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UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E
BIOQUÍMICA

Efeitos da hidroxiapatita (CaHA) e do ácido poli-lático (PDLA) na bioestimulação de

fibroblastos: Uma análise metabolômica, citotóxica e de citocinas sob a influência da

terapia com ozônio

Aluno: Ludmilla Sousa Quirino

Orientador: Dra. Letícia de Souza Castro Filice

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DRA LETÍCIA DE SOUZA CASTRO FILICE

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APRESENTAÇÃO

O envelhecimento é reconhecido como um desafio global significativo para a saúde, com foco em promover um envelhecimento saudável que estenda a expectativa de vida ao mesmo tempo que assegure boa saúde física e mental. A pele humana, como o maior órgão do corpo, sofre diversas alterações fisiológicas, moleculares e bioquímicas ao longo do tempo, particularmente uma redução na produção de colágeno, que resulta em pele mais fina, menos elástica e mais suscetível a danos.

Para combater esses efeitos do envelhecimento, diversas estratégias têm sido desenvolvidas para estimular a produção de colágeno e melhorar a aparência e funcionalidade da pele. Entre essas estratégias, destacam-se o uso de hidroxiapatita de cálcio (CaHA) e ácido poli-L-láctico (PDLA), que atuam como bioestimuladores, promovendo a produção de colágeno e a regeneração da pele. Além disso, a terapia com ozônio (O3) tem sido proposta como um método para aumentar a oxigenação celular e estimular processos biológicos que favoreçam a saúde da pele.

Neste estudo, investigamos os efeitos de CaHA, PDLA e ozônio na bioestimulação de fibroblastos humanos, na análise metabólica e de citocinas. Os resultados indicaram que esses tratamentos podem efetivamente promover a migração e proliferação de fibroblastos, além de aumentar a produção de colágeno e responder ao estresse oxidativo, sugerindo seu potencial terapêutico na regeneração da pele e no combate aos sinais de envelhecimento. Este trabalho é composto por dois capítulos principais, em que o capítulo I corresponde à fundamentação teórica seguido do capítulo II que contêm o artigo científico, dependente de publicação, com os resultados experimentais.

CAPÍTULO I

1. Contextualização sobre a pele e o envelhecimento

O envelhecimento da pele é um processo natural, complexo e multifatorial resultante da interação entre fatores intrínsecos e extrínsecos. Sendo o primeiro relacionado ao próprio organismo, como a composição genética e hormonal, enquanto o segundo abrange as influências ambientais, como a exposição à radiação ultravioleta (UV), poluição, tabagismo e a dieta. A pele envelhecida sofre várias alterações morfológicas e funcionais, incluindo a perda da elasticidade, surgimento de rugas, manchas senis e a menor hidratação (BURKE, 2018; RORTEAU et al., 2020; TOBIN, 2017; WONG et al., 2016; ZARGARAN et al., 2022).

Neste contexto, vale ressaltar ainda que os fatores intrínsecos incluem uma diminuição progressiva da capacidade de renovação celular e dos componentes estruturais da matriz extracelular (MEC), fundamentais para a integridade da mesma, devido a composição por biomoléculas estruturais como colágeno, elastina, proteoglicanos e glicosaminoglicanos (GAGs). Conforme a progressão da idade, a síntese de colágeno e elastina diminuem, enquanto a atividade das metaloproteinases de matriz (MMPs), enzimas responsáveis pela degradação de proteínas, aumenta. Consequentemente, as alterações morfológicas tornam-se visíveis caracterizando a aparência senescente (CELLENO; TAMBURI, 2009; DENGJEL; BRUCKNER-TUDERMAN; NYSTRÖM, 2020; NYSTRÖM; BRUCKNER-TUDERMAN, 2019)

Os fatores extrínsecos ao organismo, particularmente a exposição aos raios UV, desempenham um papel crucial no envelhecimento cutâneo. A radiação UVB causa danos diretos ao DNA das células epiteliais, enquanto a radiação UVA penetra mais profundamente, na derme, causando danos ao colágeno e induzindo a formação de radicais livres. Estes induzem

estresse oxidativo, que danifica proteínas, lipídios e DNA celular, acelerando o envelhecimento cutâneo. Além disso, a poluição e o tabagismo também contribuem significativamente para o envelhecimento da pele. Poluentes ambientais e compostos tóxicos do tabaco induzem a expressão de MMPs e promovem a inflamação crônica através da ativação do receptor de hidrocarboneto aril (AHR), exacerbando o dano cutâneo (TOBIN, 2017; ZARGARAN et al., 2022).

2. Estrutura da pele

A pele é o órgão mais extenso do corpo humano, atuando como uma barreira protetora do ser humano além de várias outras funções fisiológicas. Ela é composta por três camadas principais: epiderme, a derme e a hipoderme. A primeira, mais externa, um tecido epitelial estratificado pavimentoso queratinizado, composto principalmente por queratinócitos, compõe um obstáculo físico de proteção ao corpo; apresenta também melanócitos, responsáveis pela produção do pigmento melanina que minimiza os danos causados pela radiação UV, células de Langerhans, cujo papel na resposta imunológica cutânea é fundamental, além das células de Meckel que funcionam como mecanoceptores (CELLENO; TAMBURI, 2009; DENGJEL; BRUCKNER-TUDERMAN; NYSTRÖM, 2020; NYSTRÖM; BRUCKNER-TUDERMAN, 2019; WONG et al., 2016).

A segunda camada é a derme, localizada abaixo da epiderme, sendo mais espessa e composta por fibroblastos responsáveis pela síntese de colágeno, elastina e outros componentes da matriz. Além disso, a derme é subdivida em derme papilar e a reticular, contendo uma complexa rede de vasos sanguíneos, terminações nervosas e anexos cutâneos como os folículos pilosos e as glândulas sebáceas fundamentais na regulação térmica através da sudorese e da vasoconstrição e vasodilatação mantendo a homeostase térmica. Não somente, a presença das terminações nervosas contribui para a detecção dos estímulos táteis e térmicos que constroem a percepção sensorial e sensibilidade (NYSTRÖM; BRUCKNER-TUDERMAN, 2019; WONG et al., 2016).

Já a terceira camada, a hipoderme, é composta principalmente por adipócitos que armazenam energia na forma de gordura e fornecem o isolamento térmico e amortecimento contra os impactos fornecendo a proteção e prevenção a danos aos tecidos subjacentes (RORTEAU et al., 2020; WONG et al., 2016).

3. Colágeno e o processo de envelhecimento

Como citado, o colágeno é uma das biomoléculas chaves na pele, sendo o alvo de estudo deste trabalho, o entendimento do processo de síntese é fundamental para contextualização e estudos. A sua síntese começa no núcleo das células produtoras, os fibroblastos, em que os genes de colágeno (nomenclatura comumente utilizada é a *COL*, havendo variação conforme o tipo de colágeno produzido, por exemplo: *COL1A1* para o tipo I) são transcritos em pré-RNAs mensageiros que ao sofrerem *splicing,* processo de maturação de um RNA precursor, forma mRNAs maduros. Estes são transportados para o citoplasma, onde são traduzidos em polipeptídeos chamados pré-procolágenos, no retículo endoplasmático rugoso (RER) (NYSTRÖM; BRUCKNER-TUDERMAN, 2019; PAWELEC; BEST; CAMERON, 2016; SILVA; PENNA, 2012; SORUSHANOVA et al., 2019).

No RER, os pré-procolágenos sofrem modicações pós-traducionais, como a hidroxilação de resíduos de lisina e prolina, processo de adição de grupos hidroxila aos resíduos de lisina e prolina, crucial para a estabilidade da hélice tripla do colágeno. A presença de vitamina C é essencial para essa etapa por ser um cofator das hidroxilases, o que fundamenta a interação metabólica da dieta como um fator extrínseco importante no processo de envelhecimento (SORUSHANOVA et al., 2019).

Outra modificação pós-traducional é a glicosilação em que alguns resíduos de hidroxilisina são glicosilados com a adição da galactose e glicose, importante para solubilidade e posterior construção da biomolécula (DENGJEL; BRUCKNER-TUDERMAN; NYSTRÖM,

2020; GABLER et al., 2018; NYSTRÖM; BRUCKNER-TUDERMAN, 2019; SORUSHANOVA et al., 2019).

Posteriormente, a associação das três cadeias de pré-procolágeno constitui uma hélice tripla que é estabilizada pelas ligações de hidrogênio e interações hidrofóbicas, este complexo é conhecido como pró-colágeno. Este é transportado para o aparelho de Golgi, onde é embalado em vesículas secretoras e liberado no espaço extracelular. E uma vez fora da célula, as procolágeno peptidases clivam os telopeptídeos nas extremidades N e C do pró-colágeno, convertendo-o em tropocolágeno (SORUSHANOVA et al., 2019).

As moléculas de tropocolágeno podem se auto-organizar em fibrilas, um processo chamado de fibrilogênese, impulsionadas por um aumento da entropia total do sistema associada a desordem molecular na interface água-proteína devido a liberação das moléculas de água presentes na estrutura ao redor das moléculas individuais de tropocolágeno. A fibrilogênese pode ser subdivida em nucleação em que há alinhamento e formação de núcleos das fibrilas facilitado pela presença de moléculas auxiliares como a fibronectina e o colágeno tipo V. E em uma fase de crescimento linear e lateral em que após a nucleação, há aumento do diâmetro e maturação das fibrilas com a característica banda D de colágeno (PAWELEC; BEST; CAMERON, 2016; SORUSHANOVA et al., 2019).

As fibrilas de colágeno são estabilizadas por ligações cruzadas covalentes, que são formadas através da ação das enzimas lisil oxidases. Estas enzimas catalisam a oxidação de resíduos de lisina, formando aldeídos que podem reagir com outras moléculas de colágeno para formar ligações cruzadas, aumentando a resistência e a estabilidade das fibrilas de colágeno.

A regulação desse processo ocorre por vários fatores ambientais e moleculares. O pH, a força iônica e a presença de moléculas auxiliares como GAGs e pequenas proteínas ricas em leucina (SLRPs) influenciam a taxa e a qualidade da fibrilogênese. Estas moléculas podem se associar às fibrilas em crescimento, regulando seu diâmetro e a organização final da matriz extracelular (KIRK; AHMED; ROGNONI, 2021; PAWELEC; BEST; CAMERON, 2016; SHOULDERS; RAINES, 2009).

Após a formação e estabilização das fibrilas individuais, estas se associam para formar fibras de colágeno mais grossas e organizadas. Um dos tipos de colágeno mais abundantes na derme é o tipo I. As fibras de colágeno tipo I formadas se organizam em feixes paralelos, que são visíveis ao microscópio eletrônico como estruturas altamente ordenadas. Estes feixes conferem à pele e a outros tecidos conjuntivos uma resistência mecânica significativa, permitindo que resistam a forças de tração e compressão (PAWELEC; BEST; CAMERON, 2016; SHOULDERS; RAINES, 2009; WENGER et al., 2007).

A organização das fibras de colágeno em feixes é regulada por vários fatores, incluindo a densidade e a distribuição das fibrilas, a presença de proteínas reguladoras na matriz extracelular, e o microambiente mecânico ao redor das células produtoras de colágeno. Fatores de crescimento, citocinas e a interação com células circundantes também desempenham um papel importante na regulação deste processo (DENGJEL; BRUCKNER-TUDERMAN; NYSTRÖM, 2020; HE; SHARPLESS, 2017; NOWAG et al., 2024; SORUSHANOVA et al., 2019)

Dentre os fatores de crescimento, se pode citar o TGF-β (NYSTRÖM; BRUCKNER-TUDERMAN, 2019), fator de crescimento transformador beta, um dos reguladores mais importantes da síntese de colágeno. Ele aumenta a expressão dos genes do colágeno tipo I e III em fibroblastos (NYSTRÖM; BRUCKNER-TUDERMAN, 2019), promovendo a produção e a deposição de colágeno na MEC. O TGF-β também regula a atividade de MMPs e seus inibidores naturais, os TIMPs, equilibrando a síntese e a degradação do colágeno (DENGJEL; BRUCKNER-TUDERMAN; NYSTRÖM, 2020; NYSTRÖM; BRUCKNER-TUDERMAN, 2019; PAWELEC; BEST; CAMERON, 2016).

Outro fator é o IGF-1, fator de crescimento insulínico tipo 1, que promove a síntese de colágeno e a proliferação de fibroblastos. Ele atua através da ativação da via de sinalização PI3K/Akt, que é crucial para a sobrevivência celular e a síntese de proteína. Além dele o FGF-2 que estimula a proliferação de fibroblastos e a produção de colágeno, importantes no processo de cicatrização e remodelação da MEC. Da mesma forma, o fator de crescimento de fibroblastos

(FGF), particularmente o FGF-2, estimulam a proliferação de fibroblastos e a produção de colágeno assim como o fator de crescimento epidérmico (EGF) (KIRK; AHMED; ROGNONI, 2021; ROCHA; MAGALHÃES; NUNES, 2020; SHOULDERS; RAINES, 2009; SORUSHANOVA et al., 2019).

Em relação a citocinas, destaca-se o papel das interleucinas (ILs) 1, 6 e 10(PIIPPONEN; LI; LANDÉN, 2020) assim como o fator de necrose tumoral alfa (TNF-α)(PIIPPONEN; LI; LANDÉN, 2020), sendo a primeira e a segunda citocinas pró-inflamatórias. A IL-1, assim como TNF-α, pode ter efeitos duplos na síntese de colágeno, podendo em níveis elevados aumentar a produção de MMPs degradando o colágeno, mas em concentrações fisiológicas estimula sua síntese ativando os fibroblastos. Enquanto a IL-6 tem um papel modulatório na tradução desta biomolécula especialmente durante a resposta inflamatória inicial à lesão, por outro lado a IL-10 é anti-inflamatória com efeito protetor sobre a MEC inibindo a produção de MMPs consequentemente ativando a de colágeno (HÄNEL et al., 2013; PIIPPONEN; LI; LANDÉN, 2020).

A matriz extracelular (MEC), composta por colágeno e outras moléculas, é continuamente remodelada em resposta a sinais fisiológicos e patológicos. A atividade dasMMPs e TIMPs regula o equilíbrio entre a síntese e a degradação do colágeno, mantendo a homeostase tecidual. Com o envelhecimento, o equilíbrio entre a atividade das MMPs e TIMPs pode ser alterado, resultando em uma degradação acelerada do colágeno e comprometendo a integridade da MEC (BURKE, 2018; HE; SHARPLESS, 2017; PAWELEC; BEST; CAMERON, 2016; ROCHA; MAGALHÃES; NUNES, 2020).

A compreensão dos mecanismos subjacentes ao envelhecimento cutâneo e a função dos fibroblastos é essencial para o desenvolvimento de novas estratégias terapêuticas que visam melhorar a saúde da pele e mitigar os efeitos do envelhecimento e das doenças cutâneas. A contínua pesquisa nessa área tem o potencial de levar a avanços significativos na prevenção e tratamento de condições que afetam a aparência e a funcionalidade da pele, promovendo uma

melhor qualidade de vida (KIRK; AHMED; ROGNONI, 2021; ROCHA; MAGALHÃES; NUNES, 2020; TOBIN, 2017).

4. Introdução aos fibroblastos e a linhagem HFF-1

Os fibroblastos são células fundamentais na manutenção e reparo da MEC da pele, desempenhando um papel crucial na síntese de colágeno, elastina e outras proteínas essenciais para a estrutura e função cutânea. Estas células mesenquimais são responsáveis pela produção e organização dos componentes da MEC, conferindo resistência e elasticidade ao tecido dérmico. Além de sua capacidade de síntese, os fibroblastos também respondem a sinais bioquímicos e mecânicos do microambiente cutâneo, modulando sua atividade conforme necessário para processos de cicatrização e regeneração. Com o envelhecimento, a funcionalidade dos fibroblastos é comprometida por danos acumulados ao DNA, disfunção mitocondrial, e alterações epigenéticas, resultando em uma produção reduzida de colágeno e elastina. Este declínio contribui significativamente para a perda de firmeza e elasticidade da pele, características típicas do envelhecimento cutâneo (BURKE, 2018; KIRK; AHMED; ROGNONI, 2021; ROCHA; MAGALHÃES; NUNES, 2020).

5. Estimuladores de colágeno

Para combater a diminuição da síntese de colágeno associada ao envelhecimento, várias estratégias e agentes estimuladores de colágeno têm sido desenvolvidos. Dentre os principais estão o ácido poli-L-láctico (PLLA) e a hidroxiapatita (CaHA)(AHN; RAO, 2014; NOWAG et al., 2024; ZERBINATI; CALLIGARO, 2018).

O PLLA é um polímero biocompatível e biodegradável que estimula a produção de colágeno através de uma resposta inflamatória subclínica, seguida de fibroplasia. Ele é utilizado em procedimentos estéticos para aumentar o volume da pele e melhorar a firmeza e elasticidade ao promover a produção de novo colágeno por meio da indução de uma resposta inflamatória

que envolve a infiltração de neutrófilos e macrófagos nos primeiros dias após a injeção. Esta resposta é seguida pela formação de granulomas e pela deposição de colágeno tipo III, que posteriormente é substituído por colágeno tipo I. A resposta inflamatória inicial é essencial para estimular a produção de colágeno tipo I, mas pode resultar em uma inflamação crônica se não for adequadamente regulada (NOWAG et al., 2024).

Outro bioestimulador, a CaHA atua como uma matriz que favorece a fibroplasia e a formação de novo colágeno. Quando injetada na pele, as microesferas de CaHA são encapsuladas por fibrina, fibroblastos e macrófagos, o que estimula a síntese de colágeno e resulta em um efeito de preenchimento duradouro. Estudos indicam que a CaHA promove uma resposta regenerativa com deposição de colágeno e elastina, similar ao tecido natural saudável, e tende a induzir uma resposta menos inflamatória e mais regenerativa. As microesferas de CaHA servem como um scaffold (andaime) para os fibroblastos, promovendo a produção de colágeno sem desencadear uma resposta inflamatória significativa. Isso resulta em uma regeneração mais organizada da matriz extracelular, com uma deposição equilibrada de colágeno e elastina, melhorando a qualidade e a espessura da pele (ATTENELLO; MAAS, 2015; NOWAG et al., 2024).

Embora existam inúmeros estudos clínicos e pré-clínicos com esses estimuladores, há pouco ou quase nenhum estudo aprofundado a respeito dos efeitos celulares e metabólicos em análises *in vitro*. Sendo assim, este estudo teve como principal objetivo fornecer a comunidade científica, análises citotóxicas, metabólicas e do perfil de citocinas desses compostos sem processamento robusto da matéria prima como uma perspectiva inicial da compreensão dos mecanismos celulares e moleculares em fibroblastos. A fim de estudar os mecanismos dos bioestimuladores de colágeno e o processo de rejuvenescimento, a serem abordados, foi selecionada uma linhagem de fibroblastos para os ensaios abordados no Capítulo 2, tendo sido a HFF-1, derivada de prepúcio humano, isolada em 2003, amplamente utilizadas em pesquisas científicas devido a capacidade de mimetizar as funções naturais da pele em estudos de

envelhecimento, cicatrização e regeneração tecidual (CHENG et al., 2023; GE et al., 2023; LIU et al., 2022; YU; MA; ZHOU, 2023).

6. Ozônio e seu potencial estimulatório

O ozônio é um gás triatômico (O_3) conhecido por suas propriedades oxidantes potentes, que têm sido exploradas em várias aplicações médicas e terapêuticas, incluindo o tratamento de feridas, infecções e condições inflamatórias crônicas. O uso do ozônio na dermatologia tem se mostrado promissor devido aos seus efeitos antimicrobianos e de promoção da cicatrização de feridas(BORGES et al., 2017; FITZPATRICK; HOLLAND; VANDERLELIE, 2018; XIAO; WU; BI, 2021; ZENG; LU, 2018).

Este possui uma potente atividade antimicrobiana devido à sua capacidade de gerar radicais livres e oxigênio reativo, que danificam a membrana celular, proteínas e DNA de microrganismos. Estudos demonstraram que a água ozonizada pode efetivamente destruir biofilmes bacterianos, incluindo aqueles formados por *Staphylococcus aureus* e *Pseudomonas aeruginosa*, comuns em infecções cutâneas e feridas crônicas. A água ozonizada demonstrou eficácia em reduzir significativamente a viabilidade bacteriana em biofilmes após apenas 30 segundos de exposição (BIALOSZEWSKI et al., 2011).

Além disso, tem sido usado na terapia de feridas devido à sua capacidade de aumentar a oxigenação do tecido, estimular a migração de fibroblastos e promover a formação de tecido de granulação. Estudos *in vitro* indicam que fibroblastos, tratamento com soluções salinas ozonizadas (nas concentrações 8, 4, 2, 1, 0.5 and 0.25 µg/mL) resultou em um aumento significativo na migração de fibroblastos, o que é essencial para a formação de tecido cicatricial durante a cicatrização de feridas (BORGES et al., 2017).

O ozônio, quando aplicado em feridas, gera espécies reativas de oxigênio (EROs) que têm um efeito direto sobre microrganismos patogênicos. Além disso, essas EROs podem modular a resposta imunológica local, ativando macrófagos e aumentando a produção de

citocinas anti-inflamatórias e fatores de crescimento, como o fator de crescimento transformador beta (TGF-β) e o fator de crescimento endotelial vascular (VEGF). Estes fatores são essenciais para a cicatrização de feridas, promovendo a angiogênese e a deposição de colágeno (BORGES et al., 2017; CISTERNA et al., 2021).

Estudos mostraram que o ozônio pode aumentar a expressão de genes envolvidos na síntese de colágeno, estimulando os fibroblastos a sintetizarem mais colágeno, o que resulta em uma pele mais firme e elástica (BORGES et al., 2017).

O ozônio oferece uma abordagem eficaz para a promoção da saúde da pele e o tratamento de condições cutâneas, especialmente no contexto do envelhecimento cutâneo. Suas propriedades antimicrobianas, combinadas com a capacidade de estimular a síntese de colágeno e melhorar a cicatrização de feridas, fazem dele uma ferramenta valiosa na dermatologia. Estudos contínuos são necessários para elucidar completamente os mecanismos moleculares de ação do ozônio e otimizar suas aplicações terapêuticas conforme o presente estudo (BIALOSZEWSKI et al., 2011; BORGES et al., 2017; CISTERNA et al., 2021).

Diante do uso clínico dos bioestimuladores citados, o presente estudo teve como objetivo entender in vitro os mecanismos celulares e moleculares em fibroblastos. Foram realizados ensaios citotóxicos e de proliferação, além de análise metabolômica e do perfil de citocinas em cada grupo. Esses aspectos serão detalhados no Capítulo 2, juntamente com os resultados obtidos.

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CAPÍTULO II

Metabolomic and IL-6 cytokine profile responses to Calcium Hydroxyapatite and Polylactic Acid in HFF-1 bioestimulation under Ozone Therapy

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Abstract
The study investigates the effects of calcium hydroxyapatite (CaHA) and poly(DL-lactic acid) (PDLA) on human foreskin fibroblasts (HFF-1), particularly in the context of ozone (O3) therapy. The cell viability assay showed no cytotoxic effects of the bio-stimulators under the tested conditions, with optimal concentrations determined at 300 μg/ml, 100 μg/ml, and 30 μg/ml for CaHA, and 300 μg/ml, 100 μg/ml, and 30 μg/ml for PDLA. Ozone concentrations tested were 5 μg/ml, 10 μg/ml, and 15 μg/ml. The Scratch assay revealed that CaHA and PDLA, both alone and combined with ozone, promoted cell migration and proliferation. Metabolomic

analysis indicated significant activation of inflammatory and collagen synthesis pathways in HFF-1 cells treated with CaHA, PDLA, and ozone. Elevated levels of metabolites such as D-Pantothenic acid and N-(1-Deoxy-1-fructosyl)leucine suggest enhanced biosynthetic activity and oxidative stress responses, contributing to collagen production and tissue renewal. The study concludes that CaHA and PDLA, particularly when combined with ozone, have potential therapeutic applications in enhancing skin regeneration and combating signs of aging.

Keywords: CaHA, PDLA, ozone therapy, metabolomic profile

1. Introduction

The World Health Organization (WHO) recognizes aging as a significant global health challenge. Their focus is on healthy aging, which means extending life expectancy while also promoting good physical and mental health throughout those extra years. Aging is a natural, multifactorial, and heterogeneous process in the human body, characterized by numerous physiological, molecular, and biochemical changes. These alterations are influenced by the lifestyle choices made throughout an individual's life. While these changes may not be systemically significant, they are often readily apparent in physical appearance, primarily due to the decreased availability of collagen in the skin (Burke, 2018; Lemperle et al., 2020; Tobin, 2017; Wei & Ji, 2018; World Health Organization, 2020).

Human skin, a vast organ covering about 1.8 m² of body surface area and constituting about 6% of total body weight, is a complex structure with three primary layers: the epidermis, dermis, and hypodermis. (Celleno & Tamburi, 2009; Roger et al., 2019; Wong et al., 2016). The skin's structural integrity declines as we age, primarily due to decreased collagen production. Collagen, the primary protein in the dermis, provides skin tension and elasticity. In aging, accelerated degradation of collagen and reduced synthesis result in thinner, less elastic

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skin, which is more susceptible to damage and injury (Dengjel et al., 2020; Gentleman et al., 2003; Liao et al., 2008; Nyström & Bruckner-Tuderman, 2019; Pawelec et al., 2016; Shoulders & Raines, 2009; Sorushanova et al., 2019; Wenger et al., 2007).

Collagen biosynthesis with other signaling pathways in fibroblasts, such as FGFR signaling, which promotes cell proliferation and differentiation. Dysregulation in these pathways can lead to fibrotic diseases or impaired wound healing, underlining the importance of a balanced metabolic and signaling environment for optimal collagen production. Understanding these interconnected pathways opens avenues for targeted therapeutic strategies in treating fibrotic conditions and enhancing tissue regeneration (Eming et al., 2017; Nyström & Bruckner-Tuderman, 2019; Pereira et al., 2018; Rocha et al., 2020; Sorushanova et al., 2019).

Given the critical role of collagen in skin health, strategies to stimulate collagen production have become essential in combating the visible signs of aging. Various treatments have been developed, aiming to restore the skin's youthful appearance and functionality. Among these, Poly-D-lactic acid (PDLA), Calcium Hydroxylapatite (CaHA), and ozone therapy have gained popularity (Ahn & Rao, 2014; Attenello & Maas, 2015; Chen et al., 2020; Fitzgerald & Vleggaar, 2010; Keni & Sidle, 2007; Lemperle et al., 2020; Tobin, 2017; Zerbinati & Calligaro, 2018).

PDLA is a racemic mixture comprising an equimolar blend of two enantiomers: PLA (poly-L-lactic acid) and PLA (poly-D-lactic acid). These enantiomers exhibit differing spatial orientations of functional groups around a central carbon atom. Renowned for its biocompatibility and biodegradability, PDLA finds significant application in aesthetic medicine as a collagen stimulator. PDLA acts as a scaffold administered into the skin, progressively triggering the body's natural collagen production. This process enhances skin texture and volume without necessitating direct filler augmentation (Ahn & Rao, 2014; Dellacasa et al.,

2016; Fitzgerald & Vleggaar, 2010; Nofar et al., 2019; Nowag et al., 2024; Sun et al., 2011; Yu et al., 2017).

Another biocompatible material is hydroxyapatite calcium (CaHA), a resorbable biostimulatory filler employed similarly to PDLA but with the added benefit of immediate volume replacement due to its unique composition. It offers an immediate filling effect and encourages natural collagen production over time, improving skin quality and firmness (Nowag et al., 2024, 2023). CaHA and PDLA exhibit distinct mechanical properties, triggering collagen production via separate action pathways. CaHA filler facilitates volume replacement concurrently with its gradual replacement by naturally induced collagen over time. Conversely, PDLA stimulates a bodily inflammatory response that fosters collagen synthesis (Attenello & Maas, 2015; Berlin et al., 2008; Breithaupt & Fitzgerald, 2015; De Melo et al., 2017; Nowag et al., 2024; Use, 2013; Zerbinati & Calligaro, 2018).

From a different angle, ozone therapy, which entails introducing ozone gas into or onto the body, is hypothesized to elevate oxygen levels and trigger biological processes, such as skin rejuvenation and collagen production. While less conventional, proponents argue that it enhances cellular oxygenation and stimulates antioxidant mechanisms, potentially fostering healthier and more robust skin (Bialoszewski et al., 2011; Borges et al., 2017; Zeng & Lu, 2018).

Although the clinical applications and aesthetic benefits of these substances are well known, there is limited information about their mechanisms of action. This study aims to analyze the cellular and metabolic processes of clinically relevant substances in promoting collagen production. By delving into the mechanisms these substances activate in fibroblasts, we aim to significantly advance the scientific understanding in the dermatological, physiological, and pharmaceutical fields

2. Materials and methods

a. Cell culture

Human foreskin fibroblasts (HFF-1), acquired from the Rio de Janeiro Cell Bank (BCRJ/No. 0275), were cultured in Dulbecco's Modified Eagle's Medium (DMEM High Glucose, Gibco™) supplemented with 15% fetal bovine serum (FBS, Cultilab®) and 1X antibiotic-antimycotic solution (Anti-Anti, Gibco™) in a CO_2 incubator at 37°C, 5% CO2 in the Laboratory of Nanobiotechnology, Prof. Dr. Luiz Ricardo Goulart Filho, at the Institute of Biotechnology (IBTEC) of the Federal University of Uberlândia (UFU). The experiments used cells between passages 26 and 33, aiming for estimated confluence and viability above 85%.

b. Preparation and handling of PDLA, CaHA, and ozone collection

The activity of fibroblasts was analyzed upon contact with substances that stimulate collagen production in clinical aesthetic pharmacy, specifically calcium hydroxyapatite [30 μm] (CaHA) (LabTest®) at concentrations of 300 μg/ml, 100 μg/ml, and 30 μg/ml; and poly-lactic acid (PDLA) (Sigma-Aldrich®), diluted in ultrapure type I water (Mili-Q®). Both concentrations and conditions of use were followed and adapted from clinical practice protocols (Cisterna et al., 2021; Fitzgerald & Vleggaar, 2010; Goldie et al., 2018).

Furthermore, the study aimed to evaluate ozone gas (O3) as a potential enhancer of the effects of the biostimulators on collagen production and an agent capable of eliciting this physiological response. Thus, both non-associated and associated use of CaHA and PDLA, at the respective concentrations, were tested at 5 μg/ml, 10 μg/ml, and 15 μg/ml in ozonized high glucose DMEM medium. Ozone gas was collected in 10 ml syringes (Descarpack®) from the ozone generator (Ozone&Life®) for *in vitro* experiments.

For the analyses, the following combinations were tested: PDLA + O3, CaHA + O3, as well as individual treatments with PDLA, CaHA, and O3. Each combination and individual treatment was tested at three different concentrations of each biostimulator.

c. Cell Viability Assay by resazurin reduction

Initially, HFF-1 was transferred to a black microplate with a clear bottom for 96-well cell culture (CellStar®, Greiner Bio-One) with $2x10^4$ cells per well and treated with the bio-stimulators (10 μL) at the concentrations described for 24 hours, stored in a 37°C, 5% CO2 incubator. As an experimental control, untreated HFF-1 cells were cultured in high glucose DMEM supplemented with 15% FBS, and in another control, cells were treated with 10% DMSO. The assay was performed in triplicate (Riss et al., 2004).

Subsequently,20 μL of resazurin solution at a final concentration of 0.15 mg/ml was added to each well and incubated for 4 hours, with evaluation at 1 to 4 hours per protocol to determine reduction based on the untreated control (positive).

Fluorimetric analysis was conducted using the EnSpire® Multimode Plate Reader, Detection occurred at an emission wavelength of 590 nm, with excitation at 560 nm. The following formula was used for calculations:

$$\% \, resazurin = \frac{FI \, 590 \, of \, treated \, cells}{FI \, 590 \, of \, control} \, x \, 100$$

In an independent experiment, HFF-1 cells were treated with PMA (Phorbol-12-myristate-13-acetate) (Sigma®) and LPS (Lipopolysaccharide from Salmonella, reconstituted in 1x PBS), both at a concentration of 1 mg/mL, to induce an inflammatory response without compromising the viability of the cultured cells. A serial dilution was performed (1:2 to 1:128 dilutions) in an incomplete DMEM medium (without FBS).

d. Cell migration and proliferation analysis by Scratch assay

Scratch assay (Martinotti & Ranzato, 2020) is a valuable tool for understanding the cellular mechanisms involved in wound healing, cell migration, and proliferation. It provides essential insights for research in cell biology, regenerative medicine, and pharmacology. For this assay, $5x10^4$ cells were seeded per well in a 24-well microplate (Falcon®).

After cell adhesion, a scratch was strategically created perpendicular to the bottom of the well using a 200 μL pipette tip. Subsequently, the wells were gently washed with 1x PBS to remove non-adherent cells. Next, the cells were exposed to CaHA (30 μg/ml), PDLA (100 μg/ml), and ozone (10 μg/ml), with concentrations determined based on cell viability results. Cultures were maintained in a 37°C, 5% CO_2 incubator to enable assessment of collective cell proliferation and migration. Cells were visualized and photodocumented by microscopy at 0h, 8h, 24h and 48h for analysis using ImageJ® software.

The following formula was employed for calculating the closure area:

$$\% \, wound \; closure = \left[\frac{At = 0h - At = \Delta h}{At = 0h} \right] x \; 100\%$$

In this equation, At=0h corresponds to the area immediately after scratching, and At=Δh represents the area at the time point Δh. Regarding the migration rate, the ratio of the difference between initial (Wi) and final (Wf) widths to time (t) in hours is calculated as follows:

$$Migration's \, rate = \frac{Wi - Wf}{t}$$

e. Metabolic Analysis by Mass Spectrometry

HFF-1 cells were plated in a 96-well plate with 2×10^4 cells per well and exposed to the bio-stimulators (CaHA 30μg/ml and PDLA 100 μg/ml) and ozone (10 μg/ml) as previous To understand the metabolic profile of the proposed study, 100 μL of each group were aliquoted, and 1000 μL of methanol (J.T. Baker®, Avantor Performance Materials, S.A, Mexico) were added to a 2 ml microtube and vortexed for 5 minutes.

After homogenization, the samples were centrifuged for 15 minutes at 13000 xg, and the supernatant was transferred to a 2 ml microtube subjected to overnight vacuum concentration (GeneVac™ miVac Duo Concentrator, Fisher Scientific®). Subsequently, the material was resuspended in 400 μL of methanol and vortexed for 10 minutes.

Upon completion of the metabolite extraction process, the samples were filtered through a 0.22 μm filter and transferred to 2 ml vials (Clear Glass, Flat Base, Alwsci Technologies®), which were then injected into a high-resolution mass spectrometer of the Q-TOF type (model 6520B, Agilent®) with electrospray ionization (ESI) source.

The components were analyzed using a high-resolution mass spectrometry technique, considering a margin of error of less than ten ppm regarding the exact mass. MassHunter Qualitative v.10.0 software was used for raw data processing, and the Molecular Feature Extraction tool was used for the extraction of mass spectra and conversion.

Agilent Mass Profiler Professional software version B. 13.1.1 filtered and aligned the obtained molecular compounds, followed by identification through the METLIN database. These data were compared with databases and information available in the literature.

f. Cytokine analysis by flow cytometry

For cytokine analysis,the CBA Human Inflammatory Cytokines Kit (BD Biosciences®) was used. Briefly, cells were exposed to CaHA (30 μg/ml), PDLA (100 μg/ml), ozone (10 μg/ml), and 6,25 μg/ml Phorbol 12-Myristate 13-Acetate (PMA) (Sigma Aldrich®, USA), their supernatants collected and used after 24 hours of incubation.

According to the kit protocol guidelines, cytometry standard samples were prepared, and the capture microbeads were mixed correctly. Subsequently, 50 μL of these microbeads, 50 μL of human standard inflammatory cytokine sample, and 50 μL of the samples were added to the test tubes. The detection reagent, in a volume of 50 μL, was then added, and the tubes were incubated for 3 hours at room temperature, protected from light.

After the incubation period, 1 mL of wash buffer was added to each test tube and centrifuged at 200 x g for 5 minutes. The supernatant was carefully discarded, and 300 μL of buffer for resuspending the microbead pellet was added. Finally, the samples were analyzed on the flow cytometer (BD Accuri™ C6, Becton Dickson, New Jersey, USA).

Before data extraction, a threshold adjustment of 1,000,000 in FSC and 50,000 in FL4 was applied to the samples to clean the files. After this step, the files were downloaded in FCS

format and the results were analyzed using CBA Analysis Software v. 1.1.15 from BD Biosciences.

g. Statistical Analysis

After quantification in each experiment, the means were determined, and statistical analysis was conducted using GraphPad Prism® 8.0.2 software (GraphPad Software Inc., San Diego, CA, USA). This analysis included checking for normality using the Shapiro-Wilk test and applying one-way analysis of variance (ANOVA) with Tukey's post hoc test, where probability values of 0.05% were considered statistically significant.

3. Results

a. Cell Viability Assay by resazurin reduction for determining optimal concentrations of PDLA, CaHA and ozone.

The results obtained from the resazurin reduction assay demonstrate that the HFF-1 cells, which were subjected to bio-stimulators at specified concentrations **(Figure 1)**, did not exhibit any significant reduction in cell viability compared to the untreated control according with ISO 10993-3. This suggests that the bio-stimulators had no cytotoxic effect on the fibroblasts under the conditions tested. Furthermore, the assay helped to validate the viability of the cells utilized in the experiment, thus ensuring the reliability of the data for further analysis.
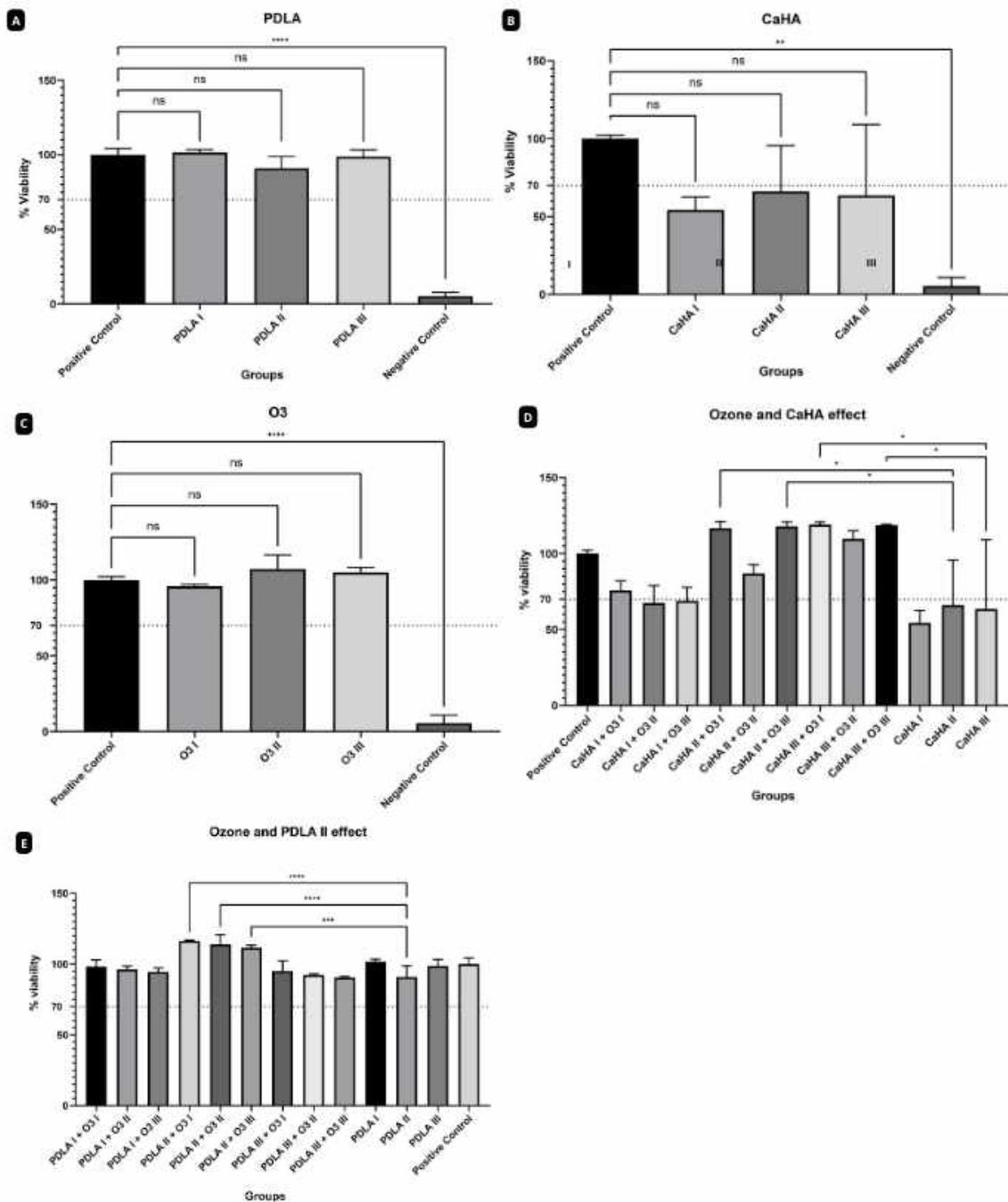
Figure 1: Statistical Analysis of bio-stimulators with and without ozone treatment. Graphs representing the statistical analysis of bio-stimulators (Panels A-E), isolated and combined with ozone. No significant differences were observed between

the positive control group (HFF-1 cells untreated and cultured in high-glucose DMEM) and the treated groups, indicating no toxic effects from the treatments.

Figure 1 displays the graphs from the bio-stimulator statistical analysis, both in isolation and when combined with ozone. According to the data, no toxic effects on HFF-1 cells were observed. Images of the wells clearly show visual impairment in the CaHA group due to saturation at different concentrations, indicating the need for a more effective solvent and the crystallization of PDLA, highlighting its amphiphilic nature. The primary objective of this study was to evaluate the compounds in their original conditions, as they are used on clinical practice, without substantial modifications to improve solubility, serving as an initial reference for the unprocessed material study.
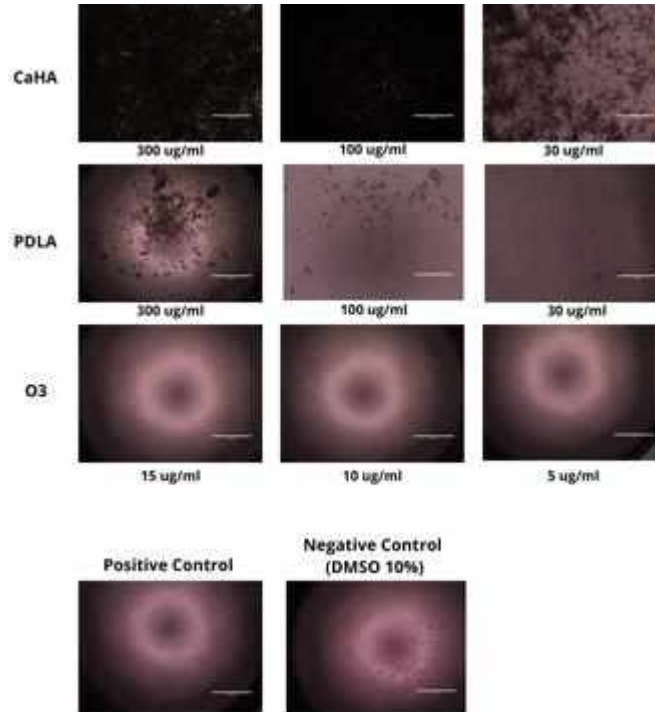

Figure 2: Microscopic imagens of bio-stimulators with and without ozone treatment. Images represent the isolated bio-stimulators in HFF-1 cells. The top row shows Calcium Hydroxyapatite (CaHA) at concentrations of 300 μg/ml, 100 μg/ml, and 30 μg/ml. The middle row shows Poly-lactic acid (PDLA) at concentrations of 300 μg/ml, 100 μg/ml, and 30 μg/ml. The bottom row depicts Ozone (O3) treatment at concentrations of 15 μg/ml, 10 μg/ml, and 5 μg/ml. The last row includes the positive control, only HFF-1, and negative control (10% DMSO) for comparison.

The ozone-treated samples did not show visual changes, which was anticipated due to the gaseous conditions. Furthermore, there was no need for image compilation in the combination treatments as there were no significant changes compared to the other groups. Image recording was performed before the assay, and resazurin was added, acting as a quality control measure. Fluorescence was assessed due to its higher sensitivity, as reported in the literature, and considering the saturation of some compounds that impeded absorbance analysis.

b. Cell migration and proliferation analysis by Scratch assay

Cell migration and proliferation was assessed by the scratch assay, treating cells with PDLA (100 μg/ml), O3 (10 μg/ml), and CaHA (30 μg/ml), as well as untreated cells for control (Figure 5).
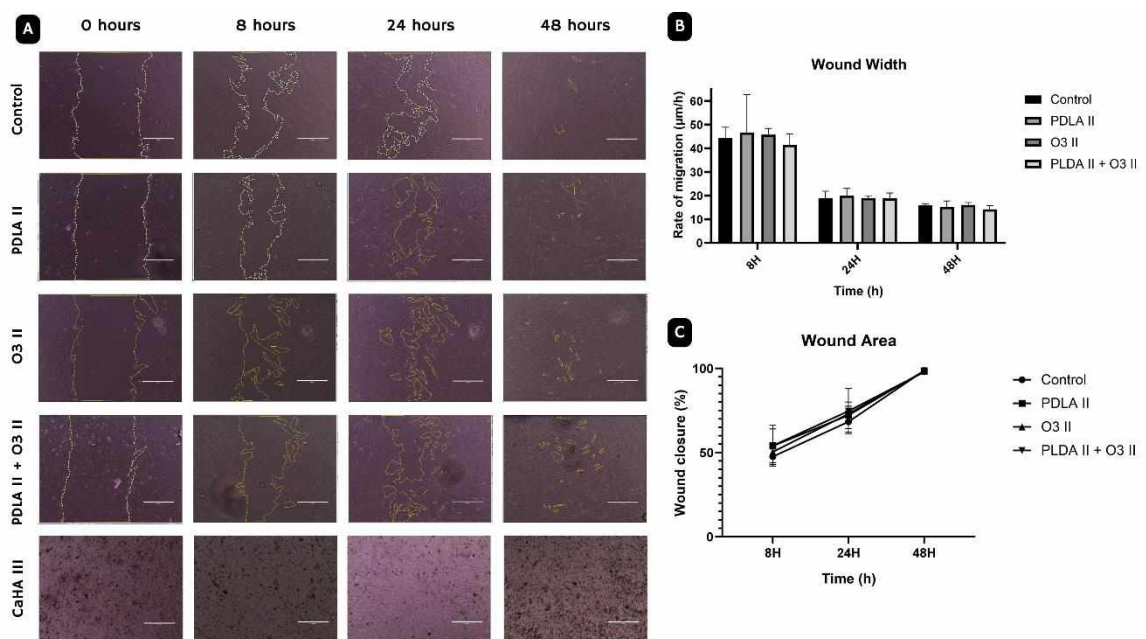
Figure 3: Cell migration was evaluated using the Scratch Assay under different experimental conditions. (A) The images show HFF-1 migration at 0, 8, 24, and 48 hours for the control, PDLA II, O3 II, PDLA II + O3, and CaHA III groups. Yellow marks indicate the boundaries of the cell-free area (scratch) at each time point. (B) Quantitative analysis is provided for the migration rate (μm/h) at each time point. (C) The percentage of wounds over time is shown for each group.

The qualitative **(Figure 3a)** observations revealed that the PDLA II, O3 II, and PDLA II + O3 II conditions exhibited significant cell migration and wound closure similar to the control. However, the CaHA III condition could not be analyzed due to granularity. Quantitative measurements **(Figure 3b-c)** showed that PDLA II, O3 II, and their combination had comparable migration rates and wound closure percentages to the control group, with over 80% closure at 48 hours. Conversely, CaHA III demonstrated significantly lower migration rates and less than 20% wound closure. These findings suggest that PDLA II and O3 II effectively promote cell migration, while CaHA III was not suitable for investigation.

c. Metabolic Analysis by Mass Spectrometry

We evaluated the metabolic profile of human fibroblasts (HFF-1) following exposure to CaHA, PDLA, and ozone using high-resolution mass spectrometry. The analyses were conducted across twelve experimental groups, each with six replicates, as detailed below:
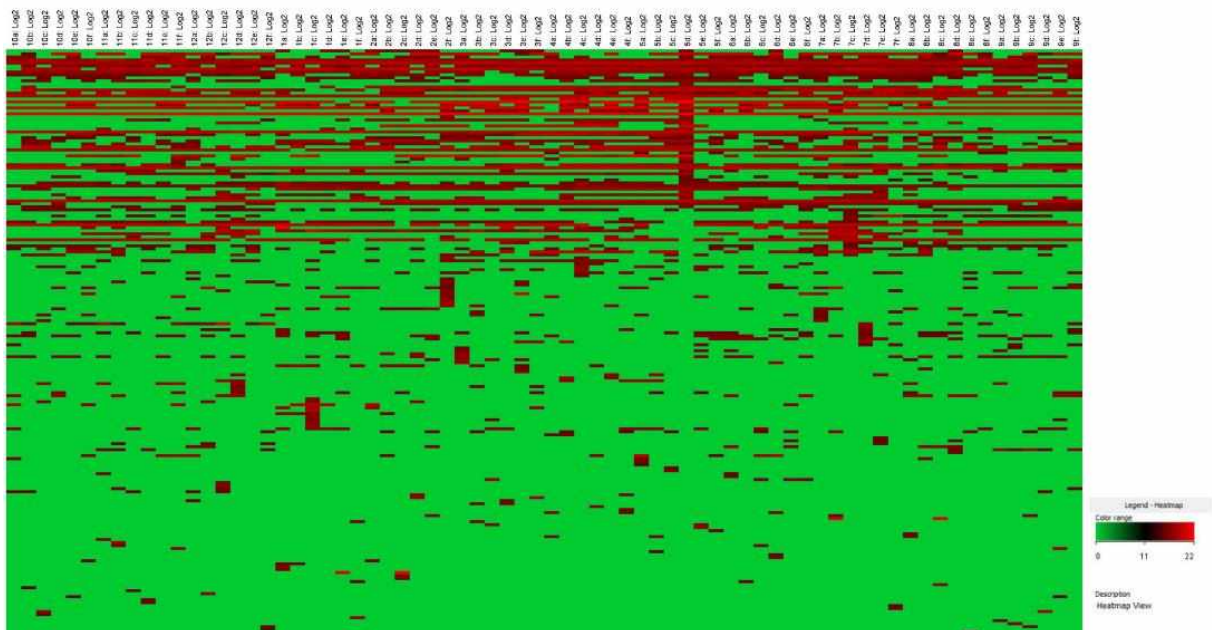

Figure 6: Metabolic Profile Analysis of HFF-1 under Different Treatments. Each group represents human fibroblasts (HFF-1) subjected to various combinations of PMA, CaHA, PDLA, and ozone. Overall Metabolite Heatmap: The heatmap

displays the expression profile of the identified metabolites across the different experimental groups. Each row in the heatmap represents a specific metabolite, while each column represents an individual sample from the experimental groups. The colors on the heatmap spectrum from green to red, indicate the intensity of metabolite expression, with green denoting low expression and red denoting high expression. The color variations mirror the log2 changes in metabolite levels between the different experimental conditions.

A total of 196 metabolites were identified during the analysis. Differentially expressed metabolites were selected for 26 combinations **(Figure 4)**, with no metabolites found in 2 combinations. The selection criteria for the metabolites **(Figure 4)** were based on statistical significance and biological relevance. Specifically, metabolites with a p-value < 0.05 were considered statistically significant. Additionally, the changes in abundance with the presence of PMA and their known involvement in inflammatory processes, collagen synthesis, or cellular stress responses were evaluated.

Metabolite	Synonyms	Biological Relevance	Regulation	HMDB	PubChem	ChEBI	KEGG	References
N-(1-Deoxy-1-fructosyl)leucine	Fructose-leucine/ 1-[(1-Carboxy-3-methylbutyl)amino]-1-deoxyfructose	Potential indicator of inflammatory or stress response	Up	37840	131752244	136629	Not found	Bernardo-Bermejo et al., 2019, 2023; García et al., 2022; Sanchez-Espirilla et al., 2023)
D-Pantothenic acid		Precursor of Coenzyme A	Up	210	6613	7916	C00864	Dattola et al., 2020; Peltier et al., 2022; Scott et al., 2022; Srivastava et al., 2014; Zhang et al., 2024
Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole	Related to protein synthesis and amino acid metabolism	Down	29746	5284474	82376	C19306	Hashimoto et al., 2004; Yamazoe et al., 2023; Yun et al., 2006
3-Methylhexanal		Indicator of oxidative stress, but lack of evidences related with human	Up	32405	140511	88822	Not found	Magnano et al., 2024; Saxena et al., 2021
Xestoaminol C	(2S,3R)-2-aminotetradecan-3-ol	Related to cellular signaling or stress response	Up	304813	14756407	73882	Not found	Abraham et al., 2008; Eming et al., 2017; Nováková et al., 2020
Prolyl-Arginine	Pro-Arg	Associated with oxidative stress	Down	29011	151004	73645	Not found	Wang et al., 2009

Table 1: Key Metabolites identified in HFF-1 stimulated with PMA, PDLA, CaHA, and O3. This table summarizes the key metabolites identified in the metabolomic profiling of HFF-1 under various stimulation conditions. The metabolites are listed alongside their synonyms, biological relevance for the study, regulation status, database identification, and literature.

The results indicate a significant activation of inflammatory and collagen synthesis pathways in HFF-1 fibroblasts treated with PDLA, CaHA, and O3. PDLA is known to induce a controlled inflammatory response that promotes collagen production. This effect is corroborated by the elevated presence of inflammatory metabolites, such as N-(1-Deoxy-1-fructosyl)leucine (Bernardo-Bermejo et al., 2019, 2023; García et al., 2022; Sanchez-Espirilla et al., 2023). Furthermore, the increase in metabolites like 3-methylhexanal (Magnano et al., 2024; Saxena et al., 2021) is related to oxidative stress, that could be related to the regenerative and healing processes **(Table 1)**.

Similarly, CaHA serves as a matrix for collagen deposition, directly stimulating fibroblasts. The elevation of metabolites related to collagen synthesis, such as D-Pantothenic acid (Dattola et al., 2020; Peltier et al., 2022; Scott et al., 2022; Srivastava et al., 2014; Zhang et al., 2024), indicates that CaHA facilitates the biosynthetic activity. Moreover, metabolites like Xestoaminol C (Abraham et al., 2008; Eming et al., 2017; Nováková et al., 2020) suggest modulation of inflammatory responses and cellular signaling, which are crucial for tissue remodeling. When O3 is combined with CaHA, there is modulation of inflammatory responses and cellular signaling, as indicated by metabolites like Xestoaminol C. Thus, CaHA, acting as a matrix for collagen deposition, benefits from inflammatory properties of O3. The controlled inflammatory response induced by O3 can enhance the efficacy of CaHA in stimulating fibroblasts for collagen production, resulting in more effective tissue remodeling.

Additionally, ozone, known for and inflammatory properties, induces the positive regulation of inflammatory compounds, suggesting the activation of these pathways. Presence of metabolites associated with oxidative stress, such as 3-methylhexanal, related to alteration in cellular redox balance, promoting adaptive responses.

The combination of ozone (O3) with PDLA showed interesting results at the metabolic level of HFF-1 fibroblasts. O3 is known to induce inflammatory and oxidative stress responses, which can potentially complement the effects of PDLA in promoting collagen synthesis. The presence of metabolites associated with oxidative stress and inflammation, such as 3-Methylhexanal, could indicate that O3 intensifies the controlled inflammatory response already induced by PDLA. This synergy can result in greater collagen production and tissue renewal, leveraging the regenerative properties of PDLA and the pro-inflammatory effects of O3, but it is necessary more studies and evidence related to this response.

Considering the lack of evidence regarding the direct link between Trp-P-1 (3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole) and protein synthesis and its known role in carcinogenesis, it would be prudent to interpret its presence in this study showing a negative regulation as due to suppressed activity with the treatments (Hashimoto et al., 2004; Yamazoe et al., 2023; Yun et al., 2006).

The treatments with PDLA, CaHA, and O3 demonstrate the induction of a controlled inflammatory response that promotes collagen production. The increase in pantothenic acid could be related to the metabolic and biosynthetic activity of the cells. Additionally, the oxidative stress-indicative metabolites suggest that these treatments promote tissue renewal through stress response mechanisms.

Therapeutically, these findings support the use of PDLA, CaHA, and O3 in aesthetic contexts to promote collagen production and tissue renewal. The induction of controlled inflammatory pathways and the modulation of cellular metabolism are key mechanisms through which these compounds exert their beneficial effects.

Moreover, PMA (Phorbol 12-myristate 13-acetate) is a well-known activator of protein kinase C (PKC) and is used to induce a cellular inflammatory response. Exposure to PMA resulted in significant changes in the metabolic profile of HFF-1 fibroblasts, evidenced by the

up-regulation of inflammatory metabolites such as N-(1-Deoxy-1-fructosyl)leucine and D-Pantothenic acid, and down-regulation of Prolyl-Arginine (Wang et al., 2009).

These changes indicate a robust activation of inflammatory pathways and an increase in biosynthetic activity, essential for collagen production. The combination intensifies the inflammatory response, further increasing collagen production due to the synergy between PKC activation by PMA and the regenerative responses induced by PDLA. Inflammatory and oxidative stress metabolites are significantly elevated, suggesting a highly reactive cellular environment conducive to tissue remodeling.

The addition of PMA to CaHA treatment enhances fibroblast stimulation. Metabolites related to collagen synthesis and inflammation are increased, indicating that PMA amplifies the effects of CaHA in promoting collagen production. The combination of O3 and PMA results in an exacerbated inflammatory and oxidative stress response. The high levels of inflammatory metabolites suggest that PMA enhances the pro-inflammatory effects of O3, creating a cellular environment that favors tissue renewal and collagen production.

To strengthen the conclusions and expand the analysis spectrum of these compounds' effects, further studies are necessary, such as gene expression profiling to identify genes related to collagen synthesis, protein expression assays, and quantification of these inflammatory markers.

d. Cytokine analysis by flow cytometry

Unfortunately, quantitative analysis of the cytokines was not possible due to a technical issue with the software. Other software programs were tested, but they also showed irregularities in extracting the results. The scientific-technical support from BD Biosciences was contacted, and as of now, the results have not been obtained.

4. Discussion

The study's findings demonstrate that calcium hydroxyapatite (CaHA) and poly(DL-lactic acid) (PDLA) at tested concentrations (300 µg/ml, 100 µg/ml, 30 µg/ml for both), along with ozone (O3) therapy (5 µg/ml, 10 µg/ml, 15 µg/ml), effectively promote the biostimulation

of human foreskin fibroblasts (HFF-1) without exhibiting cytotoxic effects. This was verified through the resazurin reduction assay, confirming cell viability across all treatment groups (Borges et al., 2017; Cisterna et al., 2021; Dellacasa et al., 2016; Nowag et al., 2024; Zerbinati & Calligaro, 2018).

Furthermore, Scratch assay results indicated enhanced cell migration and proliferation when HFF-1 cells were treated with CaHA and PDLA, both alone and in combination with ozone. Other studies demonstrated similar behavior with derived compounds, such as ozone oil promoting wound healing on fibroblasts (Xiao et al., 2021) and hydroxyapatite enhanced bone growth due to cell's migration (Lemoine et al., 2022).

The metabolomic analysis provided valuable insights, revealing a significant activation of inflammatory and collagen synthesis pathways in fibroblasts following treatment with CaHA, PDLA, and ozone. Although concrete evidence and *in vitro* studies were absent, educated inferences were made about the significance of each metabolite and its biological relevance, as outlined in the results.

In addition, the qualitative cytokine analysis using the CBA Human Inflammatory Cytokines Kit highlighted the presence of IL-6 in all treated groups, including those stimulated with PMA. IL-6's role in regulating immune and inflammatory responses, crucial for effective tissue repair and collagen synthesis, underlines the importance of the inflammatory response in enhancing collagen production and supports the therapeutic benefits of combining CaHA and PDLA with ozone therapy.

It's important to note that the presence of Trp-P-1 (3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole) in this study, showing negative regulation, does not necessarily imply a direct link between Trp-P-1 and protein synthesis. Given its known role in carcinogenesis, it would be prudent to interpret its presence as a result of suppressed activity with the treatments (Hashimoto et al., 2004; Yamazoe et al., 2023; Yun et al., 2006). These treatments with PDLA, CaHA, and O3 appear to induce a controlled inflammatory response that promotes collagen production, suggested by the increase in pantothenic acid, enhancing the metabolic and

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biosynthetic activity of the cells essential for collagen production. Additionally, the presence of oxidative stress-indicative metabolites suggests that these treatments promote tissue renewal through stress response mechanisms.

Moreover, exposure to PMA (Phorbol 12-myristate 13-acetate) results in significant changes in the metabolic profile of HFF-1 fibroblasts (Figure 10), evidenced by the up-regulation of inflammatory metabolites such as N-(1-Deoxy-1-fructosyl)leucine and D-Pantothenic acid, and down-regulation of Prolyl-Arginine (Wang et al., 2009). These changes indicate a robust activation of inflammatory pathways and an increase in biosynthetic activity, essential for collagen production. The combination intensifies the inflammatory response, further increasing collagen production due to the synergy between PKC activation by PMA and the regenerative responses induced by PDLA. The study shows that inflammatory and oxidative stress metabolites are significantly elevated, suggesting a highly reactive cellular environment conducive to tissue remodeling.

Additionally, the addition of PMA to CaHA treatment enhances fibroblast stimulation. Metabolites related to collagen synthesis and inflammation are increased, indicating that PMA amplifies the effects of CaHA in promoting collagen production. The combination of O3 and PMA results in an exacerbated inflammatory and oxidative stress response. The high levels of inflammatory metabolites suggest that PMA enhances the pro-inflammatory effects of O3, creating a cellular environment favorable for tissue renewal and collagen production. To further strengthen the conclusions and expand the analysis spectrum of these compounds' effects, additional studies are necessary, such as gene expression profiling to identify genes related to collagen synthesis, protein expression assays, and quantification of inflammatory markers.

5. Conclusion

This study aimed to investigate the effects of different concentrations of calcium hydroxyapatite (CaHA) and poly(DL-lactic acid) (PDLA), both individually and in combination with ozone (O3) therapy, on the biostimulation of human foreskin fibroblasts (HFF-1) and collagen production. The results indicated that these substances do not exhibit

cytotoxic effects at the tested concentrations (CaHA: 300 μg/ml, 100 μg/ml, 30 μg/ml; PDLA: 300 μg/ml, 100 μg/ml, 30 μg/ml; Ozone: 5 μg/ml, 10 μg/ml, 15 μg/ml). Furthermore, the Scratch assay demonstrated that CaHA and PDLA, both alone and combined with ozone, promote fibroblast migration and proliferation.

Additionally, metabolomic analysis revealed significant alteration of inflammatory and collagen synthesis pathways in fibroblasts treated with CaHA, PDLA, and ozone. Elevated levels of metabolites such as D-Pantothenic acid and N-(1-Deoxy-1-fructosyl)leucine suggest enhanced biosynthetic activity and oxidative stress responses, contributing to collagen production and tissue renewal.

Furthermore, qualitative cytokine analysis using the CBA Human Inflammatory Cytokines Kit indicated the presence of IL-6 across all treated groups, including those stimulated with PMA. This finding suggests that the treatments promote an inflammatory response that is consistent with increased collagen synthesis, as IL-6 is a key regulator of immune and inflammatory responses.

In conclusion, CaHA and PDLA, especially when combined with ozone therapy, show significant potential for enhancing skin regeneration and combating signs of aging. These findings pave the way for further investigations into the molecular mechanisms underlying these effects and support their application in clinical dermatology and aesthetic medicine. Future research should focus on validating these effects in vitro and *in vivo* and exploring the detailed molecular pathways involved, providing a solid foundation for clinical and therapeutic applications.

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