HT-NE without association to ES promoted higher proliferation than when associated to ES. Additionally, the HT-NE presented a greater increase in cell proliferation than HT in its Free form (Figure 5C and 5D). Finally, the use of HT and HT-NE promoted an increase in cell proliferation compared with only ES.

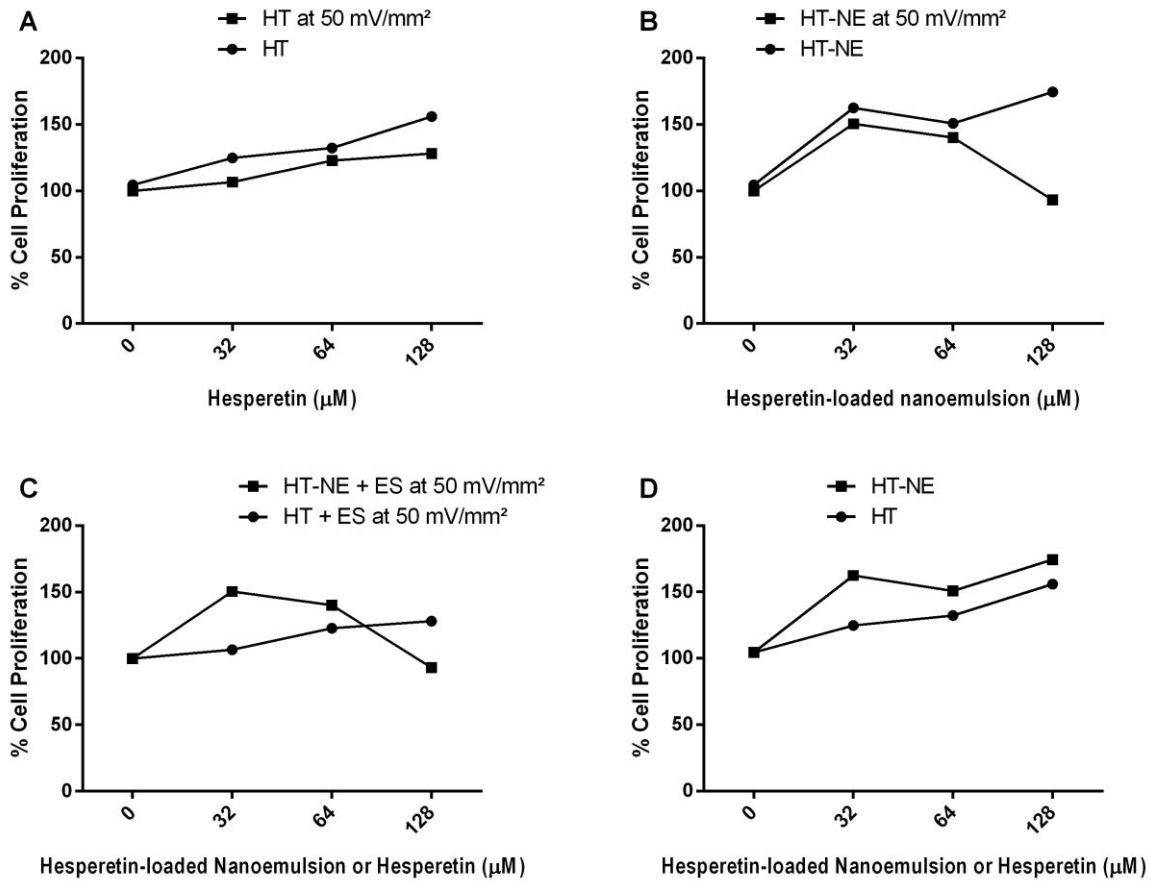


Figure 5. Comparison of the proliferation percentage between the groups treated with HT or HT-NE (32μM, 64 μM, and 128 μM) and ES (50 mV/mm²). (A) Comparison between the groups treated with HT (32μM, 64 μM, and 128 μM) and HT + ES. (B) Comparison between the groups treated with HT-NE (32μM, 64 μM, and 128 μM) and HT-NE + ES. (C) Comparison between the groups treated with HT-NE (32μM, 64 μM, and 128 μM) + ES and HT (32μM, 64 μM, and 128 μM) + ES. (D) Comparison between the groups treated with HT (32μM, 64 μM, and 128 μM) and HT-NE (32μM, 64 μM, and 128 μM).

3. Cell Nucleus Integrity after Proliferation

Figure 6 shows the cell nucleus integrity after the treatment with HT in different concentrations (32 µM, 64 µM, and 128 µM), and it is possible to see that besides the positive effect on cell proliferation, the original cell nucleus morphology was kept, however, the association of HT and HT-NE with ES can protect the cells' structures.

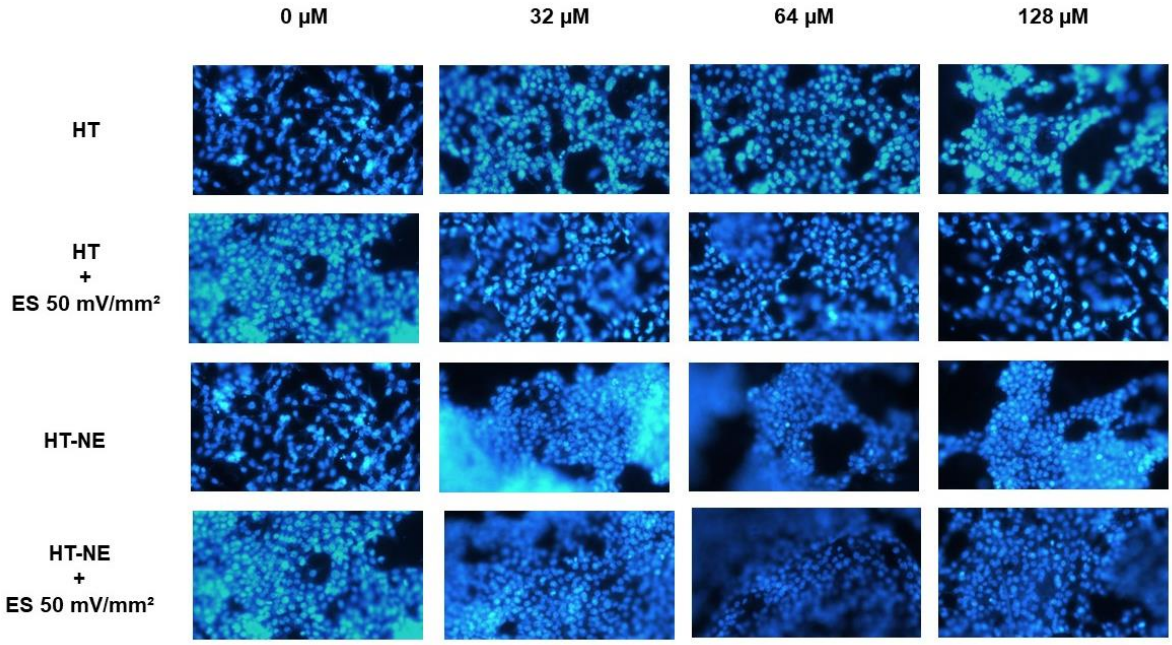


Figure 6. Cell nucleus integrity after HT or HT-NE treatment (32 µM, 64 µM and 128 µM) and ES at 50 mV/mm² for 24 h.

4. Cell Migration

Figure 7 shows the percentage area of wound after the beginning of cell migration; as we can see, all the groups presented a progressive cell migration time-dependent, being statistically significant compared with time 0h (p<0.001).

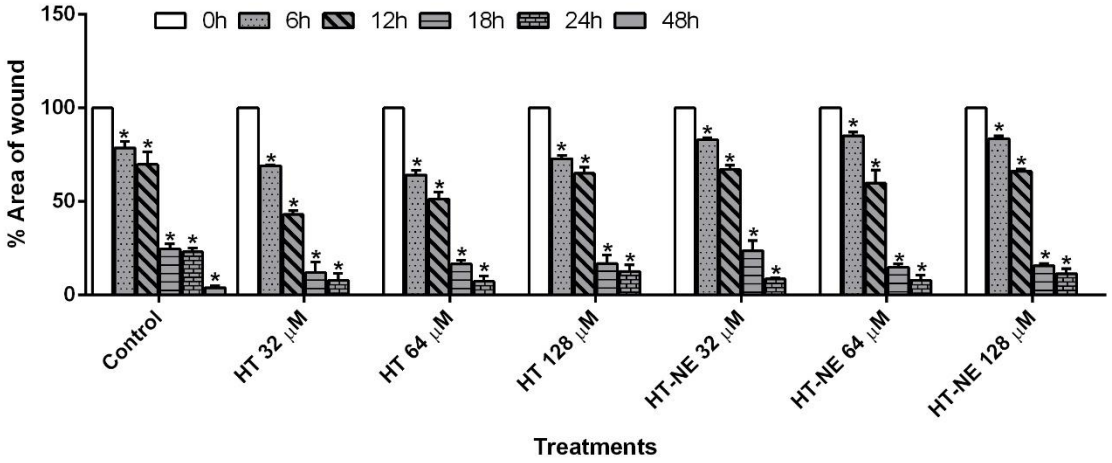


Figure 7. Percentage of the wound area compared with time 0h after the treatment of MG63 cells with HT and HT-NE in different concentrations (32 µM, 64 µM, and 128 µM) for different periods (0h, 6h, 12h, 18h, 24h, and 48h). *Statistically significant differences related with time 0h. (ANOVA, p<0.001)

Figure 8 shows the comparison between the percentage of wound area after different treatments. In Figure 8A it is presented the comparison between the groups treated with HT and the control group, the lowest concentrations (32 µM and 64 µM) presented a statistically significant decrease better than the control group in all the time points starting at 6h, and the same occurred with the highest concentration (128 µM) since 18h of treatment. In Figure B we can see the same comparison with HT-NE, where the highest concentration (64 µM and 128 µM) showed a statistically significant decrease compared with the control group since 18h of treatment, and the lowest concentration (32 µM) showed this decrease since 2h of treatment. Finally, when we compare the effect of HT and HT-NE, we notice that at 32 µM, the HT presented a greater decrease in the wound area until 18h (image C), but it was not significant in this period (18h) and did not present difference since 24h. At 64 µM, the HT presented a greater statistically significant decrease compared with the HT-NE, only until 6h (image E). While at 128 µM the groups of HT and HT-NE, presented some statistical difference, with more decrease provided from HT, just until 6h of treatment, and similar results in the other time-points (image E).

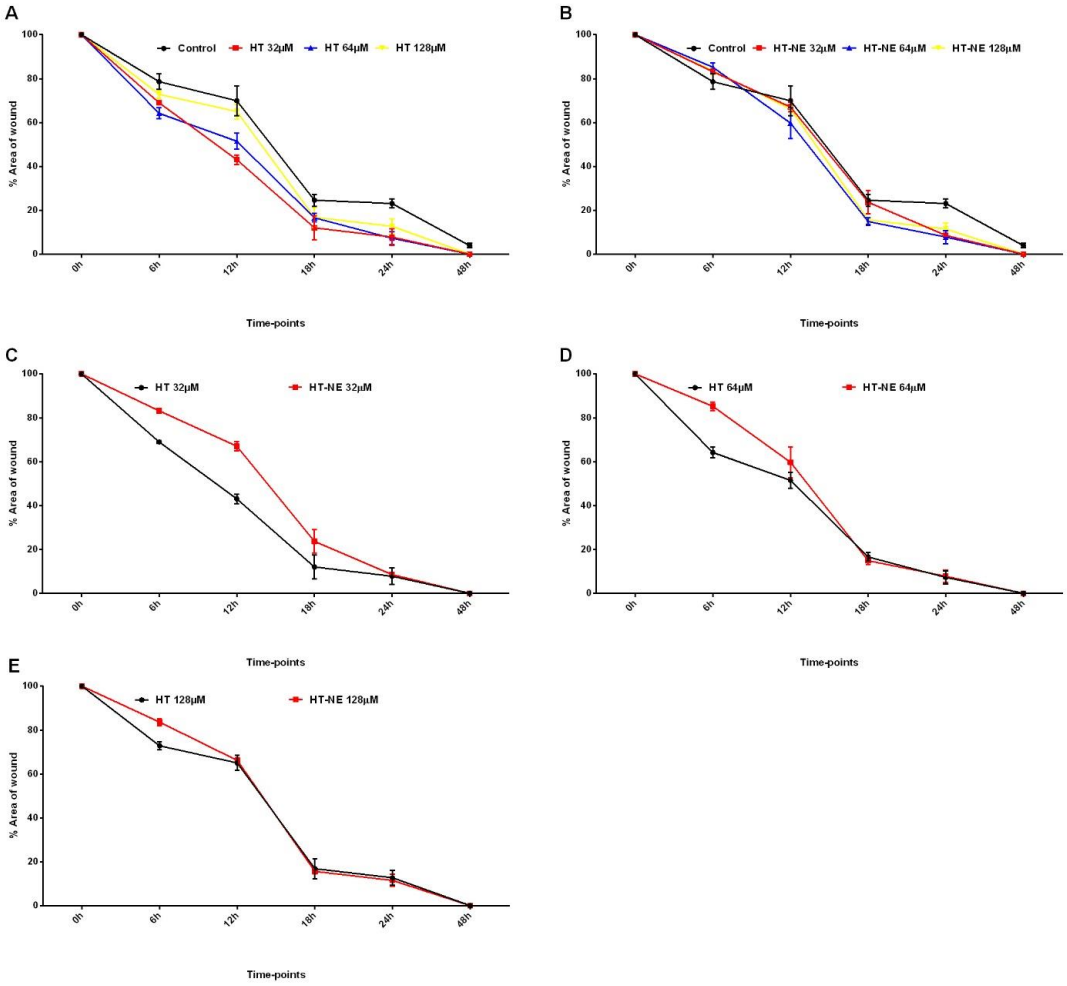


Figure 8. Comparison of the effect of different treated groups (Control, HT, and HT-NE) in the percentage of wound area after different periods (0h, 6h, 12h, 18h, 24h, and 48h).

5. ALP Activity During Cell Migration

Figure 9 shows the results of ALP activity after complete cell migration, a period of 48h of treatment, and 6 days of cell seeding. It is possible to notice that the HT and HT-NE showed an increase in ALP activity, being statistically significant to the highest concentrations of HT (64 µM and 128 µM, $p<0.05$ and $p<0.0001$) in its free form and all the concentrations of HT-NE (32 µM, 64 µM, and 128 µM, $p<0.0001$).

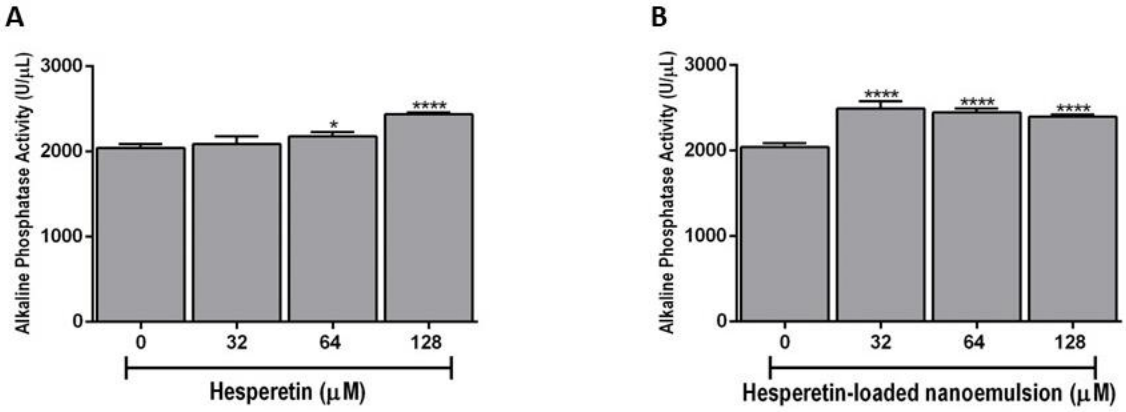


Figure 9. ALP activity of MG63 cells, after migration assay, during 6 days of growth and 48h of treatment with HT and HT-NE at 32 µM, 64 µM, and 128 µM.

Figure 10 compares the results between HT and HT-NE effects in ALP activity after cell migration, and it is clear to notice that the HT-NE presented a higher increase in ALP activity than the HT in its free form, mainly in the groups with low concentrations of treatment (32 µM and 64 µM).

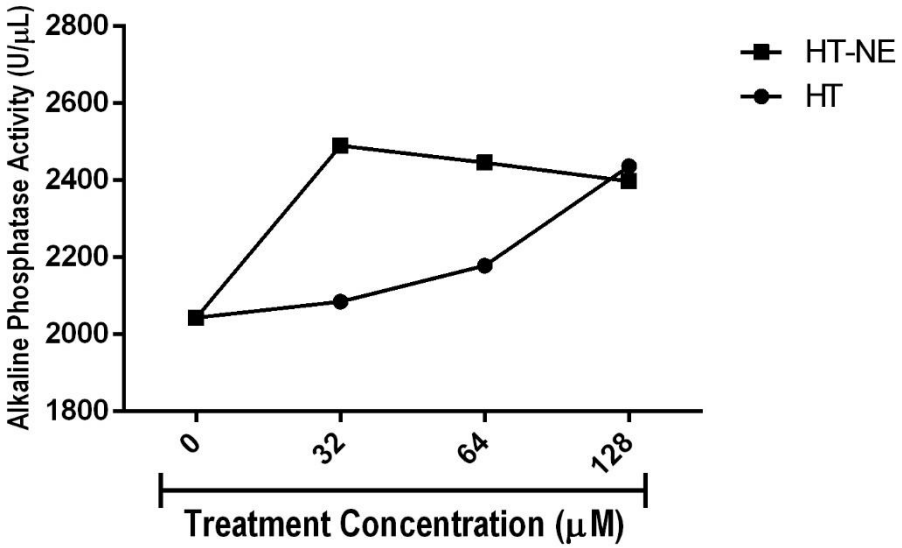


Figure 10. Comparison between the effect of HT and HT-NE at 32 µM, 64 µM, and 128 µM in the ALP activity of MG63 cells, after the treatment for 48h.

DISCUSSION

Our study demonstrated an interesting association between ES and a flavonoid, hesperetin, a natural compound with diverse biological properties such as analgesic, anti-inflammatory, neuroprotector, hepatoprotective, and cardioprotective effects and beneficial effects on bone metabolism (Nones et al., 2011; Ahmad et al., 2016; Mamun et al., 2017; Nobakht et al., 2017; Rothwell et al., 2017). It has been reported the positive effects of natural compounds, such as HT, in bone metabolism and that its effects can be enhanced by its encapsulation in nanoemulsions, systems of nanoparticles that can protect this compound against external factors, like pH, oxidation, and hydrolysis (Chen et al., 2020). On the other hand, the ES showed good effects on osteoblasts behavior, increasing important parameters to bone metabolism, such as calcium nodule formation and protein levels, such as osteocalcin (Meng et al., 2021, Meng et al., 2011). Then, an association between HT or HT-NE and ES brings some synergistic and protective effects, facilitating their use.

The membranes used in this study showed ideal biocompatibility parameters with cells, flexibility, and conductibility (Cui et al., 2021). When we reach a good cell adhesion as our cells in the membranes, we allow the occurrence of a positive effect of the electrical stimulation, enhancing the cell behavior and permitting the creation of electric and biocompatible treatments (Xue et al., 2021). As was shown before, our membrane allows the adhesion of different cells as diabetic fibroblasts, human keratinocytes, and osteoblasts (Meng et al., 2021; Rouabhia et al., 2020; Abedin-Do et al., 2021) in our study, we showed that the hesperetin does not avoid this adhesion and can show an interesting effect in this membrane. Added to the fact our membranes already present heparin in their composition, a compound that allows a better adhesion and enhances the cells' growth (Meng et al., 2008), we can suggest a good synergistic effect the HT, HT-NE, and the membrane, enhancing the proliferation in a quickly way.

In our study, we see statistically significant increases in proliferation when the cells are treated with HT and HT-NE. Although this proliferation is higher when the HT or HT-NE are applied alone, they can increase the proliferation effect of ES. Our results show that this association increases cell proliferation more than ES stimulates the cells. Besides that, in our study, we could use high level of ES (50 mV/mm^2) in a continued period of 24h, differing from another study in osteoblasts that used small periods of 6h

on different days (Meng et al., 2011). This comparison may suggest a protective effect of HT and HT-NE to the cells receiving ES, which their nucleus integrity can confirm in all groups stained by Hoechst. And corroborating the positive and synergistic effect of HT or HT-NE and ES, we have their effect on ALP activity.

The Alkaline Phosphatase activity is an important parameter of osteogeneses since it should be released at the correct time, around 7 to 14 days of cells cultivation, to generate the correct effect (Trzeciakiewicz et al., 2010; Kim et al., 2013). The evaluation of ALP activity after 6 days of culture with treatment with HT and HT-NE for 48h, demonstrated that our treatments can enhance ALP activity at the right moment, presenting a better activity of HT when it is encapsulated in the nanoemulsion. Allowing perspectives of new investigations about the effect of ES with HT and HT-NE in the ALP activity around 7 days of cell culture to compare if the ES can also be enhanced in this assay.

Finally, the migration of osteoblasts will be important to show us when the cells can react better to repair damage and fractures in bone, being able to cover distances in the millimeter range (Thiel et al., 2018). We saw important results from our study, showing that in both cases, the HT and the HT-NE generated a higher cell migration than the control group, which did not receive any treatment, allowing this way, a future and interesting investigation of their combination with ES. Besides that, we can interesting explore the effect of the nanoemulsions compared with HT in its free form when we see that in the earlier periods, the HT presented a better effect. It can be due to one of the main characteristics of nanoemulsions, that are the controlled release of the encapsulated compounds (Sabjan et al., 2020; Duarte et al., 2020; Zhang et al., 2018), which was shown with the increase in HT-NE effect in a time-dependent way.

Taking into account all our observations and the positive effect of this unprecedented association, we expect new approaches to their synergistic effect on bone, leading to the opportunity for the execution of new studies *in vitro,* becoming deeper the investigations about the effect of HT and HT-NE with ES in osteoblasts behavior, as the mineralized nodule formation, the expression of genes, proteins and signaling pathways involved in bone metabolism.

CONCLUSION

This study showed a important effect of HT in its free form and HT-NE, enhancing the effects of ES through the increase of cell adhesion on the membranes and proliferation when compared with the ES alone. Besides that, in cell migration the controlled release of HT-NE allow its effect in a long period, showing better results in ALP activity when compared with Free HT. This way the HT and the HT-NE allow future studies about their effects to ameliorate the ES application.

Aknowledgements: This study benefits CIHR (grant number 148523) and Fond Émile-Beaulieu (grant number FO134338) financial support. This research was funded by São Paulo Research Foundation, grant number 2022/09690-9).

Conflicts of Interest: The authors declare no conflict of interest.

Author Contributions: Conceptualization, Maria Júlia Mancim-Imbriani, Denise Madalena P. Spolidorio, Patricia Milagros Maquera-Huacho, and Mahmoud Rouabhia; Funding acquisition, Maria Júlia Mancim-Imbriani, Denise Madalena P. Spolidorio, Patricia Milagros Maquera-Huacho, and Mahmoud Rouabhia; Investigation, Maria Júlia Mancim-Imbriani, Atieh Abedin-Doo, Ze Zhang, and Mahmoud Rouabhia; Methodology, Maria Júlia Mancim-Imbriani1, Atieh Abedin-Doo, Ze Zhang, and Mahmoud Rouabhia; Resources, Ze Zhang, Denise Madalena P. Spolidorio, and Mahmoud Rouabhia; Supervision, Denise Madalena P. Spolidorio, Patricia Milagros Maquera-Huacho, and Mahmoud Rouabhia; Validation, Maria Júlia Mancim-Imbriani, Atieh Abedin-Doo, Ze Zhang, and Mahmoud Rouabhia; Writing – original draft, Maria Júlia Mancim-Imbriani, and Mahmoud Rouabhia; Writing – review & editing, Atieh Abedin-Doo, Ze Zhang, Denise Madalena P. Spolidorio, Patricia Milagros Maquera-Huacho, and Mahmoud Rouabhia.

References

1. Abedin-Do A, Zhang Z, Douville Y, Méthot M, Rouabhia M. Effect of Electrical Stimulation on Diabetic Human Skin Fibroblast Growth and the Secretion of Cytokines and Growth Factors Involved in Wound Healing. Biology (Basel). 2021;10(7):641.
2. Ahmad S, Alam K, Hossain MM, Fatima M, Firdaus F, Zafeer MF et al. Anti-arthritogenic and cardioprotective action of hesperidin and daidzein in collagen-induced rheumatoid arthritis. Mol Cell Biochem. 2016;423(1-2):115-127.
3. Carvalho VFM, Salata GC, de Matos JKR, Costa-Fernandez S, Chorilli M, Steiner AA, et al. Optimization of composition and obtainment parameters of biocompatible nano-emulsions intended for intraductal administration of piplartine (piperlongumine) and mammary tissue targeting. Int J Pharm. 2019;567:118460.
4. Cassidy A, Minihane AM. The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. Am J Clin Nutr. 2017;105(1):10–22.
5. Chen LH, Cheng LC, Doyle PS. Nanoemulsion-loaded capsules for controlled delivery of lipophilic active ingredients. *Adv Sci (Weinh).* 2020; 7(20):2001677.
6. Chen X, Ding HW, Li HD, Huang HM, Li XF, Yang Y et al. Hesperetin derivative-14 alleviates inflammation by activating PPAR-γ in mice with CCl4-induced acute liver injury and LPS-treated RAW264.7 cells. Toxicol Lett. 2017;274:51-63.
7. Cui S, Mao J, Rouabhia M, Elkoun S, Zhang Z. A biocompatible polypyrrole membrane for biomedical applications. RSC Adv. 2021;11(28):16996-17006.
8. Cui S, Rouabhia M, Semlali A, Zhang Z. Effects of electrical stimulation on human skin keratinocyte growth and the secretion of cytokines and growth factors. Biomed Mater. 2021; 16(6).
9. Duarte JL, Maciel de Faria Motta Oliveira AE, Pinto MC, Chorilli M. Botanical insecticide-based nanosystems for the control of Aedes (Stegomyia) aegypti larvae. Environ Sci Pollut Res Int. 2020;27(23):28737-28748. doi: 10.1007/s11356-020-09278-y.
10. Kabakov AE, Gabai VL. Cell Death and Survival Assays. Methods Mol Biol. 2018; 1709:107-127.

11. Kim SY, Lee JY, Park YD, Kang KL, Lee JC, Heo JS. Hesperetin alleviates the inhibitory effects of high glucose on the osteoblastic differentiation of periodontal ligament stem cells. PLoS One. 2013;8(6):e67504
12. Kotobuki N, Hirose M, Funaoka H, Ohgushi H. Enhancement of *in vitro* osteoblastic potential after selective sorting of osteoblasts with high alkaline phosphatase activity from human osteoblast-like cells. Cell Transplant. 2004;13(4):377-83. doi: 10.3727/000000004783983756.
13. Mamun MA, Hosen MJ, Khatun A, Alam MM, Al-Bari MAA. Tridax procumbens flavonoids: a prospective bioactive compound increased osteoblast differentiation and trabecular bone formation. Biol Res. 2017;50(1):28.
14. Mancim-Imbriani MJ, Duarte JL, Di Filippo LD, Durão LPL, Chorilli M, Spolidorio DMP et al. Formulation of a novel hesperetin-loaded nanoemulsion and its promising effect on osteogenesis. Pharmaceuticals. (*in submission*).
15. Meng S, Rouabhia M, Zhang Z. Electrical Stimulation and Cellular Behaviors in Electric Field in Biomedical Research. Materials (Basel). 2021;15(1):165.
16. Meng S, Zhang Z, Rouabhia M. Accelerated osteoblast mineralization on a conductive substrate by multiple electrical stimulation. J Bone Miner Metab. 2011;29(5):535-44.
17. Meng S, Rouabhia M, Shi G, Zhang Z. Heparin dopant increases the electrical stability, cell adhesion, and growth of conducting polypyrrole/poly(L,L-lactide) composites. J Biomed Mater Res A. 2008;87(2):332-44. doi: 10.1002/jbm.a.31735. PMID: 18181107.
18. Muhammad T, Ikram M, Ullah R, Rehman SU, Kim MO. Hesperetin, a citrus flavonoid, attenuates LPS-induced neuroinflammation, apoptosis and memory impairments by modulating TLR4/NF-κB signaling. Nutrients. 2019;11(3):648
19. Nobakht M, Trueman SJ, Wallace HM, Brooks PR, Streeter KJ, Katouli M. Antibacterial Properties of Flavonoids from Kino of the Eucalypt Tree, Corymbia torelliana. Plants (Basel). 2017;6(3):39.
20. Nones J, E Spohr TC, Gomes FC. Hesperidin, a flavone glycoside, as mediator of neuronal survival. Neurochem Res. 2011;36(10):1776-84.
21. Pandey P, Gulati N, Makhija M, Purohit D, Dureja H. Nano-emulsion: A novel drug delivery approach for enhancement of bioavailability. *Recent Pat Nanotechnol*. 2020; 14(4):276-293.

22. Rothwell JA, Knaze V, Zamora-Ros R. Polyphenols: dietary assessment and role in the prevention of cancers. Curr Opin Clin Nutr Metab Care. 2017;20(6):512
23. Rouabhia M, Park HJ, Abedin-Do A, Douville Y, Méthot M, Zhang Z. Electrical stimulation promotes the proliferation of human keratinocytes, increases the production of keratin 5 and 14, and increases the phosphorylation of ERK1/2 and p38 MAP kinases. J Tissue Eng Regen Med. 2020;14(7):909-919.
24. Sabjan KB, Munawar SM, Rajendiran D, Vinoji SK, Kasinathan K. Nano-emulsion as Oral Drug Delivery - A Review.Curr Drug Res Rev. 2020;12(1):4-15.
25. Singh Y, Meher JG, Raval K, Khan FA, Chaurasia M, Jain NK, et al. Nanoemulsion: Concepts, development and applications in drug delivery. J ControlRelease. 2017;252:28-49
26. Thiel A, Reumann MK, Boskey A, Wischmann J, von Eisenhart-Rothe R, Mayer-Kuckuk P. Osteoblast migration in vertebrate bone. Biol Rev Camb Philos Soc. 2018;93(1):350-363. doi: 10.1111/brv.12345.
27. Trzeciakiewicz A, Habauzit V, Mercier S, Barron D, Urpi-Sarda M, Manach C, et al. Molecular mechanism of hesperetin-7-O-glucuronide, the main circulating metabolite of hesperidin, involved in osteoblast differentiation. J Agric Food Chem. 2010;58(1):668-75.
28. Xue D, Chen E, Zhang W, Gao X, Wang S, Zheng Q. The role of hesperetin on osteogenesis of human mesenchymal stem cells and its function in bone regeneration. Oncotarget. 2017;8(13):21031-21043.
29. Xue G, Zhang Y, Xie T, Zhang Z, Liu Q, Li X, et al. Cell Adhesion-Mediated Piezoelectric Self-Stimulation on Polydopamine-Modified Poly(vinylidene fluoride) Membranes. ACS Appl Mater Interfaces. 2021;13(15):17361-17371. doi: 10.1021/acsami.1c02457.
30. Zhang H, Qin H, Li L, Zhou X, Wang W, Kan C. Preparation and Characterization of Controlled-Release Avermectin/Castor Oil-Based Polyurethane Nanoemulsions. J Agric Food Chem. 2018;66(26):6552-6560. doi: 10.1021/acs.jafc.7b01401.

3.3 Publicação 3*

Anti-inflammatory activity of a novel hesperetin-loaded nanoemulsion

Maria Júlia Mancim Imbriani[1,2*], Leticia P. L. Durão[2], Denise M. Palomari Spolidorio[2], Patricia Milagros Maquera-Huacho[1,2]

[1]Department of Diagnosis and Surgery, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

[2]Department of Physiology and Pathology, School of Dentistry, São Paulo State University (Unesp), Araraquara, São Paulo, Brazil

*Correspondence:

Patricia Milagros Maquera-Huacho, Department of Physiology and Pathology, School of Dentistry, São Paulo State University (Unesp), Araraquara – SP, Humaita St, 1680, CEP 14801-903 Araraquara, São Paulo, Brazil.
Email: patricia.maquera@unesp.br

Short title: Hesperetin-loaded nanoemulsion and inflammation

* Artigo seguiu as normas da revista *Plos One* para a qual pretende-se submeter.

ABSTRACT

Hesperetin is a flavonoid, that have been studied in reason of its diverse biological properties. The nanoemulsions are solutions that allow the encapsulation of drugs and compounds, offering a controlled release, the direction of these compounds to a correct active site, and the protection against oxidation, high temperatures, and pH variation. A hesperetin-loaded nanoemulsion (HT-NE) has already shown beneficial effects on bone metabolism , enhancing HT activity, through the increase of mineralized nodule formation, regulation of bone genes, enhancement of Alkaline phosphatase activity and collagen production . For this reason, the objective of this study was to evaluate the effect of the HT-NE on inflammation, to complement the future possible effects on osteoinflammatory diseases. The effects of HT-NE on inflammation were evaluated through the treatment of monocytes/macrophages U937 with non-cytotoxic concentrations of HT-NE and stimulation by *Fusobacterium nucleatum*. The evaluation of inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8 and matrix metalloproteinases (MMPs) 1, MMP-3 and MMP-9 were assessed by ELISA. The HT-NE showed non-cytotoxic effect in monocytes/macrophages at concentrations lower 256 µM, promoting the decrease of all the cytokines and MMPs evaluated.

In conclusion, the results in this work corroborated our previous finds, showed promising perspectives regarding the use of this hesperetin-loaded nanoemulsion to the treatment of osteoinflammatory diseases.

INTRODUCTION

Periodontal disease is a chronic multifactorial inflammatory disease whose inflammation is resultant of polymicrobial colonization in the form of biofilm on the tooth surface[1]. A wide range of periodontopathogen bacteria are involved in periodontal disease since the beginning of the pathological process[2-4]. Between them we can highlight *Porphyromonas gingivalis, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* and *Tanerella forsythia*[2-4]. These bacteria generated complexes immune inflammatory events such as the increase of cytokines production, for example, interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF) and the decrease in the relation of metalloproteinases matrix and the tissue inhibitor of metalloproteinase (TIMP), leading to collagen, elastin and proteoglycans degradation[4-6]. Besides that, the LPS produced by the bacteria may stimulate the immune system's cells (macrophages, monocytes, neutrophils) to activate the triggering receptor expressed on myeloid cells-1 (sTREM-1) secretion, an important receptor found in patients with periodontitis[7-8].

The conventional treatment for periodontal disease includes the scaling and planning root, and oral hygiene instructions for the bacteria reduction[9]. Additionally, new alternatives therapies are rising as adjuvant treatment when just the conventional treatment is not enough to suppress the inflammatory process and recovering the periodontal health[9]. For this reason, new bioactive and natural drugs have been attractive due to their benefic effects on human health, mainly against infectious diseases[10,11].

Hesperetin (HT) is a metabolite of hesperidin, originating by citrus fruits as orange a grapefruit, that presents diverse biological properties, such as anti-cancer, analgesic, anti-inflammatory, antioxidant and benefic effects on bone metabolism[11-15]. Previous studies showed the capacity of HT in reducing TNF-α, IL-6, IL-1β, macrophage inflammatory-2 (MIP-2), cyclooxygenase-2 (COX-2), besides of inhibiting the nuclear factor kappa beta (NF-kβ) signaling pathway[16-18]. Acting on MD2/TLR4, whose role is the identification of LPS in cells surface, the HT is able to reduce acute lung injury decreasing total protein, neutrophils quantity, and cytokines expression[19]. Finally, the HT can present hepatoprotective effect in acute liver injury caused by LPS-stimulus, through the reduction of cytokines expresion[20].

Nanoemulsions are solutions of oil in water or water in oil that present diverse advantages to encapsulated compounds' action, offering a controlled drug release, a stability of the compound, protecting against high temperatures, pH variation and

oxidation[21-24]. When encapsulated in a nanoemulsion it is possible to direct the compound to a required active site, decreasing adverse effects and allowing the utilization of higher concentrations of a drug without toxic effects[22-24]. Given that, we formulated a hesperetin-loaded nanoemulsion (HT-NE) with positive results of parameters of characterization and excellent biocompatibility with osteoblasts cells. Our previous biological results showed promising and positive effects on bone metabolism, where our formulation enhanced the beneficial properties of hesperetin compared with its free form[25].

Therefore, the objective of the current study was to evaluate the effect of HT-NE on cytokines expression and matrix metalloproteinases. Additionally, we evaluated the ability of HT-NE to regulate the NF-kβ signaling pathway.

METHODS

Cell culture

Human monocytes U937 (CRL-1593.2; American Type Culture Collection, EUA) were cultivated in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Life Technologies Inc., Brazil) supplemented with 10% Fetal Bovine Serum (FBS) and 100 µg/mL of penicillin G/streptomycin at an atmosphere of 37 °C and 5% of CO_2.

Bacteria culture

Fusobacterium nucleatum (ATCC 25586) was cultivated in anaerobiosis conditions (80% N_2, 10% CO_2, 10% H_2) for 48 h at 37º C in Brucella Agar (Brucella Agar, Neogen, Brazil) supplemented with 5% of sheep blood (Microlab, Brazil), 0.1% of hemin, 0.01% of menadione and 2 mg/mL of yeast extract. Then, colonies of *F. nucleatum* were grown for 24 h at 37 °C in Brain Heart Infusion Broth (BHI; Sigma-Aldrich, Brazil) supplemented with 0.001% of hemin, 0.0001% of menadione and 6 mg/mL of yeast extract. The bacteria was applied in the cells in a multiplicity of infection (MOI) of 100.

Cytotoxicity assay

Monocytes were cultivated in 96-well plates (2 x 10^5 cell/well) in RPMI medium with 10% FBS containing 20 ng/mL of phorbol myristic acid (PMA, Sigma-Aldrich, Brazil). The cells were incubated for 24 h for differentiation induction to macrophage-like adherent cell. After this period, the medium was refreshed by RPMI medium with 10% FBS for 48h. Immediately, the medium was changed by RPMI medium with 1%

FBS and the cells maintained for 24 h at 37 ºC in an atmosphere of 5% CO_2. Then, the macrophage-like adherent cells were treated with concentrations of HT-NE 8200 µM–0,5 µM (v/v) and their respective positive (Camptotecin10 µM) e negative (culture medium) control groups. After 24 h the cytotoxic effects were evaluated by methyl tetrazolium (MTT) (Sigma-Aldrich, EUA) assay.

The colorimetric analysis with MTT evaluates the cell metabolism through the cytochemical activity of the succinic dehydrogenase (SDH) enzyme, which represents the mitochondrial breath rate of viable cells. Each well of the experimental and control groups received a solution containing 90 µL of culture medium and 10 µL of MTT solution, which was prepared with 5 mg of MTT powder and 1 mL of sterile Phosphate-buffered saline (PBS). The cells were incubated for 4 hours at 37 °C and 5% CO_2. Then, the culture medium with MTT was aspirated, and 100 µL of acidified isopropanol (HCl 0.04 N) was applied in each well. The cell viability was evaluated considering that cells with normal mitochondrial activity were colored with an intense violet. Optical density was read at 570 nm wavelength in a spectrophotometer (Synergy H1 Multi-Mode Reader-BioTek, EUA).

Cytokines and MMPs Production by Macrophages Stimulated by *F. nucleatum*

The monocytes ($1x10^6$ cell/ well) were seeded in 12-well plates in RPMI medium with 10% FBS containing 20 ng/mL of phorbol myristic acid (PMA, Sigma-Aldrich, Brazil). The cells were incubated for differentiation induction to macrophage-like adherent cell as described previously in cytotoxicity section. The macrophage-like cells were pre-treated for 2 hours with non-cytotoxic concentrations of HT-NE (64 µM, 32 µM and 128 µM) previously prepared in RPMI with 1% FBS. Then, the cells were stimulated with *F. nucleatum* in a multiplicity of infection (MOI) of 100. Briefly, *F. nucleatum* growth in BHI broth was centrifuged at room temperature for 5 min at 10000 rpm. The supernatant was discarded and the *F. nucleatum* pellet was resuspended in RPMI medium with 1% FBS and subjected to a sequence of washing through centrifugation cycles. Then, the optical density of *F. nucleatum* was read using a spectrophotometer (BioPhotometer, Eppendorf, USA) to calculate the MOI. After pre-treatment with non-cytotoxic concentrations of HT-NE, the bacteria was added into the wells to stimulate the cells during 24 h. After this period, the supernatant was collected and stored at -20 ºC until be used. Incubated cell in culture medium with or without HT-NE treatment and stimulated or not with bacteria were used as control. Enzyme-Linked

Immunosorbent Assay kit (ELISA, Thermo Fisher, Brazil) was used to determinate TNF-α, IL-1β, IL-6, IL-8 e MMP1, MMP3 and MMP-9 expression, according to manufacturer's protocol.

RESULTS

Cytotoxicity assay

Figure 1 shows the cytotoxic effect of HT-NE in monocytes/macrophages after 24h of treatment. It is possible to note that the concentrations of 512 µM and 1025 µM evidenced a reduction of cell viability (59.78% and 63,04%, respectively, *p<0.001*).

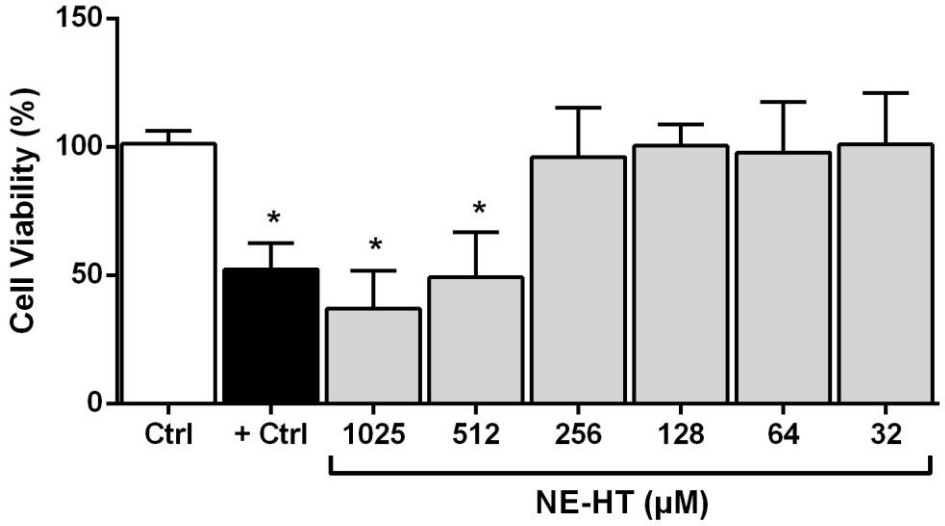


Figure 1. Cell viability percentage of monocytes/macrophages after 24 h of treatment with different concentrations of HT-NE. *, represents statistically significant differences related to the negative control group (ANOVA, *p*<0.001).

Cytokines and MMPs Production by Macrophages Stimulated by *F. nucleatum*

In Figure 2 are shown the results of cytokines and MMPs expression after the pre-treatement with HT-NE and the stimulus with *F. nucleatum*. All cytokines and MMPs showed a statistically significant reduction of their expression after the treatment with HT-NE when compared to the group stimulated by *F. nucleatum*. This inhibition was observed in all concentrations of HT-NE. The results of TNF-α expression showed a reduction of around 4, 3 and 2.5-fold to the groups treated with HT-NE at 256 µM, 128 µM and 64 µM, respectively, compared with the group stimulated by *F. nucleatum* (*p*<0.0001). To IL-1β the concentrations of 256 µM and 64 µM of HT-NE reduced the expression of the cytokine in 1.7 and 1.3-fold, respectively (*p*<0.0001 and *p*<0.05). In

the case of IL-6 the values of expression reduced in 2, 1.5 and 2-fold after the treatment with HT-NE at 256 µM, 128 µM and 64 µM, respectively (p<0.0001 and p<0.01). Added to this, the effect of HT-NE showed a reduction of IL-8 expression of 2, 2.5 and 4-fold to the concentrations of 256 µM, 128 µM and 64 µM, respectively (p<0.001 and p<0.0001). Finally, the effect of HT-NE on the MMPs reached similar values to the control group. It was observed the effect of HT-NE on MMP-1 reduction of 1.5-fold to the HT-NE concentrations of 256 µM and 128 µM, and 2-fold to 64 µM when compared with the group stimulated with *F. nucleatum* (p<0.01 and p<0.0001). Regarding MMP-3 reduction, HT-NE was able to reduce 1.5, 2, and 1.7-fold at 256 µM, 128 µM and 64 µM, respectively (p<0.01, p<0.01, and p<0.0001). Lastly, results of MMP-9 expression showed that the HT-NE treatment reduced the expression of this MMP in 1.4, 1.6 and 1.7-fold at 256 µM, 128 µM and 64 µM concentrations of HT-NE, respectively (p<0.001 and p<0.0001).

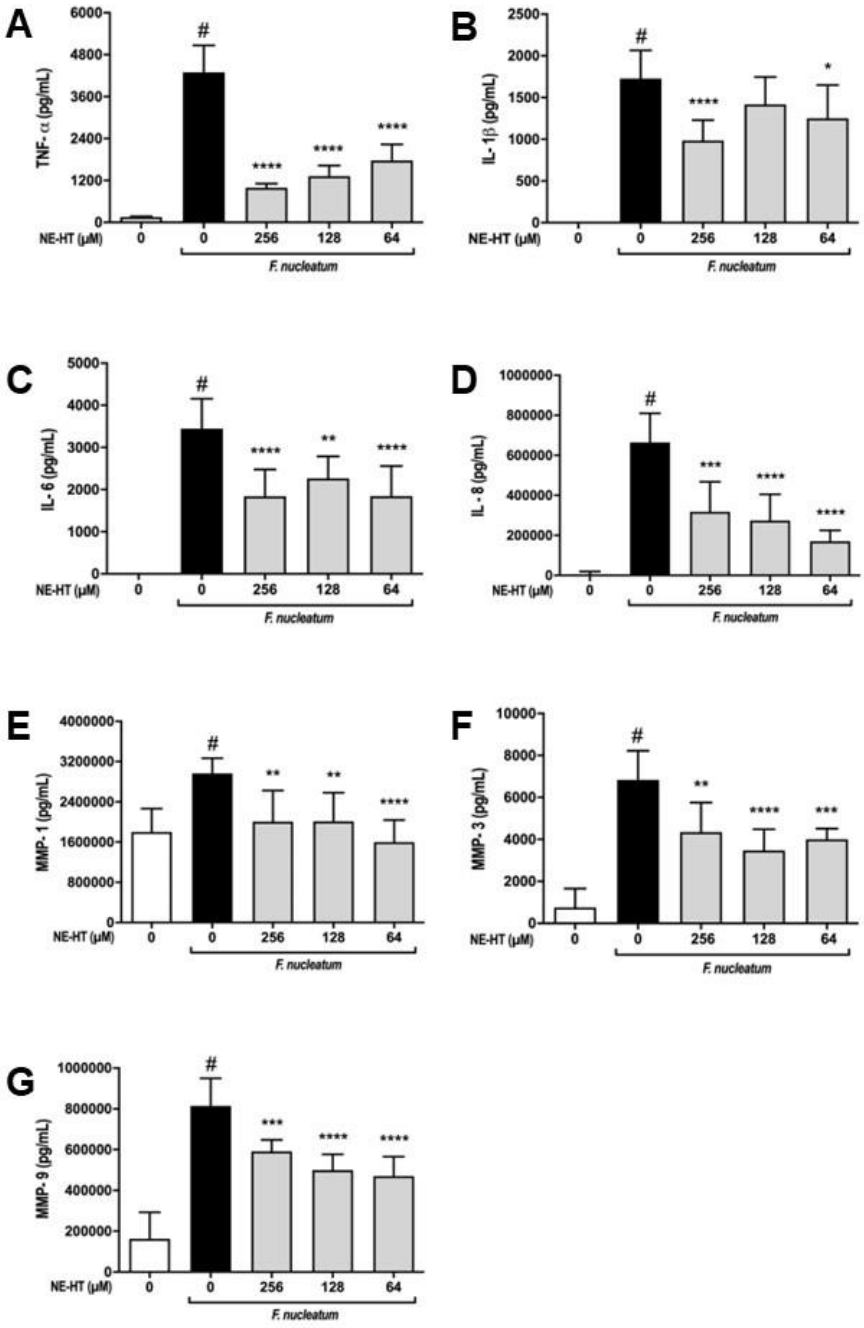


Figure 2. Expression levels of the cytokines TNF-α (A), IL-1β (B), IL-6 (C), IL-8 (D), MMP-1 (E), MMP-3 (F) and MMP-9 (G) in differentiated macrophages U937 treated with HT-NE and stimulated by *F.nucleatum* (MOI=100) for 24. #, represents a statistical increase related to the control group. *, represents a statistical decrease related to the group only stimulated by F. nucleatum. (ANOVA, ***p*<0,01; ****p*<0,001; *****p*<0,0001).

DISCUSSION

The cytokines are released resulting of a cascade of events during inflammation process, for example, the activation of signaling pathways as Myd88 (Myeloid differentiation primary response 88) that can activate NF-kβ (Nuclear factor kappa beta) releasing cytokines that will develop a wide range of roles [26]. A drug that may control the inflammatory process can be consider as an important alternative to treatment or as its adjuvant to treat inflammatory diseases. A flavonoid incorporated into a new nanoemulsion formulation may permit enhance its well-known properties against inflammation due to its advantages as a controlled release, the direction of the drug to a specific site and the possibility of collateral effects reduction[24].

The mechanism of inflammation signaling pathways may occur due to some stimulus from pathogens, such as bacteria. In case of periodontal diseases, *F.nucleatum* is a bacteria responsible for an important role in its progression [27,28]. *F. nucleatum* expresses a virulence factor, FadA, that will bind to the host cell, like macrophages/monocytes, being able the invasion of the cell for the bacteria[29]. When *F. nucleatum* invades the cell, it promotes an inflammatory environment with diverse mechanisms that allow the colonization of other bacteria[29]. For this reason, our study used the stimulus directly with bacteria, generating this way an inflammatory environment, involving the complexity of the whole bacteria, which is different to the use of only LPS (lipopolysaccharide).

The cytotoxicity assay is a primordial test that is intended for the application of a drug or medicine in clinical use. It is important to know which concentration of a drug can be used without damaging cells, allowing this way, subsequent assays to be executed. Our formulation presented a non-cytotoxic effect in higher concentrations (256 µM) when compared with another study using a nanocrystal associated to HT that presented a reduction of cell viability, at 25 µM and 10 µM, after 1 day of treatment and using the same monocytes[30]. Then it can be noted that our nanoemulsion can be biocompatible in low and higher concentrations of HT.

The stimulus led to the release of cytokines as TNF-α, Interleukin (IL)-1β, IL- 6 and IL-8, pro-inflammatory cytokines that may cause different effects to promote inflammation[31,32,33]. The TNF-α expression causes vasodilation forming edema, and leukocytes aggregation, leading to oxidative stress occurrence into inflammation sites[32]. A study showed the positive effect of hesperetin at 100 µM in the reduction of TNF-α expression, in macrophages stimulated by bacteria LPS[33]. They showed this

reduction of the expression in 2.3-fold when compared with stimulated group[34]. Comparing this result with HT-NE we can consider an increase of HT effect in our study, since we reached more expressive reduction (4 to 2.5-fold) of this cytokine using lowest concentrations of HT. The same study did not show significant reduction in IL-6 expression (less than 1-fold)[34]. Reinforcing the enhanced effect of HT-NE developed in the present study on inflammation, taking account that IL-6 plays an important role in the acute phase of inflammation, through the binding in its receptor and activation of Janus kinase-signal transducer and activator of the transcription (JAK/STAT) pathway[35].

Different to our results, a significant inhibition of IL-1β after the treatment with HT (4.6-fold) was demonstrated[34], while we founded that HT-NE was able to reduce between 1.7 and 1.3-fold the expression of IL-1β. Finally, the IL-8 main function is the attraction of neutrophils; however, it is also responsible for monocytes/macrophages differentiation and growth and for the promotion of oxidative stress[35,36]. It was not found previous results about the effect of HT or NT encapsulated on IL-8 inhibition. Therefore, we showed for the first time this effect and great performance of HT-NE in the reduction IL-8 expression in an expressive way (2 to 4-fold reduction).

Our findings can be compared with a study encapsulating narigenin, another flavonoid, in a nanoparticle system[37]. They evaluated the anti-inflammatory effect of this flavonoid in mice's macrophages and found a decrease in IL-6, IL-1β and TNF-α expression[37]. However the effects of narigerin encapsulated and in its free form did not shown statistical differences[37]. While when we compare our results with previous studies about Free HT we can notice some differences, showing the advantages of HT encapsulation. Other study encapsulating baicalein, a flavonoid extracted of *Scutellaria baicalensis* roots, corroborates with our findings[38]. They demonstrated that the encapsulation of the baicalein in a nanoparticle system was able to enhance its activity in downregulating IL-6 and IL-8 expression in gingival cells[38]. Showing the enhancement of the flavonoid effect since the nanoparticles present the controlled intracellular release[38]. Finally, a study formulated a nanosponge loaded with HT and corroborated with our findings showing the anti-inflammatory effect of the formulation in mice's macrophage[39]. A greater decrease of IL-1β and IL-6 was shown by the nanosystem when compared with HT in its free form[39].

In periodontal diseases, the inflammatory process leads to bone resorption[40], so when we study a potential treatment, the relation between bone degradation and

inflammation is an important topic to be analyzed. This way, there is the importance of MMPs expression evaluation. The MMPs are responsible for collagen fiber damage in different ways, specifically the MMPs evaluated in this study develop different roles in this process[40]. These MMPs are cytokines that can be released by the stimulus of another cytokine such as TNF-α and IL-1β, causing collagen lysis in the case of MMP -1, gelatin lysis in the case of MMP-9, and cellular matrix lysis in the case of MMP-3[33].

A previous study that evaluated the anti- inflammation property of hesperidin and hesperitin showed that the hesperidin was able to reduce MMP-1 expression, avoiding collagen degradation[41]. However, this anti-inflammatory effect was not observed after the treatment with hesperitin[41]. This is an interesting point to be discussed due to our results showed that the HT-NE was able to reduce 1.5 and 2-fold of the MMP-1 expression. On the other hand, Choi et al[42]., 2010 evaluated the efficacy of HT on MMP-3 expression by synovial cell stimulated by IL-1β, obtaining reduction rates of 1 and 1.4-fold after the treatment with HT at 10 µM. It is in according with our results where HT-NE was able to reduce MMP-3 expression up to 2-fold. Our results do not show a dose-dependent effect on the expression of MMPs. Different to these results, previous studies showed a reduction of MMPs 1, 3 and-9[43,44] with a dose-dependent effect of the flavonoid HT at concentrations of 100 µM and 150 µM. In this way, this effect suggests that, our formulation permit to encapsulate low doses of HT, and reach a better effect on reduction of cytokines and MMPs.

In conclusion, our formulation showed promising results on inflammatory conditions through the reduction of important cytokines and MMPs. Therefore, these results added to the previous demonstrated that NE-HT has the potential to be a promisor treatment for inflammatory diseases, as periodontal disease. However, more investigations about the effect of HT-NE in inflammation mechanisms and signaling pathways are necessary, *in vitro* and *in vivo*.

Acknowledgments: The authors would like to thank São Paulo Research Foundation for financial support to the research. Master's degree scholarship for Maria Júlia Mancim Imbriani (grant #2020/14155-0, São Paulo Research Foundation (FAPESP)). Scientific initiation scholarship for Letícia Pereira Lima Durão (grant #2021/15178-6, São Paulo Research Foundation (FAPESP)). Post-Doctoral fellowship for Patricia Milagros Maquera-Huacho (grant #2018/16540-8, São Paulo Research Foundation (FAPESP)). Additionally, a research scholarship for the Laboratory of Microbiology and

Molecular Biology, Department of Physiology and Pathology, School of Dentistry of Araraquara – UNESP (grant #2021/05881-1, São Paulo Research Foundation (FAPESP)).

Conflict of interests: the authors declare no conflicts of interest.

References

1. Bostanci N, Bao K, Greenwood D, Silbereisen A, Belibasakis GN. Periodontal disease: From the lenses of light microscopy to the specs of proteomics and next-generation sequencing. Adv Clin Chem. 2019;93:263-290.
2. Silva N, Abusleme L, Bravo D, Dutzan N, Garcia-Sesnich J, Vernal R. Host response mechanisms in periodontal diseases. J Appl Oral Sci. 2015;23(3):329-55.
3. Ebbers M, Lübcke PM, Volzke J, Kriebel K, Hieke C, Engelmann R et al. Interplay between *P. gingivalis, F. nucleatum* and *A. actinomycetemcomitans* in murine alveolar bone loss, arthritis onset and progression. Sci Rep. 2018;8(1):15129.
4. Kinane DF, Preshaw PM, Loos BG. Host-response: understanding the cellular and molecular mechanisms of host microbial interactions – consensus of the Seventh European Workshop on Periodontology. J Clin Periodontol. 2011;38(11):44-8.
5. Graves DT, Li J, Cochran DL. Inflammation and uncoupling as mechanisms of periodontal bone loss. J Dent Res. 2011;90(2):143-53.
6. Abdulkareem AA, Abdulbaqi HR, Milward MR. *In vitro* Homeostasis of Rat Oral Epithelial Cell Cultures Following Withdrawal of Periodontal Pathogens. Braz Dent J. 2020;31(2):135-142.
7. Lagha AB, LeBel G, Grenier D. Dual action of highbush blueberry proanthocyanidins on Aggregatibacter actinomycetemcomitans and the host inflammatory response. BMC Complement Altern Med. 2018;18(1):10.
8. Schenk M, Bouchon A, Seibold F, Mueller C. TREM-1--expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. J Clin Invest. 2007;117(10):3097-106. doi: 10.1172/JCI30602.
9. Graziani F, Karapetsa D, Alonso B, Herrera D. Nonsurgical and surgical treatment of periodontitis: how many options for one disease?. Periodontol 2000. 2017;75(1):152-188.
10. Preethi Soundarya S, Sanjay V, Haritha Menon A, Dhivya S, Selvamurugan N. Effects of flavonoids incorporated biological macromolecules based scaffolds in bone tissue engineering. Int J Biol Macromol. 2018;110:74-87

11. Nones J, E Spohr TC, Gomes FC. Hesperidin, a flavone glycoside, as mediator of neuronal survival. Neurochem Res. 2011;36(10):1776-84.
12. Ahmad S, Alam K, Hossain MM, Fatima M, Firdaus F, Zafeer MF et al. Anti-arthritogenic and cardioprotective action of hesperidin and daidzein in collagen-induced rheumatoid arthritis. Mol Cell Biochem. 2016;423(1-2):115-127.
13. Mamun MA, Hosen MJ, Khatun A, Alam MM, Al-Bari MAA. Tridax procumbens flavonoids: a prospective bioactive compound increased osteoblast differentiation and trabecular bone formation. Biol Res. 2017;50(1):28.
14. Nobakht M, Trueman SJ, Wallace HM, Brooks PR, Streeter KJ, Katouli M. Antibacterial Properties of Flavonoids from Kino of the Eucalypt Tree, Corymbia torelliana. Plants (Basel). 2017;6(3):39.
15. Rothwell JA, Knaze V, Zamora-Ros R. Polyphenols: dietary assessment and role in the prevention of cancers. Curr Opin Clin Nutr Metab Care. 2017;20(6):512-21.
16. Ma H, Feng X, Ding S. Hesperetin attenuates ventilator-induced acute lunginjury through inhibition of NF-kappaB-mediated inflammation. Eur. J.Pharmacol. 2015;769:333-341.
17. Ren H, Hao J, Liu T, Zhang D, Lv H, Song E, et al. Hesperetin Suppresses Inflammatory Responses in Lipopolysaccharide-Induced RAW 264.7 Cells via the Inhibition of NF-κB and Activation of Nrf2/HO-1 Pathways. Inflammation. 2016;39(3):964-73.
18. Duran Y, Karaboğa İ. Effect of hesperetin on systemic inflammation andhepatic injury after blunt chest trauma in rats.Biotech Histochem. 2019;18:1-8.
19. Ye J, Guan M, Lu Y, Zhang D, Li C, Li Y, et al. Protective effects of hesperetin on lipopolysaccharide-induced acute lung injury by targeting MD2. Eur J Pharmacol. 2019;852:151-158
20. Chen X, Ding HW, Li HD, Huang HM, Li XF, Yang Y et al. Hesperetin derivative-14 alleviates inflammation by activating PPAR-γ in mice with CCl4-induced acute liver injury and LPS-treated RAW264.7 cells. Toxicol Lett. 2017;274:51-63.

21. Carvalho VFM, Salata GC, de Matos JKR, Costa-Fernandez S, Chorilli M, Steiner AA, et al. Optimization of composition and obtainment parameters of biocompatible nanoemulsions intended for intraductal administration of piplartine (piperlongumine) and mammary tissue targeting. Int J Pharm. 2019;567:118460
22. Sabjan KB, Munawar SM, Rajendiran D, Vinoji SK, Kasinathan K. Nanoemulsion as Oral Drug Delivery - A Review.Curr Drug Res Rev. 2020;12(1):4-15
23. Singh Y, Meher JG, Raval K, Khan FA, Chaurasia M, Jain NK, et al. Nanoemulsion: Concepts, development and applications in drug delivery. J ControlRelease. 2017;252:28-49
24. Pandey P, Gulati N, Makhija M, Purohit D, Dureja H. Nanoemulsion: A novel drug delivery approach for enhancement of bioavailability. *Recent Pat Nanotechnol*. 2020; 14(4):276-293.
25. Imbriani MJ, Duarte JL, Di Filippo LD, Durão LPL, Chorilli M, Spolidorio DMP, et al. Formulation of a novel hesperetin-loaded nanoemulsion and its promising effect on osteogenesis. Pharmaceuticals. (*in submission*).
26. Dong Q, Li Y, Chen J, Wang N. Azilsartan Suppressed LPS-Induced Inflammation in U937 Macrophages through Suppressing Oxidative Stress and Inhibiting the TLR2/MyD88 Signal Pathway. ACS Omega. 2020;6(1):113-118. doi: 10.1021/acsomega.0c03655.
27. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998;25(2):134-44. doi: 10.1111/j.1600-051x.1998.tb02419.x.
28. Sakanaka A, Kuboniwa M, Shimma S, Alghamdi SA, Mayumi S, Lamont RJ, et al. *Fusobacterium nucleatum* Metabolically Integrates Commensals and Pathogens in Oral Biofilms. mSystems. 2022;7(4):e0017022. doi: 10.1128/msystems.00170-22.
29. Zhou J, Liu L, Wu P, Zhao L, Wu Y. Fusobacterium nucleatum Accelerates Atherosclerosis via Macrophage-Driven Aberrant Proinflammatory Response and Lipid Metabolism. Front Microbiol. 2022;13:798685. doi: 10.3389/fmicb.2022.798685.

30. Alipour M, Sharifi S, Samiei M, Shahi S, Aghazadeh M, Dizaj SM. Synthesis, characterization, and evaluation of Hesperetin nanocrystals for regenerative dentistry. Sci Rep. 2023;13(1):2076. doi: 10.1038/s41598-023-28267-y.
31. McElvaney OJ, McEvoy NL, McElvaney OF, Carroll TP, Murphy MP, Dunlea DM, et al. Characterization of the Inflammatory Response to Severe COVID-19 Illness. Am J Respir Crit Care Med. 2020;202(6):812-821. doi: 10.1164/rccm.202005-1583OC..
32. Zelová H, Hošek J. TNF-α signalling and inflammation: interactions between old acquaintances. Inflamm Res. 2013;62(7):641-51. doi: 10.1007/s00011-013-0633-0.
33. Wang T, He C. Pro-inflammatory cytokines: The link between obesity and osteoarthritis. Cytokine Growth Factor Rev. 2018;44:38-50. doi: 10.1016/j.cytogfr.2018.10.002.
34. Fordham JB, Naqvi AR, Nares S. Leukocyte production of inflammatory mediators is inhibited by the antioxidants phloretin, silymarin, hesperetin, and resveratrol. Mediators Inflamm. 2014;2014:938712. doi: 10.1155/2014/938712.
35. Vilotić A, Nacka-Aleksić M, Pirković A, Bojić-Trbojević Ž, Dekanski D, Jovanović Krivokuća M. IL-6 and IL-8: An Overview of Their Roles in Healthy and Pathological Pregnancies. Int J Mol Sci. 2022;23(23):14574. doi: 10.3390/ijms232314574.
36. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 1992;307(1):97-101. doi: 10.1016/0014-5793(92)80909-z.
37. Kumar R P, Abraham A. Inhibition of LPS induced pro-inflammatory responses in RAW 264.7 macrophage cells by PVP-coated naringenin nanoparticle via down regulation of NF-κB/P38MAPK mediated stress signaling. Pharmacol Rep. 2017;69(5):908-915. doi: 10.1016/j.pharep.2017.04.002.
38. Li X, Wang C, Wang L, Huang R, Li WC, Wang X, et al. A glutathione-responsive silica-based nanosystem capped with in-situ polymerized cell-penetrating poly(disulfide)s for precisely modulating immuno-inflammatory responses. J Colloid Interface Sci. 2022;614:322-336. doi: 10.1016/j.jcis.2022.01.091.
39. Rodrigues K., Nadaf S., Rarokar N., Gurav N., Jagtap P., Mali P., et al. QBD approach for the development of hesperetin loaded colloidal nanosponges for sustained delivery: In vitro, ex vivo, and in vivo assessment. OpenNano. 2022;7:100045. doi: 10.1016/j.onano.2022.100045.

40. Nanci A., Bosshardt D. Structure of periodontal tissues in health and disease. Periodontol 2000. 2006;40(1): 11–28.

41. Bae JT, Ko HJ, Kim GB, Pyo HB, Lee GS. Protective effects of fermented Citrus unshiu peel extract against ultraviolet-A-induced photoageing in human dermal fibrobolasts. Phytother Res. 2012;26(12):1851-6. doi: 10.1002/ptr.4670.

42. Choi EM, Lee YS. Effects of hesperetin on the production of inflammatory mediators in IL-1beta treated human synovial cells. Cell Immunol. 2010;264(1):1-3. doi: 10.1016/j.cellimm.2010.05.006

43. Liu Z, Li F, Zhang L, Yu H, Yu F, Chen J. The effect of active components from citrus fruits on dentin MMPs. Arch Oral Biol. 2017;83:111-117. doi: 10.1016/j.archoralbio.2017.07.006.

44. Taheri A, Mobaser SE, Golpour P, Nourbakhsh M, Tavakoli-Yaraki M, Yarahmadi S, et al. Hesperetin attenuates the expression of markers of adipose tissue fibrosis in pre-adipocytes. BMC Complement Med Ther. 2023;23(1):315. doi: 10.1186/s12906-023-04152-z.

4 CONCLUSÃO

Contudo, sobre os resultados apresentados, pode-se observar que:

A HT em nanoemulsão apresentou parâmetros satisfatórios de caracterização, com excelente eficiência de encapsulação, morfologia e biocompatibilidade; além de apresentar resultados biologicamente favoráveis no aumento da proliferação celular em osteoblastos, formação de nódulos de mineralização, regulação de genes importantes para o metabolismo ósseo e melhora na produção do colágeno e atividade da fosfatase alcalina;

A HT, assim como a NE-HT associadas ao estímulo elétrico mostraram resultados satisfatórios dos seus efeitos sobre osteoblastos. Sendo que a NE-HT apresentou maior efeito do que HT em sua forma livre, permitindo a adesão celular, aumentando a proliferação celular e regulando a atividade da fosfatase alcalina. Além disso, a NE-HT age melhor na migração celular do que a HT em sua forma livre;

E, por fim, os resultados da NE-HT sobre a inflamação mostram o potencial da mesma em amenizar marcadores inflamatórios, reduzindo a expressão das citocinas TNF-α, IL-1β, IL-6, IL-8 e das MMPs MMP-1, MMP-3 e MMP-9 por monócitos/macrófagos humanos.

REFERÊNCIAS*

1. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH et al. Periodontitis: consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. J Periodontol. 2018; 89(Suppl 1): S173-S182.
2. Taubman MA, Valverde P, Han X, Kawai T. Immune response: the key to bone resorption in periodontal disease. J Periodontol. 2005; 76(11): 2033-41.
3. Silva N, Abusleme L, Bravo D, Dutzan N, Garcia-Sesnich J, Vernal R. Host response mechanisms in periodontal diseases. J Appl Oral Sci. 2015; 23(3): 329-55.
4. Ebbers M, Lübcke PM, Volzke J, Kriebel K, Hieke C, Engelmann R et al. Interplay between *P. gingivalis, F. nucleatum* and *A. actinomycetemcomitans* in murine alveolar bone loss, arthritis onset and progression. Sci Rep. 2018; 8(1): 15129.
5. Kinane DF, Preshaw PM, Loos BG. Host-response: understanding the cellular and molecular mechanisms of host microbial interactions – consensus of the Seventh European Workshop on Periodontology. J Clin Periodontol. 2011; 38(11): 44-8.
6. Graves DT, Li J, Cochran DL. Inflammation and uncoupling as mechanisms of periodontal bone loss. J Dent Res. 2011; 90(2): 143-53.
7. Abdulkareem AA, Abdulbaqi HR, Milward MR. In vitro Homeostasis of rat oral epithelial cell cultures following withdrawal of periodontal pathogens. Braz Dent J. 2020; 31(2): 135-42.
8. Lagha AB, LeBel G, Grenier D. Dual action of highbush blueberry proanthocyanidins on Aggregatibacter actinomycetemcomitans and the host inflammatory response. BMC Complement Altern Med. 2018; 18(1): 10.
9. Schenk M, Bouchon A, Seibold F, Mueller C. TREM-1–expressing intestinal macrophages crucially amplify chronic inflammation in experimental colitis and inflammatory bowel diseases. J Clin Invest. 2007; 117(10): 3097–106.
10. Nanci A, Bosshardt D. Structure of periodontal tissues in health and disease. Periodontol 2000. 2006; 40(1): 11–28.
11. Matarese G, Currò M, Isola G, Caccamo D, Vecchio M, Giunta ML et al. Transglutaminase 2 up-regulation isassociated with RANKL/ OPG pathway in cultured HPDL cells and THP-1-differentiated macrophages.Amino Acids. 2015; 47(11): 2447-55.
12. Graziani F, Karapetsa D, Alonso B, Herrera D. Nonsurgical and surgical treatment of periodontitis: how many options for one disease?. Periodontol 2000. 2017; 75(1): 152-88.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf

13. Preethi Soundarya S, Sanjay V, Haritha Menon A, Dhivya S, Selvamurugan N. Effects of flavonoids incorporated biological macromolecules based scaffolds in bone tissue engineering. Int J Biol Macromol. 2018; 110: 74-87.

14. Nones J, E Spohr TC, Gomes FC. Hesperidin, a flavone glycoside, as mediator of neuronal survival. Neurochem Res. 2011; 36(10): 1776-84.

15. Ahmad S, Alam K, Hossain MM, Fatima M, Firdaus F, Zafeer MF et al. Anti-arthritogenic and cardioprotective action of hesperidin and daidzein in collagen-induced rheumatoid arthritis. Mol Cell Biochem. 2016; 423(1-2): 115-27.

16. Mamun MA, Hosen MJ, Khatun A, Alam MM, Al-Bari MAA. Tridax procumbens flavonoids: a prospective bioactive compound increased osteoblast differentiation and trabecular bone formation. Biol Res. 2017; 50(1): 28.

17. Nobakht M, Trueman SJ, Wallace HM, Brooks PR, Streeter KJ, Katouli M. Antibacterial properties of flavonoids from kino of the eucalypt tree, corymbia torelliana. Plants (Basel). 2017; 6(3): 39.

18. Rothwell JA, Knaze V, Zamora-Ros R. Polyphenols: dietary assessment and role in the prevention of cancers. Curr Opin Clin Nutr Metab Care. 2017; 20(6): 512-21.

19. Cassidy A, Minihane AM. The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. Am J Clin Nutr. 2017; 105(1): 10–22.

20. Muhammad T, Ikram M, Ullah R, Rehman SU, Kim MO. Hesperetin, a citrus flavonoid, attenuates LPS-induced neuroinflammation, apoptosis and memory impairments by modulating TLR4/NF-κB signaling. Nutrients. 2019; 11(3): 648.

21. Ma H, Feng X, Ding S. Hesperetin attenuates ventilator-induced acute lunginjury through inhibition of NF-kappaB-mediated inflammation. Eur J Pharmacol. 2015; 769: 333-41.

22. Ren H, Hao J, Liu T, Zhang D, Lv H, Song E et al. Hesperetin suppresses inflammatory responses in lipopolysaccharide-induced RAW 264.7 cells via the Inhibition of NF-κB and activation of Nrf2/HO-1 pathways. Inflammation. 2016; 39(3): 964-73.

23. Duran Y, Karaboğa İ. Effect of hesperetin on systemic inflammation andhepatic injury after blunt chest trauma in rats. Biotech Histochem. 2020; 95(4): 297-304.

24. He W, Li Y, Liu M, Yu H, Chen Q, Chen Y et al. Citrus aurantium L. and Its flavonoids regulate TNBS-induced inflammatory bowel disease through anti-inflammation and suppressing isolated jejunum contraction. Int J Mol Sci. 2018; 19(10): 3057.

25. Wang B, Li L, Jin P, Li M, Li J. Hesperetin protects against inflammatory response and cardiac fibrosis in postmyocardial infarction mice by inhibiting nuclear factorκB signaling pathway. Exp Ther Med. 2017; 14(3): 2255-60.

26. Wang WJ, Huang YC, Su CM, Ger TR. Multi-functional drug carrier micelles with anti-inflammatory. Drug.Front Chem. 2019; 7: 93.

27. Ye J, Guan M, Lu Y, Zhang D, Li C, Li Y et al. Protective effects of hesperetin on lipopolysaccharide-induced acute lung injury by targeting MD2. Eur J Pharmacol. 2019; 852: 151-8.

28. Chen X, Ding HW, Li HD, Huang HM, Li XF, Yang Y et al. Hesperetin derivative-14 alleviates inflammation by activating PPAR-γ in mice with CCl4-induced acute liver injury and LPS-treated RAW264.7 cells. Toxicol Lett. 2017; 274: 51-63.

29. Trzeciakiewicz A, Habauzit V, Mercier S, Barron D, Urpi-Sarda M, Manach C et al. Molecular mechanism of hesperetin-7-O-glucuronide, the main circulating metabolite of hesperidin, involved in osteoblast differentiation. J Agric Food Chem. 2010; 58(1): 668-75.

30. Liu L, Zheng J, Yang Y, Ni L, Chen H, Yu D. Hesperetin alleviated glucocorticoid-induced inhibition of osteogenic differentiation of BMSCs through regulating the ERK signaling pathway. Med Mol Morphol. 2021; 54(1): 1-7.

31. Rojas BF, Gutierréz-Venegas G. Flavonoids exert multiple periodontic benefits including anti-inflammatory, periodontal ligament-supporting, and alveolar bone-preserving effects. Life Sci. 2018; 209: 435-54.

32. Kim SY, Lee JY, Park YD, Kang KL, Lee JC, Heo JS. Hesperetin alleviates the inhibitory effects of high glucose on the osteoblastic differentiation of periodontal ligament stem cells. PLoS One. 2013; 8(6): e67504.

33. Xue D, Chen E, Zhang W, Gao X, Wang S, Zheng Q. The role of hesperetin on osteogenesis of human mesenchymal stem cells and its function in bone regeneration. Oncotarget. 2017; 8(13): 21031-43.

34. Bayda S, Adeel M, Tuccinardi T, Cordani M, Rizzolio F. The history of nanoscience and nanotechnology: from chemical-physical applications to nanomedicine. Molecules. 2019; 25(1): 112.

35. Kumari A, Yadav SK, Yadav SC. Biodegradable polymeric nanoparticles based drug delivery systems. Colloid Surf. B. 2010; 75: 1–18.

36. Jong WH, Borm PJA. Drug delivery and nanoparticles: applications and hazards. Int J Nanomedicine. 2008; 3(2): 133–49.

37. Abdollahi S, Lotfipour F. PLGA- and PLA-based polymeric nanoparticles for antimicrobial drug delivery. Biomed Int. 2012; 3: 11–6.

38. Santos FK, Oyafuso M, Kiill C, Gremiao MP, Chorilli M. Nanotechnology-based drug delivery systems for treatment of hyperproliferative skin diseases: a review. Curr Nanosci. 2013; 9: 159–67.

39. de Souza ALR, Kiill CP, dos Santos FK, da Luz GM, Rocha e Silva H, Chorilli M et al. Nanotechnology-based drug delivery systems for dermato mycosis treatment. Curr Nanosci. 2012; 8: 512–9.

40. Sato MR, da Silva PB, de Souza RA, dos Santos KC, Chorilli M. Recent advances in nanoparticle carriers for coordination complexes. Curr Top Med Chem. 2015; 15(4): 287-97.

41. Jafarifar Z, Rezaie M, Sharifan P, Jahani V, Daneshmand S, Ghazizadeh H et al. Preparation and Characterization of Nanostructured Lipid Carrier (NLC) and nanoemulsion containing vitamin D3. Appl Biochem Biotechnol. 2022; 194(2): 914-29.

42. Singh Y, Meher JG, Raval K, Khan FA, Chaurasia M, Jain NK et al. Nanoemulsion: concepts, development and applications in drug delivery. J Control Release. 2017; 252: 28-49.

43. Chen LH, Cheng LC, Doyle PS. Nanoemulsion-loaded capsules for controlled delivery of lipophilic active ingredients. Adv Sci (Weinh). 2020; 7(20): 2001677.

44. Chime SA, Kenechukwu FC, Attama AA. Nanoemulsions. Advances in formulation, characterization and applications in drug delivery. In: Sezer AD, editor. Application of Nanotechnology in Drug Delivery. [S.l.]: InTech; 2014 [Acesso em 10 ago. 2023]. Disponível: http://dx.doi.org/10.5772/57028.

45. Kant V, Kumari P, Jitendra DK, Ahuja M, Kumar V. Nanomaterials of natural bioactive compounds for wound healing: novel drug delivery approach. Curr Drug Deliv. 2021; 18(10): 1406-25.

46. Meng S, Rouabhia M, Zhang Z. Electrical stimulation and cellular behaviors in electric field in biomedical research. Materials (Basel). 2021; 15(1): 165.

47. Abedin-Do A, Zhang Z, Douville Y, Méthot M, Rouabhia M. Effect of electrical stimulation on diabetic human skin fibroblast growth and the secretion of cytokines and growth factors involved in wound healing. Biology (Basel). 2021; 10(7): 641.

48. Meng S, Zhang Z, Rouabhia M. Accelerated osteoblast mineralization on a conductive substrate by multiple electrical stimulation. J Bone Miner Metab. 2011; 29(5): 535-44.

49. Cui S, Rouabhia M, Semlali A, Zhang Z. Effects of electrical stimulation on human skin keratinocyte growth and the secretion of cytokines and growth factors. Biomed Mater. 2021; 16(6).

50. Rouabhia M, Park HJ, Abedin-Do A, Douville Y, Méthot M, Zhang Z. Electrical stimulation promotes the proliferation of human keratinocytes, increases the production of keratin 5 and 14, and increases the phosphorylation of ERK1/2 and p38 MAP kinases. J Tissue Eng Regen Med. 2020; 14(7): 909-19.

51. Cui S, Mao J, Rouabhia M, Elkoun S, Zhang Z. A biocompatible polypyrrole membrane for biomedical applications. RSC Adv. 2021; 11(28): 16996-17006.

52. Meng S, Rouabhia M, Shi G, Zhang Z. Heparin dopant increases the electrical stability, cell adhesion, and growth of conducting polypyrrole/poly(L,L-lactide) composites. J Biomed Mater Res A. 2008; 87(2): 332-44.

Não autorizo a publicação deste trabalho pelo prazo de 2 anos

Araraquara, 31 de outubro de 2023.

Maria Júlia Mancim Imbriani