JULIANA AMORIM DOS SANTOS

ESTRATÉGIAS PARA O REPARO TECIDUAL:

DE MODELOS COM MONOCULTURA A SISTEMAS BIOMIMÉTICOS *IN VITRO*

Strategies to enhance wound repair:

from monoculture models to *in vitro* biomimetic systems

BRASÍLIA, 2024

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PROGRAMA DE POS GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

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Orientadora: Prof. Dra. Eliete Neves Silva Guerra

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Dedico este trabalho aos estudantes de pós-graduação, para que não deixem o desafio ser maior que a coragem. Sigam firmes!

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“The only way to learn is to live. If you aim to be something you are not, you will always fail. Aim to be you. Aim to look and act and think like you.”

RESUMO

O reparo de lesões envolve um processo complexo, especializado e multifásico de resposta celular e tecidual. Complicações nesse processo podem resultar em tecido cicatricial anormal, infecções e reparo prolongado, afetando a qualidade de vida dos pacientes e impactando nos custos dos sistemas de saúde publica. Esse estudo objetivou aplicar técnicas de cultura celular para explorar estratégias emergentes em reparo de lesões. A pesquisa foi organizada em três capítulos, sendo eles: (1) O Capítulo 1 teve como objetivo avaliar os feitos da curcumina em queratinócitos e fibroblastos expostos à estímulos bacterianos e radiação ionizante. Utilizando modelos em monocultura e em co-cultura, foram realizados ensaios de fechamento de lesão, viabilidade celular, morfologia celular e expressão gênica de *PIK3CA*, *AKT*, *PTEN*, *MTOR* e *IL-6*. Os resultados demonstram que a co-cultura promove significativamente o fechamento das lesões, com a curcumina melhorando a resposta dos queratinócitos, mas não dos fibroblastos. A curcumina aumenta a viabilidade celular e altera a morfologia das células, reduzindo a área do citoplasma dos queratinócitos e ampliando a área nuclear dos fibroblastos. A análise da expressão gênica revelou que a curcumina reduziu significativamente a expressão de *AKT,* ao mesmo tempo que tendeu a elevar *MTOR* e *PTEN* em queratinócitos. Em fibroblastos, a curcumina diminui significativamente a expressão de *MTOR* e tendeu a reduzir *PIK3CA* e *IL-6* nos fibroblastos. Esses achados sugerem que a curcumina modula os mecanismos de reparo de forma diferente em queratinócitos e fibroblastos, destacando seu papel complexo na reparação celular. O estudo enfatiza o potencial terapêutico da curcumina para o reparo tecidual e a necessidade de mais pesquisas para o uso terapêutico durante a radioterapia. (2) O Capítulo 2 objetivou realizar uma revisão metodológica de protocolos atuais de bioimpressão 3D usando queratinócitos e fibroblastos humanos com foco em reparo tecidual, seguindo as diretrizes PRISMA 2020. Dezoito estudos focados em substitutos de pele foram incluídos, com 83,3% utilizando técnicas de bioimpressão por extrusão e bioinks compostos por gelatina (61,6%), fibrinogênio (38,8%) e alginato (33,3%). A distribuição geográfica mostrou maior concentração na China (44,4%) e nos Estados Unidos (27,7%). Muitos estudos validaram seus resultados implantando a pele artificial em modelos animais para avaliar o reparo. A revisão destaca a necessidade de protocolos padronizados para melhorar a replicabilidade e o potencial de aplicação clínica da bioimpressão 3D. (3) Por fim, o Capítulo 3 teve o objetivo de desenvolver um scaffold 3D biomimético capaz de fazer entrega de células epiteliais. Este estudo investigou o potencial scaffolds de colágeno bovino neutro de 70mg/mL bioimpressos carregados com queratinócitos, utilizando uma bioimpressora robótica e a bioink Lifeink® 220. Avaliaram-se a difusão de fluidos, a estabilidade dimensional e a fidelidade de impressão dos scaffolds ao longo de 7 dias. Ao utilizar um design com interconexões paralelas em comparação com um design sólido, o design interconectado apresentou melhor promoção da migração celular, mostrando aumento significativo na área coberta e maior viabilidade celular ao longo do tempo. Por outro lado, o design sólido foi capaz de formar mais unidades de expansão celular dentro do scaffold. Ao considerar o scaffold interconectado com densidade celular inicial maior que 150 cell/mm^2, esse design foi capaz de resultar não apenas em

migração para o recobrimento, como também em formação das unidades de expansão dentro do scaffold e aumento do número de celular após três dias. Assim, ambos o modelo do scaffold e a densidade celular inicial foram capazes de modular a resposta celular e otimizar o recobrimento da área. Equações matemáticas para a translação dos resultados experimentais em lesões de diferentes tamanhos foram propostas. Este estudo destaca o potencial dos scaffolds de colágeno com queratinócitos para aplicações translacionais, sugerindo que futuras pesquisas possam explorar a combinação de scaffolds otimizados com suportes mecânicos e compostos terapêuticos para aprimorar os resultados clínicos das terapias regenerativas.

Palavras-chave: Reparo tecidual; Monocultura; Co-cultura; Bioimpressão 3D; Sistemas biomiméticos; Pele e Mucosa Oral.

ABSTRACT

Wound repair involves a complex, specialized, and multi-phased process of cellular and tissue response. Complications in this process can lead to abnormal scar tissue, infections, and prolonged healing, impacting patients' quality of life and increasing public healthcare costs. This study aimed to apply cell culture techniques to explore emerging strategies in wound repair. The research was organized into three chapters: (1) Chapter 1 aimed to evaluate the effects of curcumin on keratinocytes and fibroblasts exposed to bacterial stimuli and ionizing radiation. Using monoculture and co-culture models, wound closure, cell viability, cell morphology, and gene expression assays for *PIK3CA*, *AKT*, *PTEN*, *MTOR*, and *IL-6* were conducted. The results demonstrate that co-culture significantly promotes wound closure, with curcumin improving the response of keratinocytes but not fibroblasts. Curcumin increases cell viability and alters cell morphology, reducing the cytoplasmic area of keratinocytes and enlarging the nuclear area of fibroblasts. Gene expression analysis revealed that curcumin significantly reduced *AKT* expression while tending to elevate *MTOR* and *PTEN* in keratinocytes. In fibroblasts, curcumin significantly decreased *MTOR* expression and tended to reduce *PIK3CA* and *IL-6* expression. These findings suggest that curcumin modulates repair mechanisms differently in keratinocytes and fibroblasts, highlighting its complex role in cellular repair. The study emphasizes the therapeutic potential of curcumin for tissue repair and the need for further research into its therapeutic use during radiotherapy. (2) Chapter 2 aimed to conduct a methodological review of current 3D bioprinting protocols using human keratinocytes and fibroblasts with a focus on tissue repair, following PRISMA 2020 guidelines. Eighteen studies focused on skin substitutes were included, with 83.3% utilizing extrusion-based bioprinting techniques and bioinks composed of gelatin (61.6%), fibrinogen (38.8%), and alginate (33.3%). The geographical distribution showed a higher concentration of studies in China (44.4%) and the United States (27.7%). Many studies validated their results by implanting the artificial skin in animal models to evaluate repair. The review highlights the need for standardized protocols to improve the replicability and clinical application potential of 3D bioprinting. (3) Finally, Chapter 3 aimed to develop a biomimetic 3D scaffold capable of delivering epithelial cells. This study investigated the potential of neutral bovine 70mg/mL collagen-based bioprinted scaffolds loaded with keratinocytes, using a robotic bioprinter and the Lifeink® 220 bioink. Fluid diffusion, dimensional stability, and print fidelity of the scaffolds were assessed over 7 days. Using a parallel interconnected design compared to a solid design, the interconnected design showed better promotion of cell migration, with a significant increase in the covered area and greater cell viability over time. On the other hand, the solid design was able to form more cellular expansion units within the scaffold. When considering the interconnected scaffold with an initial cell density greater than 150 cells/mm², this design resulted not only in migration for coverage but also in the formation of expansion units within the scaffold and an increase in the number of cells after three days. Thus, both the scaffold design and initial cell density were able to modulate the cellular response and optimize area coverage. Mathematical equations for translating experimental results to wounds of different sizes were proposed. This study highlights the potential of collagen scaffolds with keratinocytes for translational applications, suggesting that

future research may explore the combination of optimized scaffolds with mechanical supports and therapeutic compounds to enhance the clinical outcomes of regenerative therapies.

Keywords: Wound Healing; Monoculture; Co-culture; 3D bioprinter; Biomimetic systems; Skin and Oral mucosa.

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CAPÍTULO 2.

LISTA DE ABREVIATURAS

3D – Three-dimensional
AKT – Protein B kinase
ATB – Antibiotics-antimycotics
ATCC – American type culture collection
BNC – Bacterial nano-cellulose
CAD – Computer-aided design
CAM – Computer-aided manufacturing
CCL – Cytokine ligand
CF – Covering factor
COX-2 – Cyclooxygenase 2
DCEL – Diethylaminoethyl cellulose
DLP – Digital light processing
DMEM – Dulbecco's modified eagle's medium
DMSO – Dimethyl sulfoxide
ECM – Extracellular matrix
EGM2 – Endothelial cell growth-medium 2
FBS – Fetal bovine serum
GelMA – Gelatin methacrylate
h – Hours
HaCaT – Immortalized human keratinocytes
HDF – Human dermal fibroblast
HUVEC – Human umbilical vein endothelial cell
IL – Interleukins
ITOP – Integrated tissue and organ printing
KGM2 – Keratinocytes growth-medium 2
LA – Lesion area to be covered
LAP – Lithium phenyl(2,4,6-trimethylbenzoyl) phosphinate
LPS – Lipopolysaccharide
MMP-9, MMP-3 – Matrix metalloproteases
mTOR – Mammalian target of rapamycin
NF-κB – Necrosis factor κB
NHEK – Normal human epidermal keratinocyte
NIH – National Insitute of Health
PIBIC – Programa de Iniciação Científica
PBS – Phosphate-buffered saline
PDLLA – Poly Kε-caprolactone) diol (PCL) and poly (D, L-lactide) diol

PG – *Porphyromonas gingivalis*

PGA – Biodegradable polyglycolic acid

PI3K – Phosphoinositide 3-kinases

PID – Parallel interconnected design

PIK3CA – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PIP3 – Fosfatidilinositol-3,4,5-trifosfato

PIP2 – Fosfatidilinositol-4,5-bifosfato

PRISMA 2020 – Preferred reporting item for systematic reviews and meta-analysis guidelines

PTEN – Phosphatase and tensin homolog

PU – Biodegradable polyurethane

ROS – Reactive oxygen species

RT-qPCR – Quantitative reverse transcription polymerase chain reaction

SA – Initial scaffold area

SD – Standard deviation

SDs – Standard deviations

TGF-β – Transforming growth factor β

TNF-1 – Tumor necrosis factor-1

TSC2 – Tuberous sclerosis complex 2

UnB – Universidade de Brasília

UoM – Universidade de Michigan

β-ACTIN – Beta-actin

APRESENTAÇÃO

Às vezes, me pergunto se fui eu quem escolheu a academia ou se foi a academia que me escolheu. Embora eu tenha tido como exemplo uma avó professora na Universidade de Brasília, confesso que não pensava nessa possibilidade quando era uma criança sonhadora. A verdade é que, no meu segundo ano de graduação em Odontologia, enfrentei momentos difíceis na minha vida pessoal. Entre altos e baixos, não consegui dar o meu melhor nas matérias e acabei reprovando uma disciplina. Fora da minha zona de conforto, essa reprovação me atingiu como uma faca no coração, e achei que seria o fim do mundo.

Como uma boa oportunista (ou talvez mutualista, para que não me entendam mal), passei semanas refletindo sobre como fazer o melhor uso dessa "quebra" de fluxo. Decidi, então, me envolver com tudo que a universidade pública poderia me oferecer. Fiz matérias em outros departamentos, me dediquei intensamente a projetos de extensão, comecei a me envolver com pesquisa e assumi a representação do Centro Acadêmico. A paixão pela universidade foi uma consequência natural – afinal, como não se apaixonar pela UnB? Sem ter muita afinidade com a clínica, comecei a considerar minhas opções e senti que poderia me encaixar na tríade acadêmica: ensino, pesquisa e extensão.

Sempre fui extremamente ambiciosa em relação à minha carreira e nada era mais importante que isso. Mesmo sem saber exatamente para onde ir, sempre planejei ir longe. Escolhi com muita consciência a porta na qual bater para pedir uma oportunidade... A professora Eliete já chamava minha atenção pela firmeza com que abordava assuntos importantes como justiça, saúde e educação. Além disso, o brilho nos olhos dela ao ensinar e orientar foi decisivo na minha escolha. Eu sabia que ela era grande e suspeitava que ela queria ajudar outras pessoas a crescerem também. Com o tempo trabalhando juntas, tive certeza disso. Entendi que, após alcançar sua posição, ela se fez pronta para ajudar outras pessoas a chegarem aonde desejam.

Sou uma dessas pessoas. Felizmente! Bati na porta dela sem muita certeza da resposta. Ela me acolheu de braços abertos. Talvez eu também tenha chamado a atenção dela pela firmeza com que falo de assuntos importantes. Mas, no fundo, não importa o motivo pelo qual ela apostou em mim; o que importa é que ela apostou com vontade, me fazendo entender que eu também poderia chegar lá... E foi assim que meu sonho de ser professora universitária começou: alcançar esse objetivo para dar o braço a outras pessoas, ajudando-as a crescerem também.

Sei que cada pessoa tem seu próprio caminho e história. Ninguém é igual a ninguém. Hoje entendo isso melhor do que em 2017, quando bati na porta do laboratório. Mas, especialmente quando somos jovens e imaturos, precisamos de exemplo e inspiração. O apoio de profissionais que acreditam em nós faz toda a diferença. Nós, que somos ambiciosos, nos preparamos para encarar as oportunidades quando elas surgem, mas ainda estamos inexperientes quanto aos mecanismos necessários para isso. Estar pronto é essencial, porque a oportunidade pode não aparecer duas vezes, mas escolher quem estará ao seu lado para guiar e auxiliar nessa caminhada é igualmente importante. Ter a certeza de que, quando você não puder acreditar em si mesma, haverá pessoas que acreditarão por você.

Ninguém faz nada sozinho. Jamais esquecerei dos muitos sonhos e planos que tive ao iniciar na carreira: ganhar uma premiação na SBPqO, publicar na JDR, fazer doutorado direto, ser palestrante na JOUnB, coorientar estudantes, participar de bancas de TCC, conhecer a Universidade de Paris, fazer doutorado sanduíche na Universidade de Michigan, iniciar um pós-doutorado, e tantos outros. Como seres humanos, sempre queremos mais, mas hoje, ao olhar para trás, percebo que essa jornada repleta de desafios e aprendizados, floriu muitas conquistas e alegrias. Foi uma jornada compartilhada com uma rede de apoio incrível que sonhou e ainda sonha os meus sonhos comigo.

Quando vim para os Estados Unidos, tinha certeza de que voltaria ao Brasil após um ano para continuar meus planos profissionais. Só que vida acontece de maneiras inesperadas. O interessante é que, apesar de sempre ter sido muito ambiciosa em relação à minha carreira, foi também durante o doutorado que aprendi a valorizar meu bem-estar e minha felicidade. Hoje, sou ambiciosa pela vida. Fico feliz por estar encerrando mais uma página de conquista e sigo ansiosa por tudo o que está por vir nas próximas linhas ainda em branco.

SUMÁRIO

PREFÁCIO

O projeto de doutorado aqui apresentado foi idealizado desde a minha graduação em Odontologia, quando tive a oportunidade de participar do Programa de Iniciação Científica (PIBIC) como bolsista CNPq. Sob a orientação da Prof. Eliete Guerra, comecei a trabalhar com a equipe do Laboratório de Histopatologia Oral da Universidade de Brasília (UnB) em 2018, onde aprendi não apenas a desenvolver pesquisa, mas também a valorizar o trabalho em equipe e as parcerias, prezando pelo compartilhamento de conhecimentos e acolhendo as necessidades individuais das pessoas. Antes de me graduar tive a oportunidade de fazer uma visita técnica na Universidade de Michigan com apoio da FAPDF. Em 2020, me graduei no curso de Odontologia da UnB, no mesmo ano em que iniciei o doutorado direto no Programa de Pós-Graduação em Ciências da Saúde como bolsista CAPES.

O conteúdo desta tese foi estruturado em consonância com o meu desenvolvimento profissional. O Capítulo 1 traz resultados dos meus primeiros experimentos em laboratório, quando aprendi conceitos básicos de cultura celular. Nesse período, aprendi a isolar e cultivar células por meio de biópsias de tecido gengival e ligamento periodontal, utilizando técnicas de explante e digestão enzimática. Além disso, tive a oportunidade de trabalhar com técnicas de monocultura utilizando queratinócitos imortalizados e fibroblastos primários, bem como com a co-cultura desses dois tipos celulares, empregando insertos Transwell®. Os experimentos acompanharam minha curva de aprendizado, abrangendo ensaios de viabilidade celular, scratch, imunofluorescência e expressão gênica. Fui motivada por um grande interesse nos efeitos adversos da terapia oncológica por radiação ionizante e na aplicação de medicamentos naturais, especialmente pensando em alternativas viáveis para o Sistema Único de Saúde (SUS). Assim, o Capítulo 1 aborda o processo de reparo relacionado à mucosite oral e ao uso da curcumina.

Durante a qualificação do doutorado, em 2022, foi discutida a possibilidade de avançar a complexidade dos experimentos para incluir técnicas de cultura 3D. Nesse mesmo ano, iniciei meu período de doutorado sanduíche na Universidade de Michigan (UoM), onde fui acolhida com carinho e expertise pelos Profs. Cristiane Squarize e Rogério Castilho. Como parte do Laboratório de Biologia Epitelial da UoM, expandi minha experiência acadêmica trabalhando com uma bioprinter 3D. A máquina e eu chegamos ao laboratório quase ao mesmo tempo e trabalhamos intensamente juntas nas fases de ajustes iniciais, funcionamento dos softwares e calibração geral. Visando entender o potencial e a aplicabilidade da bioprinter 3D na pesquisa em reparo tecidual, o Capítulo 2 dessa tese apresenta uma revisão da literatura focada em modelos de substitutos de pele. Ao identificar o desafio de replicar as metodologias disponíveis na literatura, optamos por desenvolver uma revisão metodológica com base em parâmetros de revisão sistemática. Esse capítulo oferece uma visão geral do uso da bioprinter 3D na área, detalhando protocolos que utilizam essa tecnologia para imprimir modelos de pele artificial com queratinócitos e fibroblastos humanos.

Por fim, o Capítulo 3 aborda a prática com a bioprinter 3D. Como resultado de um longo processo de padronização e aplicabilidade, esse capítulo explora resultados iniciais da utilização da máquina e seu

potencial para desenvolver terapias inovadoras em reparo tecidual. Iniciando com análises de parâmetros de impressão e calibração, o trabalho avança para uma proposta preliminar de sistema de entrega de células. Esse sistema opera utilizando células previamente cultivadas em monocultura, que são posteriormente bioimpressas com componentes da matriz extracelular. Utilizando técnicas de análise de imagens, o trabalho sugere padrões de resposta celular em termos de migração, proliferação e expansão, com foco no recobrimento de áreas lesionadas.

Esta tese não seria possível sem o apoio e a colaboração de inúmeras pessoas e instituições ao longo dessa jornada acadêmica. Agradeço profundamente a todos que contribuíram, direta ou indiretamente, para a realização desta tese. Espero que os conhecimentos aqui compartilhados possam inspirar futuras pesquisas e contribuir para o avanço na área de reparo tecidual, promovendo melhorias na qualidade de vida de pacientes que dependem das descobertas e inovações científicas.

INTRODUÇÃO

A cultura celular teve sua origem no século XX, sendo inicialmente desenvolvida para estudar a maturação de tecidos, a biologia de vírus e o desenvolvimento de vacinas (Arango et al. 2013). O método de cultura laboratorial possibilita o crescimento de células eucarióticas ou procarióticas em condições controladas, fora do ambiente fisiológico natural de um organismo. Com o tempo, as técnicas de cultivo celular se diversificaram, permitindo a criação de dois principais tipos de culturas: as culturas imortalizadas e as culturas primárias. Nas culturas imortalizadas, as células são submetidas a modificações genéticas que lhes conferem a capacidade de crescer indefinidamente. Em contraste, as culturas primárias envolvem o isolamento de células sem alterações genéticas, mantendo um comportamento mais próximo ao da fisiologia natural (ThermoFisher Scientific, Cell Cultre Basics Handbook). Cada um desses tipos apresenta vantagens e desvantagens em termos de aplicabilidade técnica, custo operacional e confiabilidade dos resultados.

As aplicações experimentais das culturas celulares são vastas, abrangendo desde estudos básicos de biologia celular até a replicação de mecanismos de doenças e a investigação da toxicidade de novos compostos terapêuticos. O uso dessas técnicas *in vitro* oferece diversas vantagens, incluindo o controle rigoroso do ambiente experimental, a homogeneidade das populações celulares e a existência de protocolos técnicos bem estabelecidos. Esses fatores contribuem para a geração de dados com alta reprodutibilidade e consistência (Clift et al. 2010). Além disso, a capacidade de manipular genes e vias moleculares em modelos *in vitro* permite a criação de sistemas de estudo complexos e a exploração de potenciais terapias personalizadas (Fuji et al. 2023; Miranda et al. 2024). No entanto, as monoculturas, que envolvem o cultivo de um único tipo celular, apresentam limitações, especialmente no que diz respeito à replicação do microambiente e das interações celulares presentes nos tecidos *in vivo* (Clift et al. 2010; Arango et al. 2013).

O processo de reparo de lesões, por exemplo, depende de interações complexas entre diferentes tipos de células. A reepitelização é altamente dependente da interação entre células epiteliais e a matriz extracelular secretada por células mesenquimais (Gurtner et al. 2008; Rosselli-Murai et al. 2013). Nas mucosas, fibroblastos desempenham um papel fundamental, contribuindo para a deposição de componentes da matriz extracelular, contração da ferida e remodelação, enquanto queratinócitos são essenciais para a reepitelização da superfície lesionada (desJardins-Park et al. 2018; Ko et al. 2019). Com isso, os estudos *in vivo* em modelos animais complementam os estudos *in vitro*, fornecendo um microambiente tecidual completo para a investigação dos processos biológicos. Uma das grandes vantagens dos modelos animais é a capacidade de observar respostas complexas entre diferentes tipos de células, tecidos e órgãos em um ambiente funcional e dinâmico (Bédard et al. 2020). Entretanto, diferenças biológicas entre espécies e a dificuldade de utilizar amostras de larga escala são desafios inerentes aos modelos animais (Adhikary et al. 2021). Essas limitações frequentemente resultam em inconsistências na translacionalidade dos resultados para ensaios clínicos, impactando a progressão científica, aumentando custos e gerando desafios éticos (Hubretch and Carter, 2019; Antunes et al. 2022).

Para superar as limitações das monoculturas e dos modelos animais, as culturas tridimensionais (3D) emergiram como uma inovação crucial (Greiner et al. 2012). A cultura 3D mimetiza a arquitetura e as interações celulares dos tecidos naturais, permitindo o estudo aprofundado de processos de migração, diferenciação e sinalização. Entre as técnicas 3D, destacam-se os modelos de esferoides, organoides e scaffolds bioimpressos (Bédard et al. 2020; Lee et al 2022). Em particular, a bioimpressora 3D, conhecida como bioprinter, tem se destacado por sua capacidade de criar estruturas teciduais complexas de forma precisa e controlada (Ravanbakhshm, 2021). Utilizando geralmente matrizes de gelatina, alginato e/ou colágeno, essa tecnologia permite a incorporação de células específicas, como queratinócitos e fibroblastos, em padrões que mimetizam a organização tecidual nativa (Ma et al. 2018). No que diz respeito ao reparo da pele e da mucosa oral, a bioprinter oferece técnicas promissoras para a criação de substitutos que replicam a arquitetura e função desses tecidos (Albanna et al. 2019, Zhang et al 2023). A capacidade de bioimprimir construtos que mimetizam a composição e organização celular desses tecidos tem implicações tanto para a replicação de modelos de estudo *in vitro* complexos, como para o desenvolvimento de tratamentos inovadores de transplantes artificias e terapia baseada em entrega de células. Scaffolds bioimpressos em 3D oferecem um ambiente ideal para a regeneração tecidual, proporcionando suporte e nutrição para a proliferação e diferenciação celular. Além disso, esses scaffolds podem ser customizados para atender às necessidades específicas de cada tipo de lesão, maximizando a eficácia dos tratamentos.

Embora a combinação de técnicas tradicionais de cultura celular com abordagens inovadoras de bioimpressão 3D represente um avanço significativo, a padronização desses modelos e a criação de construtos fiéis à realidade ainda apresentam desafios importantes (Bishop et al. 2017). A heterogeneidade dos modelos atuais e a falta de protocolos bem definidos são obstáculos para a reprodução e aplicação consistente desses construtos em pesquisas. No entanto, uma vez estabelecidos protocolos funcionais e padronizados para a pele e mucosa oral *in vitro*, uma ampla gama de modelos de estudo poderá ser desenvolvida (Ma et al. 2018). Esses modelos permitirão a aplicação de estímulos para simular diversas condições de doenças e lesões, incluindo forças mecânicas, lesões térmicas, indução por quimio-radioterapia, infecções, exposição à fumaça de cigarro, entre outros. Além disso, a impressão de substitutos para pele e mucosa com bioprinters poderá melhorar as análises de biocompatibilidade de novos produtos cosméticos e adequar os requisitos para o screening de novas drogas, reduzindo também a dependência de modelos animais. Modelos de doenças também poderão ser desenvolvidos ao incluir linhagens de células neoplásicas ou culturas primárias de explantes de pacientes com síndromes. Potencialmente, construtos 3D específicos para cada paciente permitirão avaliações mais precisas, considerando o perfil genético e resposta celular individual (Loewa et al. 2023). Abordagens personalizadas como essas poderiam ser particularmente úteis na tomada de decisões para tratar condições recorrentes, não responsivas e raras, ou como alternativas para enxertos autólogos.

Por exemplo, a via PI3K-PTEN-AKT-mTOR é amplamente reconhecida como alvo promissor para novas terapias, desempenhando papel central na regulação de processos como migração, sobrevivência e proliferação celular. A sinalização de PI3K resulta na fosforilação e ativação de AKT por meio de

fosfatidilinositol-3,4,5-trifosfato (PIP3), enquanto a proteína PTEN atua antagonicamente ao desfosforilar PIP3 em fosfatidilinositol-4,5-bifosfato (PIP2) (Zhao, 2007). A inibição de PTEN, portanto, surge como uma abordagem promissora para modular a sinalização de PI3K, com potencial aplicação tanto em terapias de reparo de lesões mucosas quanto em tratamentos antitumorais (Castilho et al. 2013; Borges et al. 2020a; Squarize et al. 2013). Em paralelo, compostos naturais como a curcumina têm atraído atenção por seus efeitos duais, capazes de modular diversas respostas biológicas. Estudos *in vitro* indicam que a curcumina pode atuar como um potente agente anti-inflamatório e antioxidante em células não neoplásicas, enquanto outros trabalhos sugerem seus efeitos inibidores de mTOR como alternativa antineoplásica (Borges et al. 2017; Borges et al. 2020b; Zhu et al. 2018). A utilização de modelos 3D, particularmente com o emprego de bioprinter, facilitaria a análise da segurança de medicamentos potenciais, bem como a definição de doses ótimas para suas aplicações. Além disso, esses modelos permitiriam uma exploração mais detalhada das vias de sinalização cruciais para diferentes processos biológicos, ampliando o entendimento e as possibilidades terapêuticas.

Segundo o Instituto Nacional de Saúde (NIH), a ciência translacional engloba um amplo espectro de atividades voltadas a transformar descobertas científicas em aplicações práticas que melhorem a saúde e o bem-estar humano. Essa abordagem visa preencher a lacuna entre as descobertas laboratoriais e sua implementação clínica, promovendo avanços na saúde da comunidade (National Institue of Health, NIH). Em 2022, Faupel-Badger et al. (2022) defenderam um esforço colaborativo entre instituições educacionais, órgãos financiadores e agências governamentais para formar uma nova geração de cientistas capacitados a traduzir descobertas científicas em soluções práticas de saúde de forma eficiente. Os autores destacam a importância de cientistas atuarem em equipes interdisciplinares, compreendendo os cenários regulatórios e as dinâmicas de mercado, a fim de introduzir inovações com sucesso. Além disso, ressaltam a necessidade de fomentar parcerias estratégicas para o compartilhamento de recursos e a aceleração da pesquisa, especialmente em áreas inovadoras.

Nesse contexto, esta tese de doutorado tem como principal objetivo aplicar técnicas de cultura celular para explorar estratégias emergentes no reparo de lesões, partindo da monocultura a sistemas biomiméticos 3D com bioprinter. Entre as técnicas *in vitro* utilizadas, destacam-se a reprodução de modelos para estudo, análises celulares em resposta ao uso de terapêuticos, fabricação de substitutos 3D de pele e a criação de scaffolds 3D para a entrega de células. Esta pesquisa foi realizada com o apoio de diversas instituições brasileiras, e contou com a colaboração entre a Universidade de Brasília e a Universidade de Michigan, refletindo a importância da cooperação interdisciplinar e internacional na busca por avanços científicos e formação de recursos humanos.

OBJETIVOS

Objetivo Geral

Aplicar técnicas de cultura celular para explorar e desenvolver estratégias emergentes no reparo de lesões, começando com a utilização de modelos de monoculturas e co-culturas, avançando para modelos biomiméticos 3D e sistemas de entrega celular.

Objetivos Específicos

(1) Comparar as respostas de migração celular entre monoculturas e co-culturas de queratinócitos e fibroblastos humanos após a aplicação de radiação ionizante e estímulos bacterianos.
(2) Utilizar monoculturas de queratinócitos e fibroblastos para investigar processos básicos de reparo tecidual em células isoladas após a aplicação dos estímulos e tratamento com curcumina, incluindo viabilidade celular, morfologia, e expressão gênica.
(3) Buscar e relatar protocolos de substitutos de mucosa oral e pele utilizando técnicas com bioprinter 3D que incorporem queratinócitos e fibroblastos humanos.
(4) Disponibilizar uma visão geral dos estudos com bioprinter 3D, no que diz respeito à localização geográfica, instituições afiliadas e áreas de concentração. Ainda, explorar e relatar os protocolos para que possam ser replicados em futuras pesquisas.
(5) Utilizar uma bioprinter 3D para criar sistemas biomiméticos com cultura celular.
(6) Calibrar a bioprinter 3D para viabilizar a impressão padronizada de construtos.
(7) Introduzir o desenvolvimento de sistemas biomiméticos 3D para a entrega de queratinócitos humanos, avaliando potenciais estratégias para otimizar a promoção do reparo tecidual e regeneração com bioprinter 3D.

CAPÍTULO 1 (Intended submission: Phytotherapy Research; IF = 6.1)
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Curcumin modulates keratinocytes and fibroblasts repair under bacterial stimuli and ionizing radiation

ABSTRACT

Radiotherapy is one of the most applied antineoplastic modalities. However, the toxicity in the surrounding region implicates adverse effects and impacts patients' quality of life during oncology treatment. Curcumin, known for its anti-inflammatory and antioxidant properties, has been suggested as a potential alternative for the management of radiotoxicity due to its versatile repair role. Thus, this study aims to evaluate the effects of curcumin on keratinocytes and fibroblasts exposed to bacterial stimuli and ionizing radiation. For the experiments, immortalized human keratinocytes and primary culture of human gingival fibroblasts were used in both monoculture and co-culture settings. The stimuli were composed of bacterial stimuli with lipopolysaccharide of *Escherichia coli* and *Porphyromonas gingivalis* protein extract followed by ionizing radiation. To evaluate curcumin effects, wound closure, cellular viability/mitochondrial activity, cell morphology, and gene expression (qRT-PCR: *PI3KCA*, *AKT*, *PTEN*, *MTOR*, *IL-6*) assays were conducted. Co-cultivation markedly promoted faster wound closure compared to monoculture conditions. Curcumin treatment enhanced keratinocyte wound healing at 12h in co-culture ($p<0.05$) and 24h in monoculture settings ($p<0.05$); however, it did not aid in fibroblast response, tending to a slower closure, especially at 24h. Curcumin was able to increase the mitochondrial activity of monocultures of keratinocytes and fibroblasts compared to the stimuli without curcumin ($p<0.05$). The cell morphology analyses revealed a decrease in keratinocytes cytoplasm area ($p<0.05$) and enlargement of fibroblasts nuclear area ($p<0.05$) upon curcumin treatment. Also, curcumin downregulated *AKT* expression ($p<0.01$) and presented a trend of elevating *MTOR* and *PTEN* in keratinocytes, suggesting repair through Akt-independent mechanisms. Conversely, in fibroblasts, a significant downregulation of *MTOR* was found ($p<0.01$) and a tendency to decrease *PIK3CA* and *IL-6*. Curcumin demonstrates the potential to enhance different processes related to wound healing of keratinocytes and fibroblasts. Curcumin-dependent effects indicate a complex role in modulating cellular repair mechanisms, dependent on cell type and stress conditions. This study delves into curcumin's effects on the wound healing process after ionizing radiation and bacterial stimuli. Furthermore, it highlights the potential and the importance of additional research into curcumin application as a therapeutic agent for tissue repair.

KEYWORDS: Curcumin, Wound healing, Ionizing radiation, Oral mucositis, PI3K pathway.

INTRODUCTION

Radiotherapy is among the most effective antineoplastic modalities for solid tumors. During oncology treatment with radiotherapy, the exposed tissue activates several overlapping pathways (Gruber et al. 2016). While aiming to induce tumoral cell death, the ionizing radiation affects the surrounding region, activating inflammation and signal amplification to restore homeostasis. Immediate reactive oxygen species (ROS) are generated, signaling the expression of transcription factors and inflammatory cytokines, such as necrosis factor κB (NF-κB), transforming growth factor β (TGF-β), and interleukins (IL) (Citrin et al. 2017). In the oral mucosa, especially non-keratinized mucosa, this process directly injures the epithelial cells and the underlying fibroblasts, leading to oral mucositis that may vary from mild, erythematous lesions with a burning sensation to severe ulcerations with pseudo membrane formation (Sonis et al. 2004, Mougeot et al. 2019). The overlay process, associated with bacterial colonization and the periodicity of antineoplastic therapies, impacts the inflammatory response and prolongs tissue repair (Bowen et al. 2019, Lala et al. 2019, Sonis et al. 2009).

Oral mucositis affects up to 90% of patients undergoing radiotherapy for head and neck carcinomas, severely diminishing their quality of life and increasing the risk of systemic infections (Brown et al. 2019). Frequently, these clinical aspects require specific supportive care, impacting the antineoplastic therapy continuation (Pulito et al. 2020). Since 750,000 new cases of head and neck cancer are estimated yearly (GLOBOCAN, IARC Global Cancer Observatory), effective treatments for oral mucositis are a current topic of interest. The MASCC/ISOO recommends topical treatments such as benzydamine mouthwash and photobiomodulation while also endorsing efforts on novel alternatives with natural compounds (Elad et al. 2020). The scientific community is actively exploring new strategies to modulate inflammatory pathways, reduce bacterial loads, and promote wound healing (Pulito et al. 2020; Soni et al. 2022).

Curcumin extract, derived from turmeric (*Curcuma longa L.)*, emerges as a promising compound due to its properties in multiple signaling pathways (Liczbinski et al. 2020; Farhood et al. 2018). Recognized as an agent with diverse therapeutic actions, curcumin is believed to exert a dual-modulatory effect when different cell types, drug doses, and experimental methods are taken into consideration. With a dose-dependent effect, non-cytotoxic concentrations of curcumin (e.g. 0.2-20μM) are suggested to modulate the inflammatory process and enhance wound healing. Conversely, higher concentrations (e.g. 25-50μM) promote selective cytotoxic response, inducing cell cycle arrest and inhibiting neoplastic progression (Rujirachotiwat, and Suttamanatwong 2021; Borges et al. 2020; Boretti et al. 2023).

The primary effects of curcumin are linked to its ability to mediate anti-inflammatory and antioxidant pathways (Farhood et al. 2018; Kumari et al. 2022). Curcumin disrupts NF-κB activation, suppresses cytokine production, and influences other crucial cell survival pathways. By modulating NF-κB, curcumin reduces levels of pro-inflammatory matrix metalloproteinases (MMP-9, MMP-3) and cytokines (TNF-1, IL-1, IL-8), while also decreasing the production of prostaglandins and thromboxane by binding to cyclooxygenase 2 (COX-2) (Farhood et al. 2018). As an antioxidant, curcumin primarily reduces ROS formation, thereby mitigating tissue damage and subsequent inflammatory responses (Kumari et al. 2022).

In this sense, curcumin is a promising adjuvant in cancer therapy due to its low toxicity and protective effects in non-neoplastic cells paired with the ability to enhance the effects of radiotherapy and chemotherapy in neoplastic cells. The potential of curcumin has been clinically explored for oral mucositis, showing promising results in reducing severity and promoting pain relief (Normando et al. 2019; Wu et al. 2024). However, the modulatory effects of curcumin at cellular levels in radio-induced lesions warrant further investigation. Thus, this study aims to evaluate the effects of curcumin in human keratinocytes and gingival fibroblasts exposed to ionizing radiation and bacterial stimuli, focusing on the repair modulation.

MATERIAL AND METHODS

Experimental design

This is an *in vitro* study that investigates both monoculture and co-culture conditions of immortalized human keratinocytes and primary human gingival fibroblasts (Figure 1A). To stimulate the cells, bacterial stimuli, and ionizing radiation were applied, followed by treatment with either curcumin or vehicle control (Figure 1B). In the monoculture setup, analyses included wound closure, cell viability, cell morphology, and gene expression. In the co-culture setup, the focus was on wound closure assays (Figure 1C).

Cell culture

Immortalized human keratinocytes were obtained from the American Type Culture Collection (ATCC). The primary gingival fibroblasts were collected in previously approved research conducted under the Ethical and Research Committee of the University of Brasília (CAAE No. 52278221.6.0000.0030) and have been characterized by Monteiro et al. (2024). For the experimental studies, both cell lines were preserved at -80°C and grown in 100 mm culture dishes. The growth medium used was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic (penicillin and streptomycin). The cells were maintained in an incubator with a 5% CO_2 atmosphere at 37°C. Every 3-4 days, the culture media was replaced to ensure optimal growth conditions. When the cells reached approximately 80% confluence, they were treated with trypsin and reseeded onto fresh plates either for experimental protocols or for continued propagation. The primary gingival fibroblasts were utilized for experiments up to their 6^{th} passage to ensure cellular integrity and validity of the experimental results.

Co-culture

The keratinocytes and fibroblasts co-cultures were established using 0.4 µm Transwell® permeable supports (Corning Costar, Corning ®, USA. Reference #3401) in 12-well plates. After initial maintenance of the cell culture, the cell lines were seeded separately, with one strain on the bottom of the wells (lower compartments) and the other one on the microporous membrane (upper compartments). The cells were immersed in DMEM (10% fetal bovine serum and 1% antibiotics) and incubated under optimal conditions. After 24 hours, ensuring complete adhesion of the cells to the bottom of the compartments, the seeded Transwell® insert was positioned over the lower compartment to proceed with the experiments. For the arrangement with keratinocytes in the lower compartment and fibroblasts in the upper compartment,

keratinocytes were plated at a density of $2x10^5$ cells per well and fibroblasts at a density of $1x10^5$ cells per membrane. Conversely, for the arrangement with fibroblasts in the lower compartment and keratinocytes in the upper compartment, both fibroblasts and keratinocytes were plated at a density of $1.5x10^5$ cells. All the analyses were performed on the lower compartments.

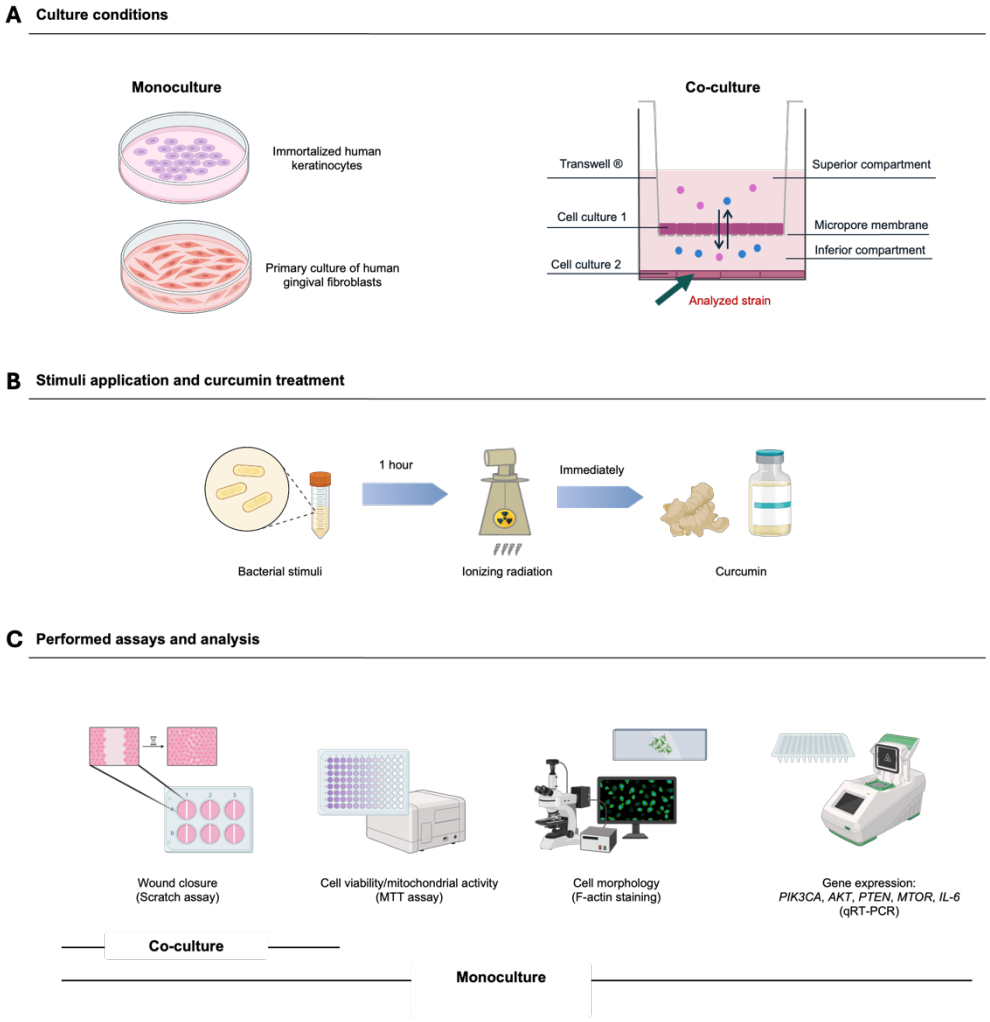

Figure 1. Experimental design. (A) For the experiments, immortalized human keratinocytes and primary culture of fibroblasts were used in monoculture and co-culture settings conditions. (B) The bacterial and ionizing radiation stimuli were applied in all samples. Immediately after, the cells were treated with curcumin or vehicle. (C) After 24h of treatment, the cells underwent wound closure, cellular viability, cellular morphology, and gene expression assays.

Ionizing radiation and bacterial-induced stimuli

The stimuli were applied as previously described (Monteiro et al. 2024). Briefly, the cells were incubated with lipopolysaccharide (LPS) of *Escherichia coli* 0111:B4 (10 µg/mL or 1 µg/mL) and *Porphyromonas gingivalis* (PG) protein extract (5 µg/mL) for 1 hour. In sequence, the cultures were submitted to 8 Gy of ionizing irradiation using a 6 MV linear photons beam (Siemens Primus linear accelerator – Malvern, Pennsylvania, USA). LPS was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Pg protein

extract was prepared in the University of Campinas, São Paulo, Brazil, as described by Albiero et al., and donated to the Laboratory of Oral Histopathology of the University of Brasília.

Curcumin treatment

Pure curcumin (Sigma-Aldrich, St. Louis, Missouri, USA. Reference: #08511) was purchased in powder form and prepared by diluting it in Dimethyl Sulfoxide (DMSO). The cells were treated with curcumin 2.5μM, as previously defined through viability test (Borges et al. 2020).

Experimental groups

For better comprehensiveness through the manuscript, the control *in vitro* model and treatment groups were entitled as follows: (1) **Control group**: cells stimulated with ionizing radiation and bacterial stimuli in the vehicle**;** (2) **Curcumin group**: cells stimulated with ionizing radiation and bacterial stimuli treated with curcumin.

Scratch assay

Prior to the experiments, the dishes were coated with 2 mL of a fibronectin solution at a concentration of 10 μg/mL and left to incubate overnight at 4°C, rinsed, and air-dried under sterile conditions. Keratinocytes and fibroblasts were seeded and incubated for 24 hours to allow cell attachment and a confluent monolayer formation. Then, a wound was introduced in each well by dragging a p200 pipette tip across the cell layer to create perpendicular vertical and horizontal lines. The cells were washed with PBS and exposed to the described stressful condition. Immediately after, treatments were applied according to the following experimental groups for both keratinocytes and fibroblasts: (1) Control in monoculture; (2) Curcumin in monoculture; (3) Control in co-culture; (4) Curcumin in co-culture. Imaging was taken at 0h, 12h, and 24h. The initial wound size at time 0 was set as the 100% reference point. Subsequent images were used to calculate the percentage of wound closure relative to the initial wound size using Zeiss Axio Observer D1 (Göttingen, Germany) with a connected digital camera (AxioCam MRc Zeiss, Göttingen, Germany).

Cell viability

Cell cultures were seeded into 96-well plates at a seeding density of $5x10^3$ cells/well and incubated for 24h. Subsequently, the experimental stimuli protocol and curcumin treatment were administered across nine biological replicates for each group. Following another 24h incubation, each well received a 10 μL dose of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) solution (Sigma-Aldrich, based in St. Louis, Missouri, USA). The plates were then incubated for 4 hours in a light-protected environment, and the reaction was stopped by the addition of a modified isopropanol solution (25 mL of isopropanol mixed with 104 μL of concentrated hydrochloric acid). Cell viability was quantitatively assessed by measuring the optical density at a wavelength of 570 nm using a Thermo Plate TP Reader device (manufactured by Thermo Fisher Scientific, headquartered in Waltham, Massachusetts, USA), comparing the absorbance levels between experimental groups.

Cytoskeleton morphology

Cell cultures were plated onto 18x18mm glass coverslips (Menzel Glaser, Thermo Scientific) that were set into 6 well plates at a concentration of $5x10^4$ cells/well, followed by 24h of incubation. The experimental protocols were then administered to each well in triplicate and incubated for additional 24h. The coverslips were gently rinsed twice using phosphate-buffered saline (PBS), fixed with 1 mL of 4% paraformaldehyde for 10 minutes, and subjected to two further PBS washes. Subsequently, the cells were permeabilized with 1 mL of 0.1% Triton X-100 and received two additional PBS washes. For staining of actin, phalloidin (P5282, Sigma-Aldrich, St. Louis, Missouri, USA) was applied for 40 minutes, succeeded by two washes with PBS. Nuclei were counterstained using DAPI Fluoromount-G® (SouthernBiotech). High-resolution imaging was performed with a SP8 LIGHTNING confocal microscope (Leica Microsystems, Wetzlar, Germany), and the analysis of cytoplasmic area and nuclear area was carried out using the ImageJ software (Schneider et al. 2012).

Gene expression

The gene expression was evaluated by the quantitative reverse transcription polymerase chain reaction (RT-qPCR). The gene expressions of *PTEN, PIK3CA, AKT,* and *MTOR* and the pro-inflammatory cytokine *IL-6* were obtained by RT-qPCR with PowerUp® SYBR® Green Master Mix (Applied Biosystems) and specific primers (Supplementary Table 1). For that, the cells were seeded (Fibroblasts $2x10^5$ and keratinocytes $5x10^5$ cells/well) into 6 well dishes and incubated for 24 hours. The stimuli protocol was applied, followed by curcumin treatment. After 24h, total RNA was extracted using TRizol® (Invitrogen), following the manufacturer's instructions. RNA integrity and concentration were determined by NanoVue Plus (GE Healthcare Life Sciences, UK, EU). Then, 400 ng of total RNA was treated with DNase I (Invitrogen). The cDNA templates were obtained by reverse transcription in 20 µL reactions, containing MultiScribeTM Reverse Transcriptase, 10X RT Buffer, 25X dNTP mix,10X RT Random Primers, and RNase-Free Water using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). The following reactions were prepared in triplicates using 96 well dishes with 10 µL of final volume, 0.2 µL of each primer (forward and reverse), 5 µL of Power Up SYBR Green Master Mix (2X), 0.1 µL of RNase-Free water, and 4.5 µL cDNA. Analysis of all qPCR reactions was performed by StepOne Software v2.1 (Applied Biosystems). Beta-actin (*β-ACTIN*) was used as a housekeeping gene to normalize the reaction. The relative quantification was analyzed using the comparative CT method, and the statistics were calculated after performing the equation $2^{-\Delta\Delta Ct}$, where $^{\Delta\Delta}Ct = \Delta Ct_{treatment} - \Delta C_{tcalibrator/control}$ (Schmittgen & Livak, 2008). Experiments were performed in technical triplicates for each sample.

Statistical analyses

Statistical analysis was conducted using GraphPad Prism version 10.2.3 (GraphPad Software, La Jolla, California). The Shapiro-Wilk test was employed to determine the normality of the data distributions, with a p-value <0.05 suggesting a non-parametric distribution. Depending on the data's distribution, comparisons between the two groups were performed utilizing either the parametric t-test data or the non-

parametric Mann-Whitney test. When examining multiple groups, one-way ANOVA followed by Dunnett's multiple comparisons post-test was utilized for data presenting normal distributions. In contrast, Kruskal-Wallis's test, followed by Dunn's multiple comparisons post-test, was applied for data with non-normal distributions. Results are presented as mean ± standard deviation (SD). Statistical significance was determined at a p-value<0.05.

Schematic figures

Schematic illustrations were produced using registered and licensed versions of BioRender and the Microsoft Office 365 PowerPoint suite.

RESULTS

The interaction between keratinocytes and fibroblasts is essential for wound closure after ionizing radiation and bacterial stimuli

After applying bacterial stimuli and ionizing radiation, keratinocyte and fibroblast responses were evaluated using scratch assays under both monoculture and co-culture conditions. The results showed a significant improvement in wound healing when keratinocytes and fibroblasts interacted, with both cell types exhibiting significantly accelerated wound closure in co-culture at 12 and 24 hours (Figures 2A and 2B).

Specifically, for keratinocytes, the co-culture environment reduced the wound area to 84.88% of the initial size after 12h, whereas keratinocytes in monoculture only achieved 94.86% of wound area ($p<0.01$). At 24h, the wound area in co-culture further diminished to 55.16%, while the monoculture demonstrated a less substantial decrease, leaving the wound area at 86.23% ($p<0.01$) (Figure 2A). In contrast, for fibroblasts in co-culture, almost half (54.75%) of the wound area was closed within the first 12h, while the monoculture showed slower wound healing, with the area covered reaching 82.09% of the initial area ($p<0.0001$). At 24h, co-culture remained at 1.31% of the area, and monoculture reached only 20.38% ($p<0.001$) (Figure 2B).

When the cells were treated with curcumin, the keratinocyte response followed the previous pattern when comparing co-culture and monoculture (Figure 2C). At 12h, co-culture conditions with curcumin showed a reduction of the initial area to 71.42%, while 91.52% was found in monoculture ($p<0.01$). At 24h, co-culture and monoculture treated with curcumin reduced the area to 43.84% and 71.98% ($p<0.05$), respectively. When curcumin was added to the co-culture and monoculture of fibroblasts, the pattern was the same (Figure 2D). At 12h, the initial area was reduced to 58.36% in co-culture and 79.29% in monoculture ($p<0.01$), while at 24h, the reduction reached 3.04% of the initial area in co-culture and 31.21% in monoculture ($p<0.001$).

These observations corroborate the critical role of cellular interactions in wound healing, with the co-culture environment notably enhancing the efficacy of the process with or without curcumin.

Curcumin reduces the wound area of keratinocytes after ionizing radiation and bacterial stimuli

To further investigate the effects of curcumin in keratinocytes and fibroblasts under ionizing radiation and bacterial stimuli, the comparisons were set between curcumin treatment and control group for both co-culture and monoculture. In co-culture at 12h, curcumin significantly reduced the wound area of keratinocytes

to 71.42%, compared to the control (without curcumin), 84.88% ($p<0.05$). However, no significant differences were found between treatment with curcumin and control at 24h, although the wound area of curcumin-treated keratinocytes remained smaller (43.84% and 55.16%, respectively). In monoculture, there was no difference at 12h with curcumin reducing the initial area to 91.52% and the control without curcumin to 94.86%, and a significant difference was found at 24h (71.98% with curcumin and 86.23% without it, $p<0.05$) (Figure 3A).

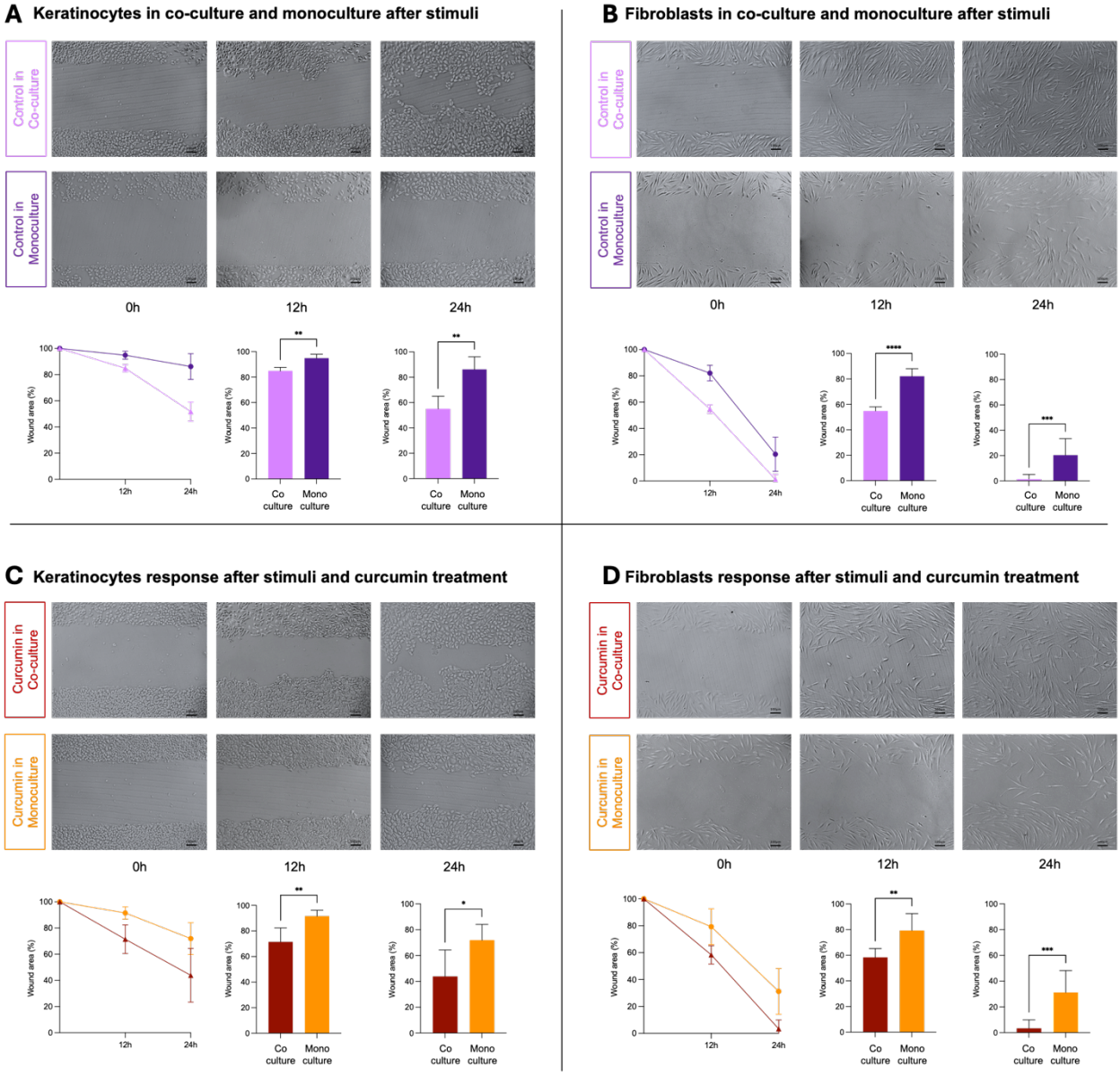

Figure 2. Comparison of co-culture and monoculture wound closure. Response of monocultures and co-cultures of (A) keratinocytes and (B) fibroblasts after ionizing radiation and bacterial stimuli. Response of previously stimulated monocultures and co-cultures of (C) keratinocytes (D) and fibroblast treated with curcumin. Statistical analysis: Mean ± SD, significant when p-value<0.05. Parametric distribution: Unpaired t-test (B 12h, C 12h and 24h, D 12h); non-parametric distribution: Mann-Whitney test (A 12h and 24h, B 24h, D 24h). Abbreviations: h (hours).

In fibroblasts, no differences were found between curcumin and control without curcumin at any time points for both co-culture and monoculture. In co-culture, curcumin showed a closure to 58.36% of the initial area compared to 54.75% without curcumin at 12h. At 24h, the wound area dropped to 3.04% with curcumin and 1.31% without it. When comparing monoculture, curcumin reduced the wound area to 79.12% at 12h and to 31.21% at 24h, while the group control reduced it to 82.09% and 20.38%, respectively (Figure 3B).

Curcumin increases viability and induces morphological modifications in keratinocytes and fibroblasts

After 24h of treatment with curcumin, monocultures of keratinocytes and fibroblasts previously stimulated by ionizing radiation and bacterial extracts were evaluated regarding cellular viability and morphology. Whereas curcumin increases the mitochondrial dehydrogenase activity in both keratinocytes and fibroblasts culture, the morphological modifications were different for keratinocytes and fibroblasts.

Specifically, keratinocytes treated with curcumin presented an increase of 28% in cellular viability compared to the stimuli without curcumin ($p<0.05$). The cytoplasmic mean area of keratinocytes treated with curcumin was 1093.92 μm^2, which was significantly smaller than the control group (1193.89 μm^2) ($p<0.01$). However, no difference was found for the nuclei area (Figure 3C).

In fibroblasts, curcumin significantly increased cell viability by 33.81% ($p<0.05$). While maintaining no difference in the cytoplasmatic area, fibroblast treated with curcumin presented a larger mean nuclear area of 232.54 μm^2 compared to 186.29 μm^2 in the control group ($p<0.001$) (Figure 3D).

Curcumin modulates gene expression in keratinocytes and fibroblasts

Differences in the relative expression of *PIK3CA*, *MTOR*, *AKT*, *PTEN*, and *IL-6* have been observed in keratinocytes and fibroblasts, comparing monocultures under ionizing radiation and bacterial stimuli with and without curcumin (Figures 4A and 4B).

In keratinocytes, curcumin significantly decreased *AKT* ($p<0.01$), showing a relative expression of 0.05 in treated cells, compared to 1.03 in the control group. *PIK3CA* remained close between the groups, with a relative expression of 1.09 in the curcumin group and 1.16 in the control group. *MTOR* presented a slight tendency of upregulation in the curcumin group (1.38) compared to the control (1.04), as well as PTEN (1.74 and 1.07, respectively) and *IL-6* (1.24 and 1.05, respectively), although no statistical significance was found (Figure 4A).

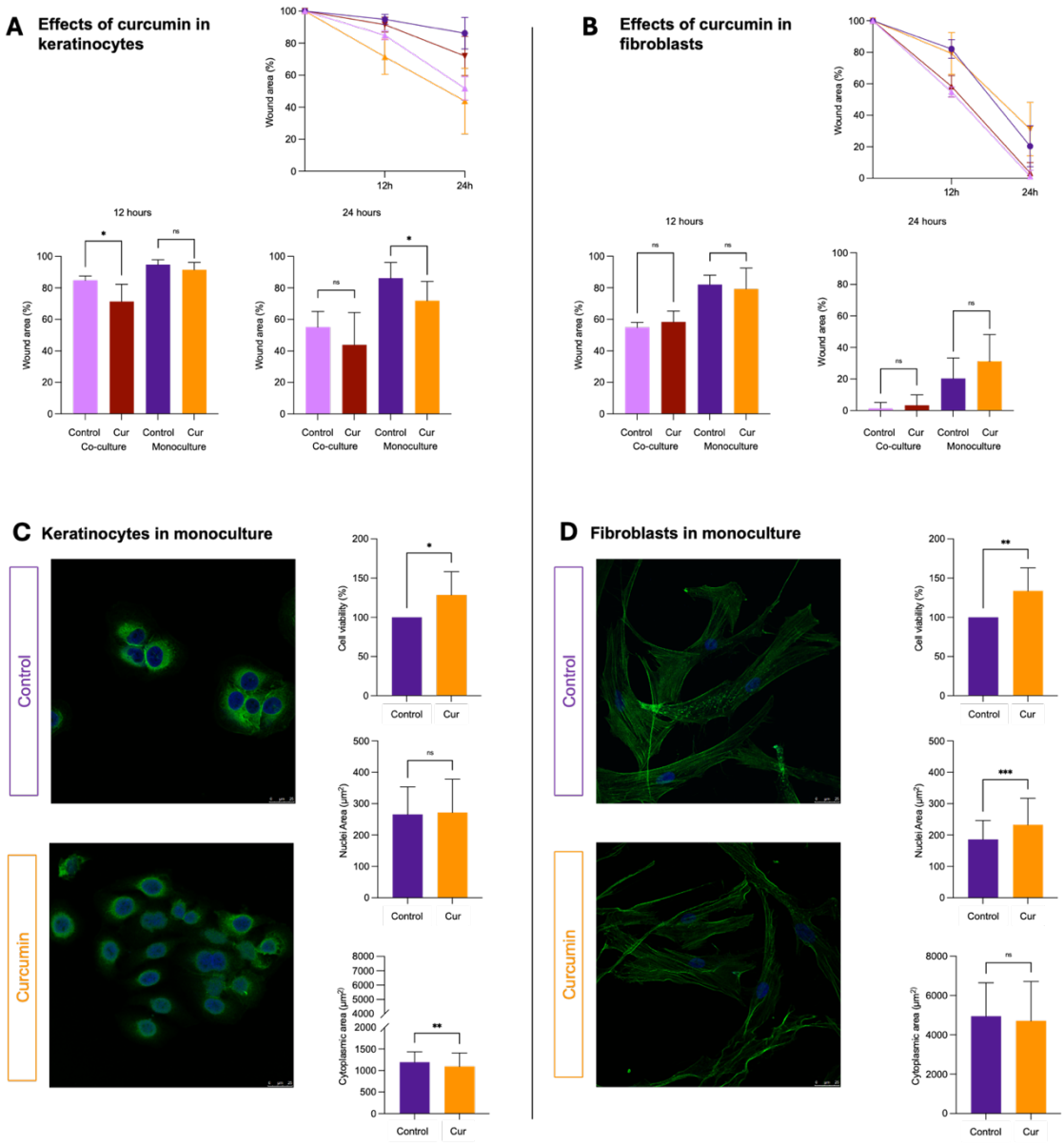

Figure 3. Cellular effects after ionizing radiation and bacterial stimuli with or without curcumin treatment. Response to curcumin treatment compared to stimuli in both co-culture and monoculture settings for (A) keratinocytes and (B) fibroblasts. (C) Cell viability, cytoplasmic area, and nuclei area of keratinocytes and (D) fibroblasts. Statistical analysis: Mean ± SD, significant when p-value<0.05. Parametric distribution: Unpaired t-test (A 12 and 24h co-culture, B 12 co-culture and monoculture, 24h monoculture; C and D cell viability). Non-parametric distribution: Mann-Whitney test (A 12h and 24h monoculture, B 24h co-culture; C and D cytoplasmic area and nuclei area).

Differently, curcumin significantly decreased *MTOR* in fibroblasts, presenting a relative expression of 0.71 compared to 1.00 in the control group ($p<0.01$). Although without significance, curcumin showed a tendency to decrease *PIK3CA* (curcumin: 0.48; control: 1.10) and *IL-6* (curcumin: 0.56; control: 1.04) while tending to increase *PTEN* (curcumin: 1.14; control: 1.02) and maintain *AKT* (curcumin: 0.92; control: 1.01 (Figure 4B).

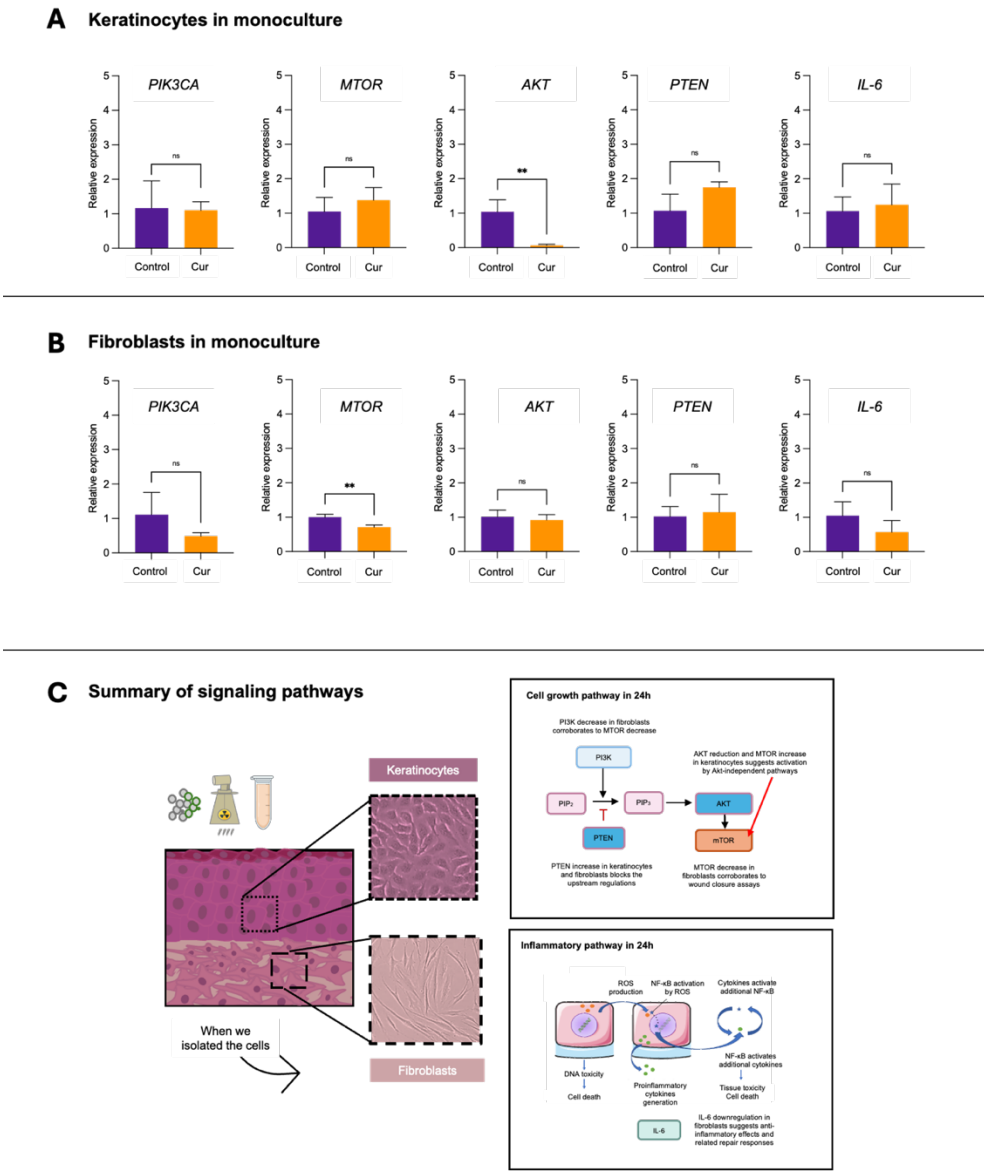

Figure 4. Effects of introducing curcumin after ionizing radiation and control stimuli in monocultures. Relative gene expression of *PIK3CA*, *MTOR*, *AKT*, *PTEN*, and *IL-6* in (A) keratinocytes and (B) fibroblasts. (C) Summary of signaling pathways findings. Statistical analysis: Mean ± SD, significant when p-value<0.05. Parametric distribution: Unpaired t-test (A and B).

Given the results, it may be proposed a potential pathway expression model in keratinocytes and fibroblasts treated with curcumin following exposure to ionizing radiation and bacterial stimuli (Figure 4C). For keratinocytes, the observed *AKT* downregulation, alongside a tendency of *PTEN* and *MTOR* upregulation, suggests that *MTOR* may be regulated via Akt-independent mechanisms, which could support the results observed in the other assays. Conversely, in fibroblasts, the significant reduction in *MTOR* expression, coupled with a tending decline in *PIK3CA* expression and *PTEN* upregulation, indicates that curcumin likely does not promote repair through the PI3K-AKT-MTOR cell growth pathway, aligning with

findings from the wound closure assays. Additionally, the slight reduction in *IL-6* levels hints at a modulation of inflammatory processes, which could influence other aspects of cell repair and intercellular interactions.

DISCUSSION

For years, natural compounds, such as curcumin, have been suggested as radioprotectants and radiomitigators due to their antioxidant, anti-inflammatory, and DNA-protective properties (Jagetia et al. 2007). Clinically, Ryan et al. (2013) conducted a pilot study that indicated the topical use of a curcumin-based cream reduced the severity of radiation dermatitis in breast cancer patients. In terms of managing radio-induced oral mucositis, clinical trials using bio-enhanced formations (Soni et al. 2022) and mouthwashes (Patil et al. 2015) showed curcumin's beneficial effects in reducing symptom severity in head and neck cancer patients receiving radiation therapy. Additionally, a recent meta-analysis of clinical trials reinforced curcumin's effects in improving oral mucositis clinical outcomes after radiotherapy and radiochemistry (Wu et al. 2024). Considering its well-tailored profile but the biostability and biodistribution limitation, efforts in several formulations of topical curcumin, such as emulsion, fibers, films, hydrogels, and nanoformulations, have aimed to improve its clinical applications (Mohanty et al. 2017).

The present study evaluates the effects of curcumin on the repair response of keratinocytes and fibroblasts after ionizing radiation and bacterial stimuli. Initially, the results highlight the impact of epithelial and mesenchymal interactions on cellular migration and proliferation, both with and without curcumin. Furthermore, the results demonstrate the effects of curcumin on different aspects of the repair process, delving into migration, cell viability, morphology, and gene expression.

When cells were co-cultured, the wound areas of both keratinocytes and fibroblasts were significantly reduced at 12h and 24h compared to isolated cell culture conditions, reinforcing the importance of their interaction. While keratinocytes activate fibroblasts through signaling molecules like IL-1, IL-19, TGF-β, and cytokine ligand (CCL)26, fibroblasts contribute to matrix deposition and wound contraction secreting growth factors and mediators that aid keratinocyte function (desJardins-Park et al. 2018; Ko et al. 2019; Russo et al. 2020). Also, ionizing radiation directly damages cellular DNA and indirectly affects surrounding cells, hindering the tissue repair process (McBride et al. 2020). The interaction between epithelial and mesenchymal cells is critical to orchestrate tissue repair, and several intracellular and intercellular pathways might be initiated to restore tissue integrity and homeostasis (desJardins-Park et al. 2018; Eming et al. 2014; Amiri et al. 2022).

Although co-cultured conditions improved wound closure, it was also evident that keratinocytes were more sensitive to ionizing radiation and bacterial stimuli than fibroblasts, regardless of isolated or co-cultured conditions. By including curcumin as a treatment in monocultures under ionizing radiation and bacterial stimuli, keratinocytes improved their wound closure significantly after 24h. In the same way, curcumin was able to improve cell viability and reduce cytoplasmatic areas of keratinocytes. The literature shows that exposing oral keratinocytes to 8 Gy of ionizing radiation significantly reduces cell viability and considerably increases the production of pro-inflammatory cytokines IL-1α and IL-8. However, in fibroblasts, a significant reduction in cell viability is observed only after exposure to 20 Gy of radiation, which also leads to elevated

expression of IL-6 (Tobita et al. 2010; Colley et al. 2013). A reduction in cytoplasmic volume relative to the nucleoplasm can also be indicative of active cellular proliferation, as the cell cytoskeleton, composed of microfilaments, microtubules, and intermediate filaments, integrates and orchestrates functions responsible for many cellular mechanisms, such as cell motility, migration, differentiation, and proliferation (Cheng et al. 2017; Udayashankar et al. 2016).

Despite the limited evidence of curcumin on non-neoplastic epithelial cells, research demonstrating its role in modulating proliferation pathways, such as mTOR complex 1 and PTEN in neoplastic cells, points out a potential to influence the epithelial response through different activations (Borges et al. 2020). In our study, treatment with curcumin decreased *AKT* expression and tended to improve *MTOR*, *PTEN*, and *IL-6* in keratinocytes exposed to ionizing radiation and bacterial stimuli. Concurrently, the study by Chen et al. (2022) applied similar doses of curcumin (2.5 and 5μM) on UV-irradiated keratinocytes showing its potential to protect photodamage. However, curcumin decreased cell progression while it downregulated a variety of inflammatory biomarkers, such as NF-κB, MMP1, IL-1b, and IL-6.

In fibroblasts, curcumin was not able to improve wound closure either in co-culture or monoculture after ionizing radiation and bacterial stimuli. Curiously, cells treated with curcumin presented a slower closure tendency at 24h compared to the group without curcumin. Although it did not significantly improve closure outcomes, curcumin was able to increase cell viability and nuclei area of fibroblast, which could be indicative of cell cycle progression and increased transcription facilitated by larger nuclei (Webster et al. 2009). Mandroli et al. (2016) demonstrated the ability of different concentrations of curcumin to improve pulp fibroblasts' viability and induce proliferation. Additionally, using a lower concentration of curcumin (1 μM), Rujirachotiwat, and Suttamanatwong (2021) demonstrated curcumin effects on maintaining cellular viability and significantly upregulated gene expression of *TGF-β1, TGF-βRI, TGF-βRII*, or *VEGF* in wounded gingival fibroblasts. In another view, low doses of curcumin (5-25 μmol/L) were explored for hypertrophic scaring. Curcumin was able to impair cellular proliferation and migration of dermal fibroblasts. Its effects on inhibiting *α*SMA expression and modulating the TGF-β1/Smad3 pathway indicate the potential to regulate fibroblast activation and inflammatory response (Fei et al. 2023). In parallel, Webber al. (2019) showed that the topical inhibition of mTOR prevents and halts scar formation by downregulating ps6. The ability of curcumin to inhibit *MTOR* expression in fibroblasts while improving viability but not wound closure could suggest its effects on this modulation, especially for the late response to ionizing radiation and bacterial stimuli.

The overall research using curcumin suggests its potential to modulate different responses and pathways depending on the conditions and cellular type. Indeed, as a natural modulator of multifaceted singling pathways, several factors might be associated (Liczbiński et al. 2020). By modulating mTOR, a variety of signaling pathways could be involved, including receptor tyrosine kinases and inflammatory cytokines (Panwar et al. 2023). The phosphorylation of tuberous sclerosis complex 2 (TSC2) prevents TCS activity enhancing mTORC1. It can occur via AKT or MAPK/ERK, and its target is p90RSK. Crucially, mTORC1 responds to both external and internal stress signals, adjusting cell growth and survival in response to low oxygen, depleted ATP levels, and DNA damage, which are expected results from ionizing radiation. When cells experience lower energy levels, the metabolic regulator AMPK is activated. AMPK can inhibit

mTORC1 by directly phosphorylating Raptor or indirectly through the phosphorylation of TSC2. These mechanisms collectively ensure that mTORC1 modulates cell growth and survival following the cellular energy status and environmental conditions (Menon et al. 2014; Sengupta et al. 2010; Gwinn et al 2008; Panwar et al. 2023). As a notable limitation, the RT-qPCR analysis in our study was employed with an exploratory intent. Nevertheless, the evidence of curcumin's effects on non-neoplastic cells, especially under lesion conditions, is still limited, and more research is necessary to fully understand its mechanism of action.

CONCLUSIONS

Curcumin modulates wound-healing responses in keratinocytes and fibroblasts exposed to ionizing radiation and bacterial stimuli. While enhancing wound closure of keratinocytes, treatment with curcumin improved cellular viability and modulated the morphology of both cells. Additionally, this natural compound induced different gene expression patterns, suggesting its therapeutic versatility and potential effects as an alternative treatment to improve clinical outcomes and patients' quality of life. This study highlights the need for further research on gene expression and pathways activation and additional effects of curcumin on mucosal cells and tissues exposed to ionizing radiation and oral microbiome.

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APPENDIX

Supplementary Table 1. Primers' sequences and characteristics used for RT-qPCR.

Name	Sequence	Scale
PIK3CA_F	5'CCACGACCATCATCAGGTGAA3'	100 nm
PIK3CA_R	5'CCTCACGGAGGCATTCTAAAGT3'	100 nm
mTOR_F	5'GCAGATTTGCCAACTATCTTCGG3'	100 nm
mTOR_R	5'CAGCGGTAAAAGTGTCCCCTG3'	100 nm
AKT1_F	5'TCCTCCTCAAGAATGATGGCA3'	100 nm
AKT1_R	5'GTGCGTTCGATGACAGTGGT3'	100 nm
PTEN_F	5'TTTGAAGACCATAACCCACCAC3'	100 nm
PTEN_R	5'ATTACACCAGTTCGTCCCTTTC3'	100 nm
β-ACTIN_F	5'TCACCCACACTGTGCCCATCTACG3'	100 nm
β-ACTIN_R	5'CAGCGGAACCGCTCATTGCCAATG3'	100 nm
IL-6_F	5'CCTGAACCTTCCAAAGATGGC3'	100 nm
IL-6_R	5'TTCACCAGGCAAGTCTCCTCA3'	100 nm

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3D bioprinting skin equivalents: a methodological review of human keratinocyte and fibroblast-loaded models

ABSTRACT

Three-dimensional (3D) bioprinting is a promising technological approach for developing reliable tissue substitutes, essential for translational research. This methodological review aims to retrieve current protocols in 3D bioprinting involving human keratinocytes and fibroblasts for wound healing research, providing a general profile to guide future studies and to improve replication methodologies. Following the PRISMA 2020 guidelines, a comprehensive search was conducted on MEDLINE/PubMed, EMBASE, and Web of Science. The inclusion criteria were 3D bioprinter constructs with human keratinocytes and fibroblasts focusing on wound healing. Two blinded authors undertook the selection. Initially, they screened titles and abstracts, followed by full-text documents. Data extractions were conducted by one review and cross-checked by two others utilizing customized table sheets. Figures and graphics were developed using licensed accounts of Microsoft Suit, BioRender, and GraphPad Prism. Eighteen studies met the inclusion criteria, primarily focusing on skin substitutes, with no studies found on oral mucosal models. The geographic distribution of studies was predominantly concentrated in China (44.4%) and the United States (27.7%), followed by South Korea (11.1%). Collaborative international efforts were noted in 27.8% of the studies. Most studies (83.3%) used extrusion-based bioprinting techniques, while gelatin-based components were the most frequently used bioink material (61.6%). Other common materials included fibrinogen (38.8%), and alginate (33.3%). The studies utilized a variety of customized bioinks, such as those incorporating human serum, plasma fibronectin, and silk to enhance bioink functionality. The constructed skin substitutes included epidermal layers primarily composed of cultured keratinocytes and dermal layers containing cultured fibroblasts, with some studies incorporating endothelial cells and follicle papilla cells for additional complexity. Layers were printed either separately or together, with some studies adding acellular structural components. In terms of analysis, most studies assessed morphology, cell viability, histology, proliferation, protein and gene expression, and transepidermal electrical resistance. A significant number of studies (61.1%) validated their results by subcutaneously implanting the artificial skin in animal models, primarily using mice to evaluate wound closure, contraction, and other healing parameters. Current studies demonstrate the global interest and collaborative efforts in advancing research within skin wound healing using 3D bioprinting. This review highlights the need for standardized protocols to enhance the replicability and translational potential of 3D bioprinting technologies for skin modeling and clinical applications.

Keywords: 3D bioprinter; *In vitro* substitutes; Wound healing; Skin; Tissue engineering; Methodological Review.

INTRODUCTION

The scientific development of clinical innovations involves an extensive process of cell culture, animal models, and clinical trials. While cell culture models enable valuable scientific advances, *in vitro* monocultures limit the translation of the evidence. Animal models more accurately replicate integrated microenvironments *in vivo*. However, the low-throughput nature of techniques impacts the success rate of clinical trials (Adhikary et al. 2021; Loewa et al. 2023; Urzì et al. 2023). Therefore, bioprinting three-dimensional (3D) cell scaffolds stand now at the forefront for reliable tissue substitutes of particular interest to translational research. This technology creates biological constructs with a hierarchical architecture that closely resembles human tissues (Murphy and Atala, 2018). By recapitulating the complexity of tissues with low species bias, 3D bioprinting technology holds the potential to bridge this gap bringing an expedited translation from the bench to the bedside.

Raised from prototyping principles and printing materials, 3D bioprinting can develop biomimetic environments of native tissues. The approaches involve sequential or simultaneous layer-by-layer printing of multiple materials to support different cell types. The flexibility of incorporating biocomponents to create functional bioinks allows for the customization of complex constructs (Murphy et al. 2014). Typically, hydrogels are applied as the main component due to their high-water content and remarkable resemblance to the natural extracellular matrix (ECM), providing an enriched environment for cell viability, proliferation, and differentiation. The integration of nanoparticles and nanofibers can improve printability and functionality while adding growth factors, drugs, and shRNAs might modulate pathways of interest. In addition, utilizing sacrificial supports or suspended hydrogel techniques facilitates detailed 3D designs with refined structures and perusable channels (Ma et al. 2018; Bishop et al. 2017; Ravanbakhsh et al. 2021).

Notably, 3D bioprinting holds considerable promise for advancing research in skin and oral mucosa wound healing research. The skin and oral mucosa tissues are composed of several layers that coordinate interdependent functions (Toma et al. 2021). In both healthy and pathological states, overlapping mechanisms nourish a complex microenvironment with cell-cell and cell-ECM essential interactions. While epithelial cells usually promote reepithelization, the mesenchymal components give structural and defensive support with essential vascular and immunological responses (Diaz-Garcia et al. 2021). Thus, the layer-by-layer customization including physiological ECM alongside cells, vascular, and immunological elements of choice is promising to study a variety of mucosa conditions *in vitro* (Bourland et al. 2018). Bioprinter technology tunes reliable mucosa substitutes expediting the understanding of cell-cell, cell-ECM interactions, and modulatory biological processes, as well as revolutionizing personalized wound dressings and grafts (Mazzocchi et al. 2019).

Although the diverse upcoming 3D bioprinter technology proposes valuable perspectives, there are still methodological challenges implicating in advancing translational research. In this view, to improve health outcomes, a comprehensive overhaul of educational frameworks to better prepare scientists and guide future studies is essential. Collaborative efforts among educational institutions, funding bodies, and government agencies can significantly contribute to transforming scientific discoveries into real-world health solutions. Furthermore, the formation of cross-disciplinary teams adept at navigating scientific, regulatory, and business

dynamics is crucial for successfully bringing innovations to market (Faupel-Badger et al. (2022). This comprehensive approach is essential for innovating methodologies in several fields, including 3D bioprinting and wound healing research.

Thus, this methodological review aims to retrieve and summarize current protocols for 3D bioprinting of skin and oral mucosa substitutes incorporating human keratinocytes and fibroblasts for wound healing research. The final goal is to provide a comprehensive landscape of the global distribution of this field, offer a general view of the applied methodologies, and guide future research in replicating and building upon existing protocols available in the literature.

MATERIAL AND METHODS

Design and protocol registration

This methodological review used the Preferred Reporting Item for Systematic Reviews and Meta-analysis guidelines (PRISMA 2020) as references (Page et al. 2020). The protocol was published on the Open Science Framework (https://osf.io/xqnwd/). Due to methodological heterogeneity during the screening part, the protocol was updated to ensure consistency. Both versions are available.

Research Question

Study, Data, Methodology, Outcome

The research question was defined based on the SDMO acronym (Munn et al. 2018):

Study – clinical and laboratory.

Data – bioprinted constructs with human keratinocytes and fibroblasts.

Methodology – 3D bioprinting.

Outcome – skin or oral mucosa substitute for wound healing.

Research question: What are the current protocols for 3D bioprinting of skin and oral mucosa substitutes incorporating human keratinocytes and fibroblasts for wound healing research?

Eligibility criteria

Inclusion criteria considered (1) Studies using bioprinter; (2) Culture of human keratinocytes and fibroblasts within a matrix; (3) Wound healing and repair analysis for either skin or oral mucosa. Exclusion criteria considered (1) Reviews, conference abstracts, and book chapters, (2) Protocols not involving printing with a bioprinter, (3) Dressing-scaffolds testing not involving tissue substitute, (4) Wrong cells or acellular-scaffold component, (5) Wrong outcome, (6) Not available. There were no data or language restrictions.

Information sources and selection

On January 12th, 2024, the search strategy was adapted and applied to the following electronic databases: MEDLINE/PubMed (via the National Library of Medicine), EMBASE, and Web of Science Core Collection (WoSCC). Additionally, the gray literature was assessed through Google Scholar (Appendix 1). The

references were imported to the EndNote Web platform for collection and duplicate removal. Sequentially, two independent authors (JAS and MMM) assessed the titles and abstracts using Rayyan software (Ouzzani et al. 2016) to initially match the inclusion criteria. The same authors investigated the full text of selected articles under blinded conditions to confirm inclusion and exclusion criteria. Articles that did not fulfill the inclusion criteria were excluded (Appendices 2 and 3). Disagreements during the selection process were solved by a discussion between the two authors. When necessary, a third author was involved (CSB) to make the final decision.

Descriptive summary

One author (JAS) collected and organized data from the selected studies using personalized sheet documents. A second author (MMM) and a third author (CSB) cross-checked the information to confirm the accuracy. Disagreements between them were resolved by discussion. The initial information summary considered: (1) Author, Year, and Country; (2) Journal; (3) Journal's impact factor; (4) Keywords; (5) Title; (6) Aim; (7) Type of tissue and focus; (9) Bioprinter technique; (10) Main matrix components; (11) Applied assays and analysis (Appendix 4). Based on that initial assessment, separate tables and figures were structured. All tables and figures were created using licensed Microsoft Office Suite, BioRender, and GraphPad Prism accounts.

RESULTS

Information sources and selection

From the database search, 2,696 records were initially identified. Following the removal of duplicates, 2,163 studies remained for the screening phase. Upon screening the titles and abstracts, 1,961 studies were excluded as they did not meet the inclusion criteria. Thus, 202 studies were selected for a full-text eligibility assessment. Of these, 184 reports were subsequently excluded, each for specific reasons. Finally, 18 studies published between 2018 and 2023 were included in this methodological review (Albanna et al. 2019; Baltazar et al. 2023; Cavallo et al. 2023; Choi et al. 2023; Dai et al. 2022; Desanlis et al. 2020; Hafezi et al. 2020; Huyan et al. 2020; Jiao et al. 2022; Jin et al. 2021; Jorgensen et al. 2020; Jorgensen et al. 2023; Lee et al 2021; Li et al. 2023; Liu et al. 2022; Seol et al. 2018, Somasekharan et al. 2021; Zhang et al. 2023). The selection process is summarized in the flow chart presented in Figure 1A and Appendix 3.

General characteristics of the studies

Keywords

A word cloud was generated using individual terms from the keywords across the 18 included studies (Figure 1B). The most frequently reported term was "SKIN," which appeared in 17 studies, followed by "BIOPRINTER" (12 studies), and "3D" (10 studies). Subsequently, the terms "WOUND" (9 studies), "HEALING" (8 studies), "TISSUE" (6 studies), and "ENGINEERING" (5 studies) were also prominently reported. The "MATRIX", "FULL-THICKNESS", "BIOINK", "PRINTING", "CELLS", "EXTRACELLULAR", "REGENERATION", "HYDROGEL", "DERMAL", and "ALGINATE" were reported by two to three studies.

Furthermore, specific hydrogels and biomaterials, such as "Silk", "polyurethane", "methacrylate", and "chitosan", along with methodological terms like "coculture", "keratinocytes", and "fibroblast" were also mentioned (Appendix 4). This consistency between the identified keywords and the review objectives indicates alignment with the research question, as the terms relate to the focus of the investigations. Additionally, it is noteworthy that all the included studies exclusively reported on skin outcomes. Despite a comprehensive literature screening, no studies focused on oral mucosal models using 3D bioprinting with keratinocytes and fibroblasts were found.

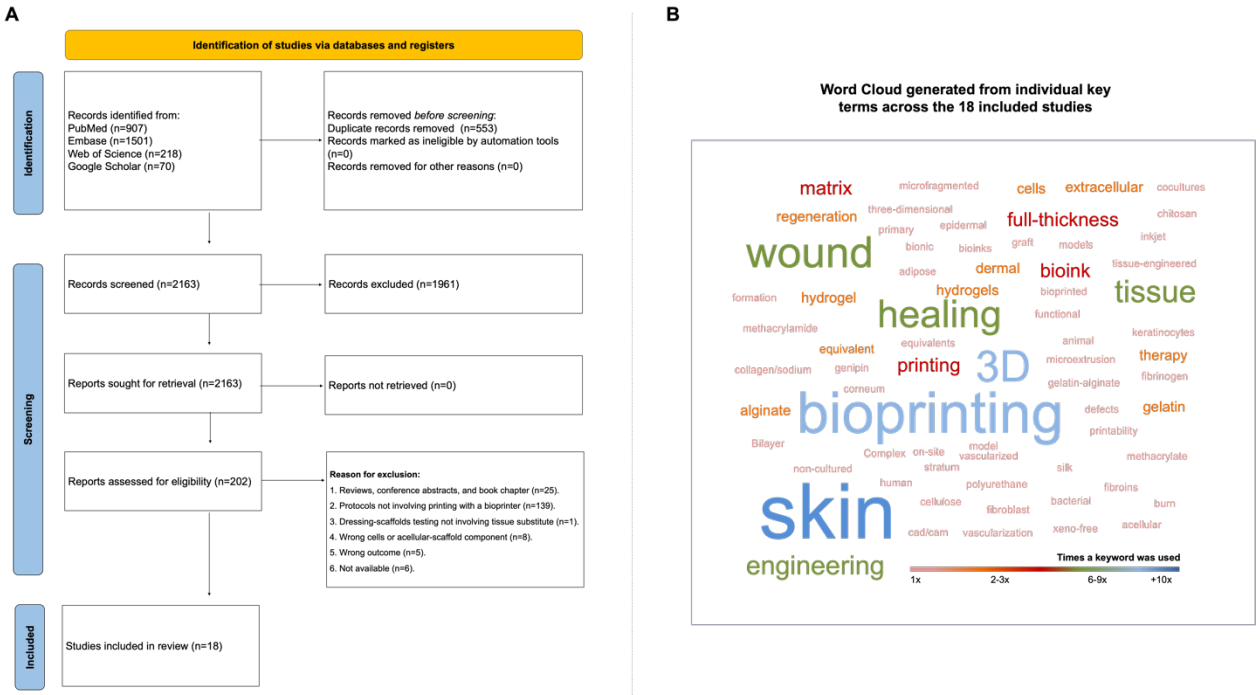

Figure 1. (A) Flow diagram of literature search and selection criteria adapted from PRISMA (Page et al. 2021). (B) Word cloud of keyword across included studies.

Geographical distribution

The geographical distribution of the studies was predominantly concentrated in Asia, Europe, and North America. Out of the 18 studies, 5 (27.8%) were conducted through international partnerships, while the remaining 13 (72.2%) were undertaken by researchers in single countries. The highest number of studies were carried out in China (8 studies, 44.4%) and the United States (5 studies, 27.7%), followed by South Korea (2 studies, 11.1%). The United States demonstrated a considerable number of international collaborations, partnering with China on two occasions, and with Russia and South Korea on one occasion each. Additionally, China formed a partnership with Portugal. Other contributing countries included France, India, Italy, Taiwan the United Kingdom (Figure 2).

In this view, we analyzed the distribution of bioprinter models across different continents based on the studies that reported this information. Figure 2 also illustrates the diverse range of bioprinter technologies

being utilized around the world, with Asia demonstrating the broadest variety of models. The company Cellink, with models BioX™ and Inkredible™, was featured in four studies spanning multiple continents. In Asia, several companies were prominently featured such as Regenovo which appeared twice with two different devices, Bio-Architect PRO and Bio-Printer-WS. EnvisionTEC was mentioned with the 3D printer and 3D-Bioplotter. NBR Tech Ltd was cited with the Digital Light Processing (DLP) NBR system. And the RegenHU with the 3D Discovery bioprinter. In North America, the ITOP system was highlighted in three studies, including partnerships with Russia and South Korea. In Europe, LabSkin Creations/TOBECA appeared once.

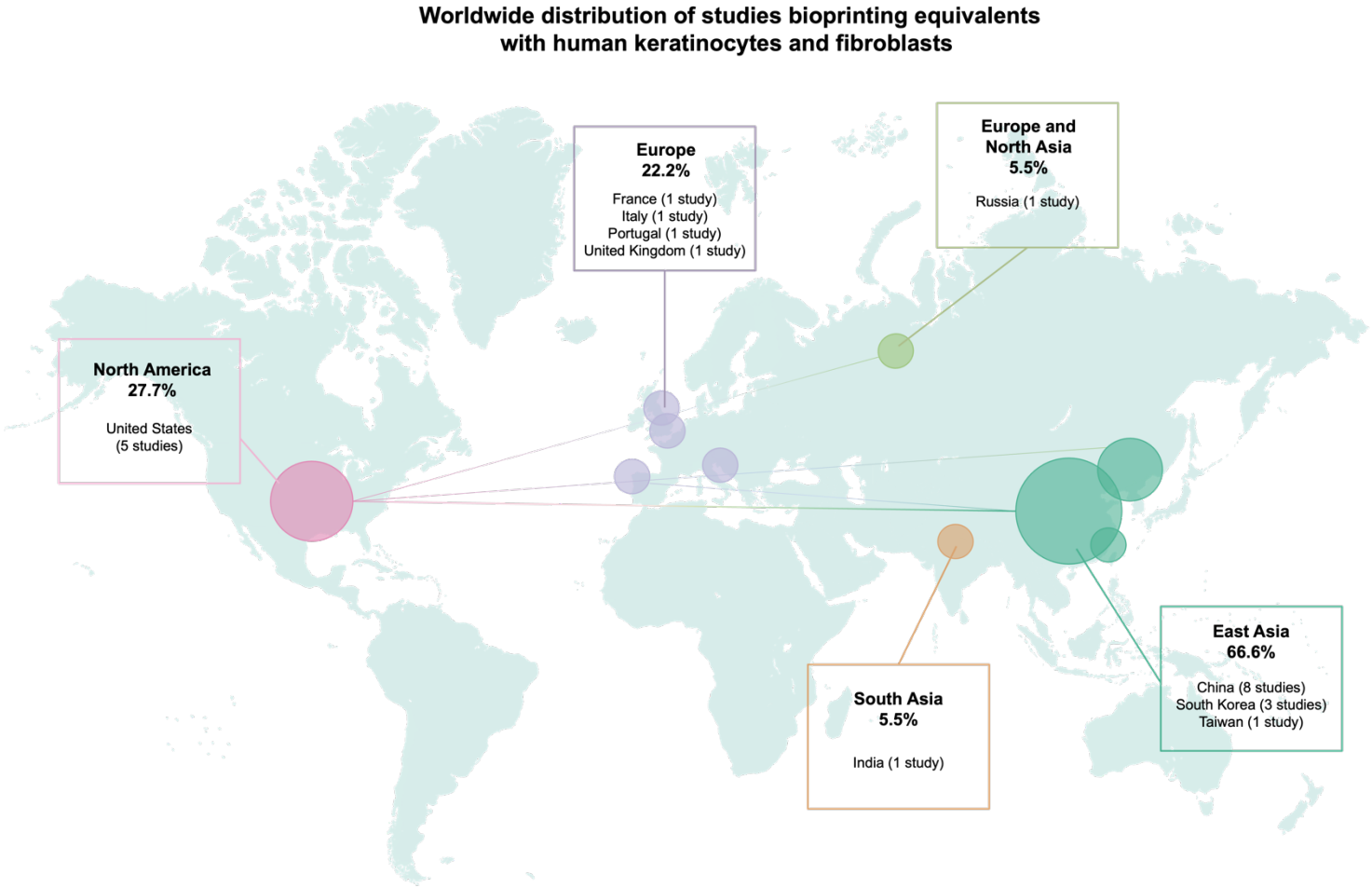

Figure 2. Geographic map showing the worldwide distribution of studies, international collaborations, and types of 3D bioprinter devices utilized.

Affiliations and main areas

To further analyze contributions to advancing research in this field, we identified the first authors, institutions, and departments affiliated with more than one publication (Appendix 5). A total of 17 first authors were identified, with Adam M. Jorgensen standing out as the only first author contributing to two studies, one from 2020 and another from 2023. While we recognize other authors with consistent contributions across

studies, detailed data on them were not collected. The affiliations are enclosed with 38 institutions, totaling 57 different departments or schools across the 18 included studies. Wake Forest University has four publications, involving the Institute for Regenerative Medicine (School of Medicine) and the School of Biomedical Engineering and Science (Virginia Tech) (Albanna et al. 2019; Jorgensen et al. 2020; Jorgensen et al. 2023; Seol et al. 2018). Additionally, the Wake Forest Baptist Medical Center was affiliated with three studies, all from the Department of Plastic and Reconstructive Surgery (Baltazar et al. 2020; Jorgensen et al. 2020; Jorgensen et al. 2023). Furthermore, Pohang University and Xi'an Jiaotong University were each affiliated with two studies. Pohang University's affiliations were within the Departments of Mechanical Engineering (Lee et al. 2021; Seol et al. 2018), while Xi'an Jiaotong University's affiliations were within the School of Mechanical Engineering (Huyan et al. 2020; Jiao et al. 2022).

Among the 36 identified institutions, the majority were universities, making up 50% of the total. Hospitals constituted 22.2%, followed by university hospitals and national research centers, both at 13.8%. Companies accounted for 5% of the institutions (Figure 3A). Regarding the main areas of the 57 departments or schools identified, Biomaterials, Engineering, and Technology led the field, accounting for 42.1% of the departments. Departments focused on Surgery represented 19.3%, while those in Health Science/Healthcare made up 10.5%. Other specialized areas included Pathology and Physiology (5.2%) and Burns (5.2%). Additional areas, such as Chronic Diseases, Immunology, Orthopedics, and Pharmacy collectively accounted for 7%, whereas Biology, Biophysics, and Chemistry together represented 3% (Figure 3B). The complete affiliation collection and classifications can be found in Appendix 6.

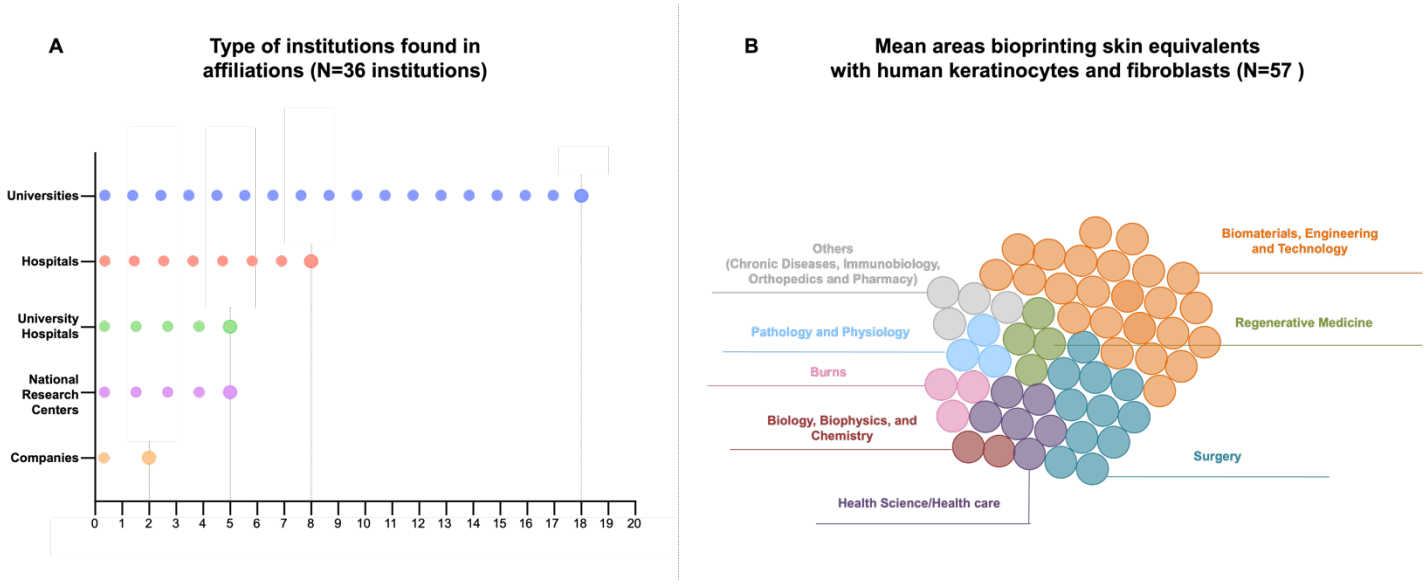

Figure 3. (A) Distribution of study affiliated institution types, including universities, hospitals, university hospitals, national research centers, and companies. (B) Distribution of research areas based on the affiliations of the studies bioprinting skin equivalents with human keratinocytes and fibroblasts.

Methodological characteristics of the studies

A variety of protocols were applied across the studies. Despite the use of similar biomaterials, bioprinting techniques, and types of cells, the specific bioink formulations varied. Different protocols related to mixing ratios and bioprinting parameters were described. Table 1 outlines the main objectives of each study, the bioprinting technique used, the main matrix components, and the main conclusions. Table 2 provides detailed protocols, including mixing ratios, cell-laden production methods, and bioprinting parameters. Additionally, a summary of the bioink components' properties and applications is provided in Table 3.

Bioprinter techniques

Three main bioprinting techniques were identified across the 18 studies as Extrusion, Inkjet, and Digital Light Processing (DLP) (Figure 4A). Extrusion emerged as the most extensively applied technique, utilized in 15 studies (Baltazar et al. 2023; Cavallo et al. 2023; Dai et al. 2022; Desanlis et al. 2020; Hafezi et al. 2020; Huyan et al. 2020; Jiao et al. 2022; Jin et al. 2021; Jorgensen et al. 2020; Jorgensen et al. 2023; Li et al. 2023; Liu et al. 2022; Seol et al. 2018, Somasekharan et al. 2021; Zhang et al. 2023), and in combination with Inkjet in one study (Lee et al 2021). Inkjet (Albanna et al. 2019) and DLP (Choi et al. 2023) were each applied individually in one study.

Bioink components

Among the identified studies, all utilized customized bioinks, while Baltazar et al. (2023) also included a commercial bioink in their formulation (VitroGel® Hydrogel Matrix). Gelatin-based components were the most prevalent, used in 12 (61.6%) studies, in various forms such as isolated gelatin, gelatin methacrylate composites, and polyurethane gelatin. Fibrinogen was applied in 38.8% of the studies, followed by alginate and sodium alginate (33.3%). Collagen type I and hyaluronic acid were each used in 4 studies (22.2% each) while glycerol was utilized in 3 studies (16.6%) (Figure 4B). Other components were employed to further customize or crosslink the bioinks, including human serum and plasma fibronectin (Baltazar et al. 2023), lithium phenyl-2,4,6-trimethyl benzoyl phosphonate (LAP) (Choi et al. 2023; Li et al. 2023), silk (Choi et al. 2023), poly (ε-caprolactone) diol (PCL) and poly (D, L-lactide) diol (PDLLA) (Dai et al. 2022), chitosan (Hafezi et al. 2020), porcine acellular dermal matrix (Jin et al. 2021), bacterial nano-cellulose (Li et al. 2023), and diethylaminoethyl cellulose (Somasekharan et al. 2021).

Gelatin was always combined with at least one additional component, such as alginate (Densanlis et al. 2020; Huyan et al. 2020; Liu et al. 2022; Somasekharan et al. 2021), fibrinogen (Jorgensen et al. 2020; Jorgensen et al. 2023; Seol et al. 2018), methacryloyl composites (Choi et al. 2023; Jin et al. 2022; Li et al. 2023; Zhang et al. 2023), or polyurethane (Dai et al. 2022). Collagen was mixed with fibrinogen (Albanna et al. 2019), human serum with plasma and VitroGel® (Baltazar et al. 2023), sodium alginate (Jiao et al. 2022), or used in isolation (Lee et al. 2021).

Only one study fabricated a xeno-free substitute by extracting all components from human samples (Baltazar et al. 2023). Also, despite initial tests involving sodium alginate, Jiao et al. (2022) ultimately opted

for a formulation without this component, choosing to use 1% type I collagen for constructing their skin substitute.

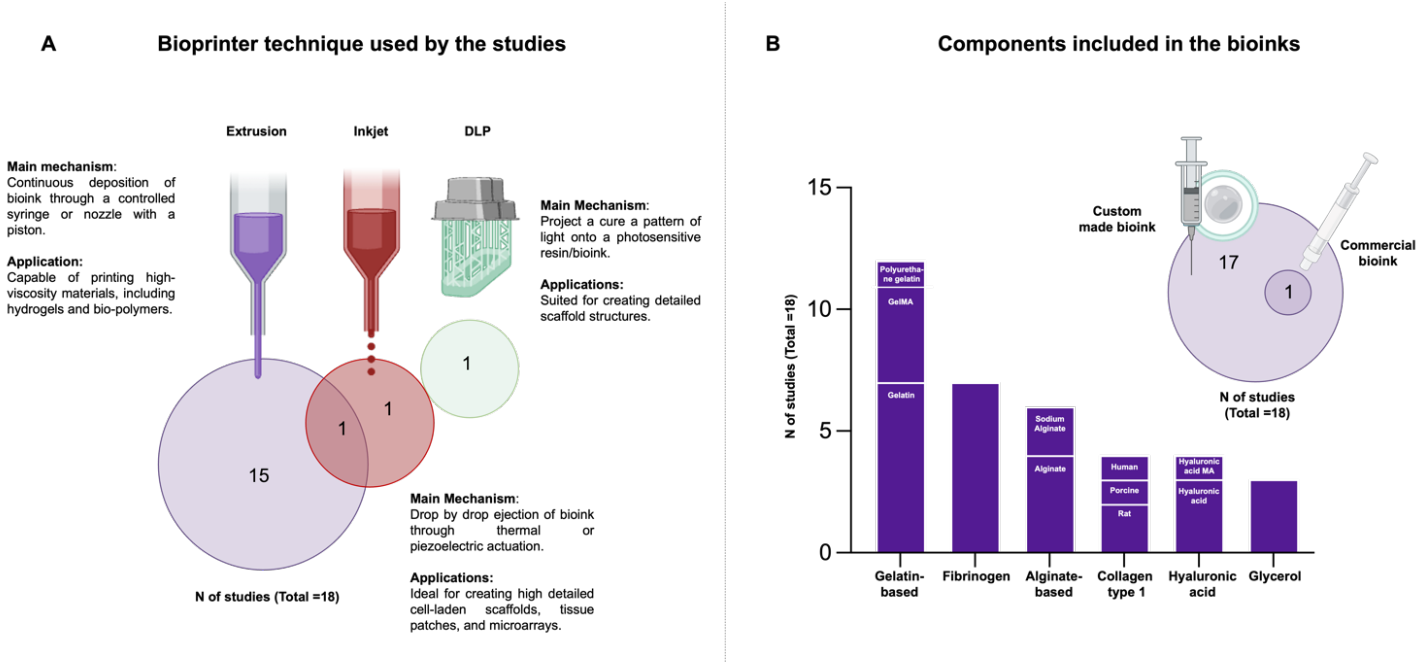

Figure 4. (A) Distribution of bioprinting techniques utilized across the studies, highlighting the prevalence of different methods. (B) Frequency of bioink components used, including the distinction between custom-made and commercial bioinks.

Skin substitute layers

The inclusion criteria for the studies required the incorporation of both keratinocytes and fibroblasts within a matrix to construct skin substitutes. The cell-laden bioinks could be either printed together or separately, with the inclusion of additional cells and components also being considered. Consistency with these criteria was evident during data collection, allowing the identification of specific sections dedicated to explaining the artificial skin structure across the studies.

Cellular and structural layers

Figure 5A shows that the epidermal layers primarily consisted of cultured keratinocytes alone in 15 studies (83.3%) and were combined with melanocytes in 2 studies (11.1%). One study (5.5%) used tissue explants for their cell-laden (Densanlis et al. 2020). Conversely, the dermal layers were composed of cultured fibroblasts alone in 9 studies (50%). To provide a vascular element, endothelial cells were utilized in 8 (44.4%), from where one also added pericytes (5.5%). Follicle papilla cells were included in two studies (11.1%). As with the epidermis, Densanlis et al. (2020) used explants in the cell-laden. The epidermis and dermis were printed as the only layers in 55.5% of the studies (10 studies). Three studies added acellular layers as basal or top components (16.6%) and two others included a hypodermis layer (11.1%). The

epidermis and dermis were printed together in 2 cases (11.1%), while a mesh structure between those two layers was considered in one study (5.5%).

To prepare the cell-laden, keratinocytes and fibroblasts were either purchased or obtained from biopsies. Five studies (27.7%) isolated keratinocytes from original biopsies (Baltazar et al. 2023; Dai et al. 2023; Densanlis et al. 2020; Jorgensen et al. 2020; Jorgensen et al. 2023), while seven studies used commercially purchased epidermal or neonatal primary keratinocytes (Choi et al. 2023; Hafezi et al. 2020; Huyan et al. 2020; Jiao et al. 2022; Lee et al. 2021; Seol et al. 2018; Somasekharan et al. 2021). Immortalized keratinocytes (HaCaT) were used in five studies (27.7%) (Cavallo et al. 2023; Jin et al. 2021; Li et al. 2023; Liu et al. 2022; Zhang et al. 2023), and one study did not report this information (Albanna et al. 2019). Regarding fibroblasts, seven studies (38.8%) isolated them from original biopsies (Baltazar et al. 2023; Dai et al. 2022; Densanlis et al. 2020; Jin et al. 2021; Jorgensen et al. 2020; Jorgensen et al. 2023; Liu et al. 2022), while ten studies used dermal or neonatal primary fibroblasts (Cavallo et al. 2023; Choi et al. 2023; Hafezi et al. 2020; Huyan et al. 2020; Jiao et al. 2022; Lee et al. 2021; Li et al. 2023; Seol et al. 2018; Somasekharan et al. 2021; Zhang et al. 2023), and one study did not report this information (Albanna et al. 2019). Table 2 describes the details and cell densities of each cell type.

Epidermis and Dermis bioink components

Figure 5B shows that most studies prepared separate cell-laden for printing the epidermis and dermis layers, with 13 studies (66.6%) using the same materials for both layers. Four studies utilized different mixes for each layer, where the epidermis was composed of supplemented medium mixes (Baltazar et al. 2023; Lee et al. 2021), a higher concentration of GelMA (Jin et al. 2021), or GelMA combined with hyaluronic acid and LAP (Zhang et al. 2023). In these studies, the main difference between the layers was an additional inclusion of extracellular matrix substances in the dermis, including dermal matrix (Jin et al. 2021), microfragmented adipose extracellular matrix (Zhang et al. 2023), and collagen type I (Baltazar et al. 2023; Lee et al. 2021). Additionally, two studies prepared a unique cell-laden for epidermis and dermis components. Densanlis et al. (2020) utilized micro-explant pieces isolated from skin biopsies, reporting it challenging to achieve perfectly separated cell populations after printing. Hafezi et al. (2021) prepared one cell-lade with primary epidermal keratinocytes and dermal fibroblasts being able to maintain high cell viability for at least seven days post-printing. Table 2 provides details on the preparation of each cell-laden.

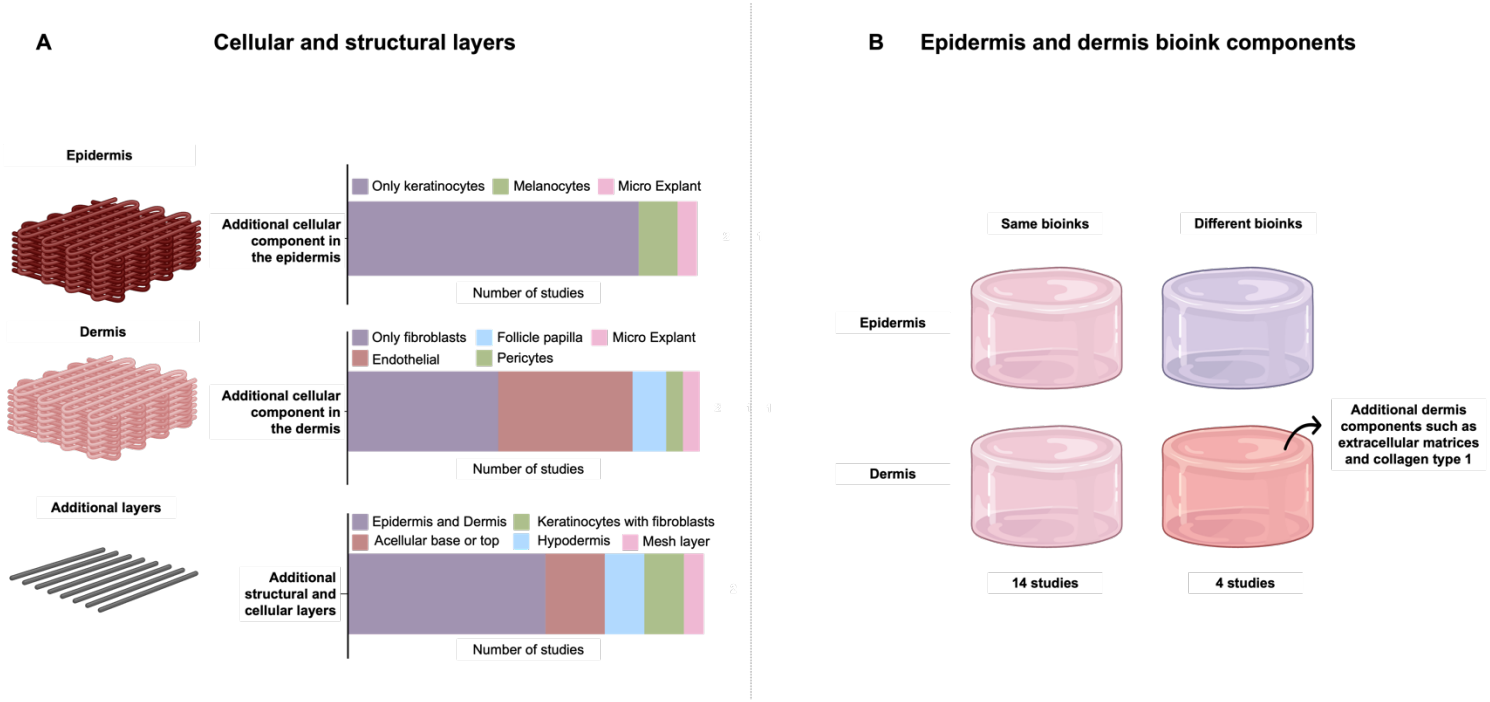

Figure 5. (A) Key components used to construct the cellular and structural layers of the skin substitutes using bioprinting process. (B) Distribution of studies based on the use of similar or different bioinks for bioprinting the epidermal and dermal layers.

Analysis focus and assays

All studies focused on bioprinting skin substitutes, but they differed in their specific analytical objectives and performed different assays (Figure 6). For the skin equivalents, the primary analyses centered around morphology, cell viability, histology, proliferation, cell tracking/distribution, trans-epidermal electrical resistance, and protein and gene expressions. Five studies (27.7%) employed the air-liquid interface strategy to facilitate epithelial differentiation. Most of the studies (61.1%) incorporated animal validations, using mice (55.5%) and porcine models (5.5%). These animal studies assessed various parameters such as wound closure, wound contraction, histology, protein and gene expressions, bioink toxicity, vessel perfusion, and collagen quantification. Additionally, 11 studies (61.1%) examined the properties of the bioinks including printability, rheological and mechanical characteristics, degradation, compression and tensile properties, swelling, water absorption, morphology/microstructure, cross-linking behavior, and biocompatibility. Imperatively, all of those also included analysis of the skin equivalent, animal validation or both. Appendix 4 details the specific assays performed in each study.

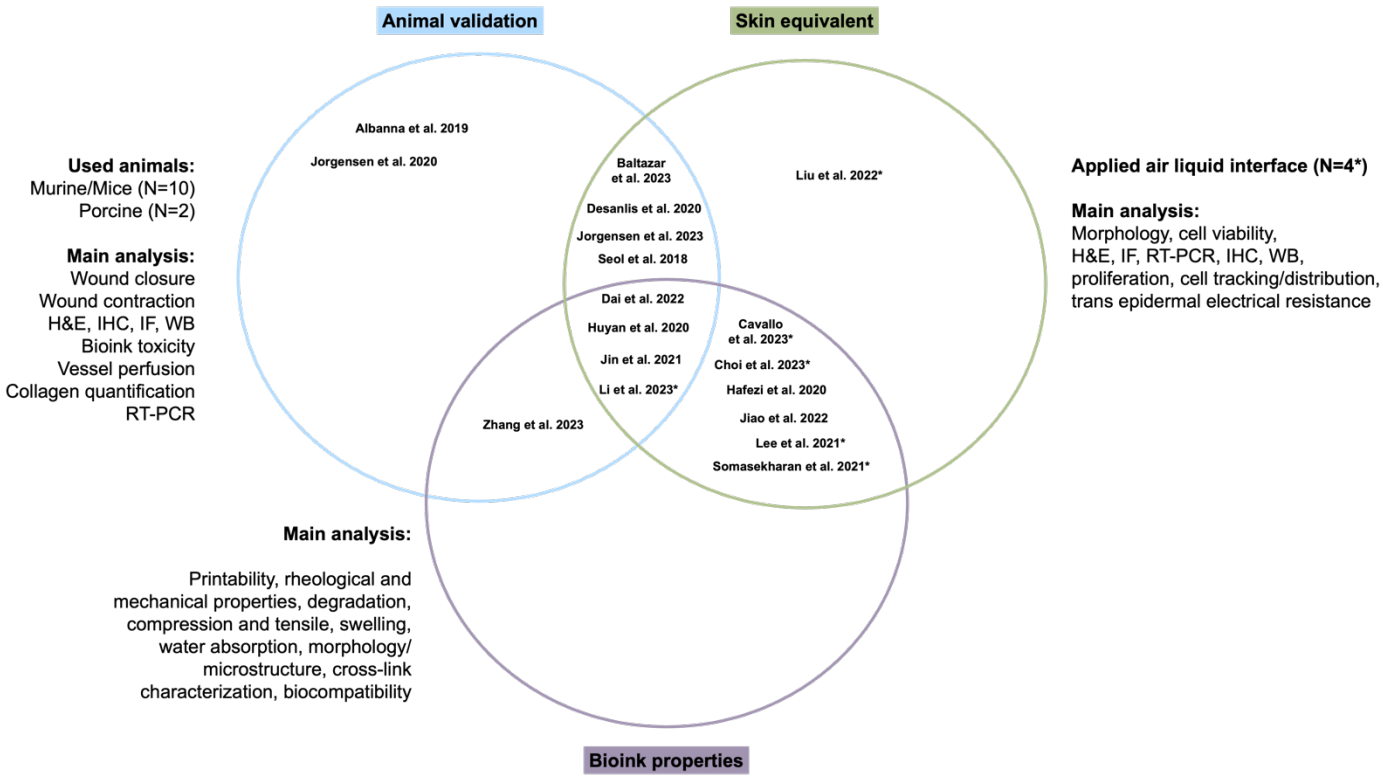

Figure 6. Venn diagram summarizing the types of analysis conducted to evaluate bioink properties, the skin equivalents themselves, and animal validations. The diagram highlights the number of studies focusing on each of these three domains, along with corresponding references.

DISCUSSION

Three-dimensional bioprinter technology is a diverse and emerging field that offers valuable perspectives for translational research. Many reviews summarize the properties, biological effects, and applications of a variety of hydrogels and biomaterials in use currently for skin dressings and tissue substitutes (Sivaraj et al. 2021; Miraj et al. 2022; Li et al. 2021; Yadav et al. 2024). However, while the customization of the protocol makes 3D bioprinting valuable for different applications, its heterogeneity implicates significant methodological challenges. To the best of our knowledge, there is no systematic review that summarizes methodological protocols. By providing a general profile of the applied methodologies, this review seeks to guide future research in replicating available protocols worldwide (Figure 7).

The keywords identified among the studies reveal a pattern aligned with the research priorities and expertise of leading centers in 3D bioprinting keratinocytes and fibroblasts-loaded constructs. The word cloud featuring the terms "SKIN," "3D BIOPRINTING," "WOUND HEALING," and "TISSUE ENGINEERING" shows a primary focus on skin-related outcomes. This is also evident in the principal contributing departments, including Biomaterials, Engineering and Technology, Surgery, Health Science and Healthcare, Regenerative Medicine, and Burns. Notably, out of the 18 studies, two were spearheaded by the same first author from Wake Forest Institute, enduring their leadership with global collaborations. Wake Forest-associated institutes

have been advancing this field since 1999 when they first reconstructed an organ using 3D printing. In general, this multidisciplinary nature of bioprinting research integrates expertise from various scientific and medical domains, which is imperative to foster innovation and comprehensive strategies for developing functional tissues. The involvement of universities, hospitals, national research centers, and companies indicates collaborative efforts between public and private sectors, which can accelerate the translation of research findings into clinical applications (Faupel-Badger et al. 2022). By continuously fostering interdisciplinary collaborations and leveraging the global diversity of expertise, the field of bioprinting in skin regeneration is poised for transformative advancements soon.

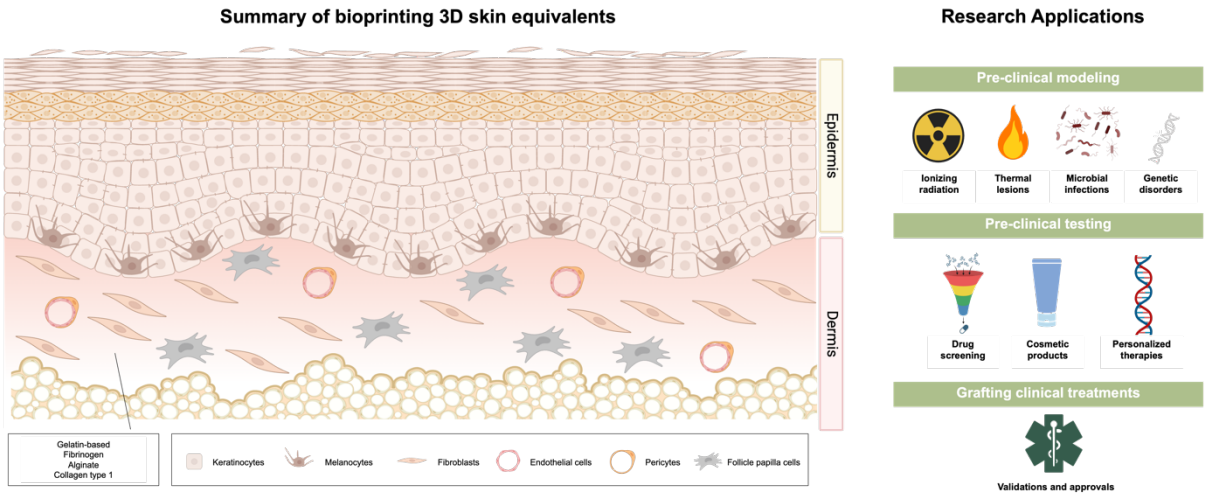

Figure 7. Skin substitute model illustrating the cellular components and most utilized materials across the studies with potential research applications that could emerge from standardizing functional models.

The skin consists of three main layers: the epidermis, dermis, and subcutaneous layer. The epidermis, primarily composed of juxtaposed keratinocytes is the outermost layer and is divided into five sub-layers. The stratum corneum consists of keratin-filled cells. Beneath it, the stratum granulosum contains cells with keratin granules, while the stratum spinosum is known for its spiny appearance due to desmosome connections. The stratum basal is a single layer of constantly dividing cells, also containing melanocytes and Merkel cells (Diaz-Garcia et al. 2021). The dermis is thicker than the epidermis and contains collagen and elastin fibers that lend strength and elasticity to the skin. The dermis also houses blood vessels, nerve endings, hair follicles, and sweat glands. The deepest layer, the subcutaneous, consists mainly of fat and connective tissue (Mamun et al. 2024). Thus, to build functional skin substitutes, tissue engineering must successfully create well-defined dermis and epidermis layers (Stanton et al. 2022). The dermis is typically engineered using hydrogels infused with fibroblasts. Over this dermal layer, an epidermis is bioprinted using keratinocytes that might undergo a stratification process to form the multiple layers typical of the natural tissue (Randall et al. 2018). Additionally, some studies have incorporated stem cells into the process. This

approach is beneficial by their ability to differentiate into various cell types required for both epidermal and dermal layers, including vascularization features (Nagano et al. 2023; Wang et al. 2023).

Overall, the protocol proposed in this review demonstrated reliable and effective methods for constructing skin substitutes. Collectively, the studies bioprinted 3D skin constructs involving keratinocytes and melanocytes, alongside epithelial stratification strategies to form the epidermis. For the dermis and subcutaneous layers, a combination of fibroblasts, endothelial cells, pericytes, and pre-adipocytes were incorporated within a variety of hydrogels. Most of the studies utilized animals to validate their findings, taking a high step at research levels (Albanna et al. 2019; Baltazar et al. 2023; Dai et al. 2022; Desanlis et al. 2020; Huyan et al. 2020; Jin et al. 2021; Jorgensen et al. 2020 Jorgensen et al. 2023; Li et al 2023; Seol et al. 2018). The high heterogeneity across the protocols highlights the flexibility and potential for customization depending on specific research objectives. However, this heterogeneity also poses a limitation in defining standard protocols for replication, thus demonstrating the need for consistency across research studies to ensure reproducibility and comparability of results. The replication of successful protocols across laboratories and research institutes could benefit consistency for the development of efficient solutions in wound healing.

Among the bioprinting techniques identified, extrusion-based bioprinting emerged as the most frequently reported. The extrusion technique involves the continuous deposition of bioinks through a pressurized nozzle, making it versatile and capable of handling high-viscosity hydrogels and cell-laden bioinks, which explains its widespread use (Malekpour et al. 2022; Wu et al., 2023). Cellink's extrusion devices were the most cited commercial bioprinters, featured in four studies (Baltazar et al., 2023; Cavallo et al., 2023; Hafezi et al. 2020; Somasekharan et al. 2021). Cellink has significantly influenced the field since they introduced the first commercial bioink in 2015 (Arumugam et al., 2024). The earliest study identified utilized the Integrated Tissue and Organ Printing (ITOP) system (Seol et al., 2018), an extrusion method equipped with multiple cartridges for individually delivering cell-laden hydrogels, an XYZ stage/controller, a dispensing module, and a closed chamber (Kang et al. 2016). This custom-made system was used in three studies, showing its application in various collaborations (Seol et al. 2018; Jorgensen et al. 2020; Jorgensen et al. 2023).

In addition to extrusion-based methods, inkjet bioprinting offers high-resolution and rapid printing by ejecting low-viscosity bioinks drop by drop (Nishiyama et al. 2009; Li and Liu et al. 2020). Lee et al. (2021) benefited from both extrusion and inkjet techniques, using inkjet bioprinting to deposit keratinocytes onto previously extrusion-printed dermis layers. This approach yielded a uniform distribution of epidermal layers, which is a crucial factor for effective skin tissue generation. Furthermore, a Digital Light Processing (DLP) technique was proposed to create a skin substitute for the first time (Choi et al. 2023). DLP is known for its exceptionally high resolution, achieved by using light to polymerize photosensitive bioinks (Gong et al. 2022). Choi et al. (2023) reported that DLP facilitated the creation of a biomimetic environment with excellent biological and mechanical properties.

Currently, a significant challenge in 3D bioprinting of skin substitutes is the absence of an ideal bioink that offers both excellent biocompatibility and adjustable mechanical properties (Jin et al. 2021). To address this, many studies utilize mixed hydrogels derived from natural and synthetic polymers to enhance bioink

applications. Gelatin-based components were the most used, followed by fibrinogen, alginate, collagen, and hyaluronic acid. Although collagen, resembles the natural ECM and provides essential cell attachment ligands for adhesion and proliferation, it has poor mechanical properties, making it challenging to print in isolation. Particularly in extrusion-based protocols, the incorporation of gelatin, alginate, and hyaluronic acid improves bioink viscosity and printability (Malda et al. 2013; Zhu et al. 2019; Heidenreich et al. 2020; Yadav et al., 2024). Fibrinogen undergoes crosslinking polymerization with thrombin to form fibrin, which supports cell adhesion and growth while allowing rapid gelation to maintain the 3D structure of printed constructs (Cavallo et al. 2023). Additionally, incorporating components such as glycerol, photo-initiators, human serum, silk, chitosan, and bacterial nanocellulose can enhance mechanical properties, cellular interactions, and biodegradability, tailoring bioinks for specific applications in skin outcomes (Li et al. 2023; Hafezi et al. 2020).

Controversially, our methodological review identified a significant gap in the exploration of the oral mucosa tissues using a 3D bioprinter. Despite the structural similarities between the skin and oral mucosa, the oral cavity is divided into masticatory, lining, and specialized mucosa. Areas exposed to mechanical forces exhibit protective keratinized epithelium firmly connected to the underlying collagen-rich lamina propria. Facilitating functional movements, flexible areas lined with a non-keratinized epithelium, and a lighter-elastic connective tissue. The specialized mucosa presents both keratinized and nonkeratinized epithelium while the tongue is equipped with taste buds (Squier et al. 2001; Toma et al. 2021). In between conventional monoculture and animal modeling, current 3D oral models are proposed with *ex vivo* equivalents and manual scaffolds. Basso et al. (2017, 2018) standardized keratinocytes onto an ex vivo oral mucosal equivalent produced with a porcine acellular dermal matrix. Cardoso et al. (2020) utilized a collagen matrix loaded with fibroblasts. Although using ECM biomimetics, these proposed methodologies present manual limitations or do not resemble the natural oral mucosa layers. Lee et al. 2023 compared non-printed and printed methods, showing that manually produced samples differed in cell distribution and shape from each other, posing important limitations. Thus, the bioprinter efficiency would enable precision and reproducibility of high throughput analysis for a range of oral conditions *in vitro*.

Standardizing 3D bioprinter protocols of both skin and oral mucosa equivalents could be essential for advancing future applications and translational research. Once functional *in vitro* tissues are established and reproducible, a broad range of modeling becomes feasible (Ma et al. 2018; Mazzocchi et al. 2019). These models can be stimulated to mimic oral diseases and conditions, such as chemo-radio induction, thermal injuries, infections, and genetic disorders. Reliable substitutes could meet the requirements for cosmetics biocompatibility or drug screening without the ethical concerns associated with animal testing. Furthermore, 3D patient-specific models would enable precise evaluations based on genetic profiles and facilitate personalized skin grafts (Loewa et al. 2023). Enhancing the accuracy of existing 3D models also holds promise for integrating Artificial Intelligence in understanding tissue biology, potentially leading to the identification of novel therapeutic targets and personalized treatment strategies (Lee and Wu et al. 2023). Thus, we believe that continued efforts to replicate protocols with multidisciplinary teams will accelerate the translation of these innovations from the laboratory to clinical applications.

CONCLUSIONS

This methodological review provided a comprehensive analysis of current protocols utilized in 3D bioprinting to generate skin substitutes incorporating human keratinocytes and fibroblasts for wound healing research. The primary focus of the literature was on developing bioprinted skin substitutes, highlighting a significant research gap in the oral mucosa domain. 3D bioprinting technology provides a high level of automation and precision to enhance the quality and functionality of the engineered skin offering promising laboratory models and grafting solutions. Extrusion-based bioprinting techniques were the most frequently applied, with a variety of hydrogels serving as the primary bioink components. This review highlights the necessity for standardized protocols and multidisciplinary collaborations to enhance the replicability and translational potential of 3D bioprinting technologies, thereby expediting the transition of scientific discoveries into clinical applications.

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TABLES

Table 1. Studies characteristics and conclusions (n=18).

Author, Year, and country	Title	Study focus	Bioprinter technique (Model, Company)	Main Matrix components	Main conclusions
Albanna et al. 2019, United States and China	In situ bioprinting of autologous skin cells accelerates wound healing of extensive excisional full-thickness wounds	Describe the design and a proof-of-concept validation of a novel mobile skin bioprinting system	Inkjet (NR)	Fibrinogen and Collagen type I rat tail	The treatment with autologous fibroblasts and keratinocytes, delivered directly to specific locations of the wound based on wound size and topology, resulted in the acceleration of wound healing and the formation of normal skin in situ
Baltazar et al. 2023, United States and China	3D bioprinting of an implantable xeno-free vascularized human skin graft	Demonstrate the fabrication of a bioprinted vascularized skin graft generated under strict xenogeneic-free	Extrusion (BioX™, Cellink)	Human male AB serum, human plasma fibronectin, human Collagen type I, and VitroGel Hydrogel Matrix	This protocol for isolation, culture, and cryopreservation of primary cells, bioink/scaffold development, construct maturation *in vitro* was successful, as well as the stable engraftment of a bioengineered xeno-free vascularized skin construct
Cavallo et el. 2023, Italy	Fibrinogen-based bioink for application in skin equivalent 3D bioprinting	Propose a bioink based on a fibrinogen and alginate blend to form a hydrogel by the enzymatic polymerization of fibrinogen with thrombin and by the ionic crosslinking of alginate with divalent calcium ions	Extrusion (BioX™, Cellink)	Fibrinogen and alginate	The fibrinogen-based bioink allowed fibroblast encapsulation for 3D bioprinting skin equivalents. Proliferative fibroblast and keratinocyte suggest it useful for wound healing applications
Choi et al. 2023, Korea	A digital light processing 3D-printed artificial skin model and full-thickness wound models using silk fibroin bioink	Print a 3D artificial skin model using silk and fibroin to mimic the structural and cellular	Digital Light Processing 3D printer (NBR Tech. Ltd)	Gel-GMA, Silk-GMA dissolved in lithium phenyl-2,4,6 trimethyl benzoyl phosphonate	Cells printed with Silk-GMA + Gel-GMA bioink demonstrated significant cell growth and proliferation. DLP ensures

		compositions of the human skin			the high biological activity with high resolution with fast printing speed
Dai et al. 2022 Taiwan	A bioprinted vascularized skin substitute with fibroblasts, keratinocytes, and endothelial progenitor cells for skin wound healing	3D bioprint a bilayer dermo-epidermal skin substitute using polyurethane gelatin multimaterial bioink with proper mechanical stability	Extrusion (Bio-Printer-WS, Regenovo)	Polyurethane (PU) gelatin, poly (ε-caprolactone) diol (PCL), poly (D, L-lactide) diol (PDLLA)	The interaction of three types of human cells in the 3D dermo-epidermal substitutes fabricated by bioprinting using the PU-gelatin bioink may facilitate the healing of extensive skin wounds
Desanlis et al. 2020, France	Validation of an implantable bioink using mechanical extraction of human skin cells: First steps to a 3D bioprinting treatment of deep second degree burn	Describe different steps and landmarks on experimental data obtained from mechanically extracted human skin cells and clinical grade implantable formulated bioink	Extrusion (LabSkin Creations/TOBECA)	Gelatin, alginate, and fibrinogen	The isolation protocol was proven to enable the achievement of proliferative keratinocytes and fibroblasts. However, the use of micro-explants directly as part of the bioink showed a growth delayed with not perfectly separated cell populations
Hafezi et al. 2020, United Kingdom	Bioprinting and preliminary testing of highly reproducible novel bioink for potential skin regeneration	Print keratinocytes and human dermal fibroblasts cells embedded in chitosan-Genipin-based inks by applying an extrusion bioprinting technique without damaging the cells	Extrusion (BioX^{TM}, Cellink)	Chitosan–polyethylene glycol and sodium alginate	The rheological properties of chitosan-genepin bioinks enhanced printability and geometric accuracy. The design of bioinks resulted in high cell viability for at least 7 days within the 3D constructs
Huyan et al. 2020, China	Pilot study of the biological properties and vascularization of 3D printed bilayer skin grafts	Fabricate a 3D-printed artificial skin including vascular features and hydrogel hybrid materials printed by an extrusion printing process	Extrusion (Custom-built)	Gelatin and sodium alginate	The printing process achieved more than a 90% cell survival rate for all three skin-specific cell types. The protocol facilitated the manufacture of a bilayer structure which was able to maintain the printed skin structure over time and integrated with host tissue within a wound. Constructs with cells had the clear advantage of

					micro vessels regeneration
Jiao et al. 2022, China and Portugal	Properties of collagen/sodium alginate hydrogels for bioprinting of skin models	Propose blending collagen and sodium alginate to obtain hydrogels with excellent mechanical and biological properties for a bilayer skin construct	Extrusion (NR)	Collagen type 1 rat tail and sodium alginate	Hydrogels with 1% collagen showed biological properties, micropores and degradation properties suitable for fibroblasts and keratinocytes growth and skin wound healing. This bioink was suitable for biofabrication of a bi-layer skin construct with good mechanical stability and promising properties for bioprinting of a skin model to be used in future wound healing research applications
Jin et al. 2021, China	Three-dimensional bioprinting of a full-thickness functional skin model using a cellular dermal matrix and gelatin methacryla-mide bioink	Investigate a combination of acellular dermal matrix and GelMA for 3D bioprinting of skin, in which GelMA is used as a structural bioink, and acellular dermal matrix is printed as the matrix bioink, to satisfy the biological requirements of the cells, as well as the structural requirements of the skin tissues	Extrusion (Bio-Architect PRO, Regenovo)	GelMA and porcine acellular dermal matrix	The good biocompatibility and tunable mechanical properties of acellular dermal matrix and GelMA bioinks made the bioink suitable for 3D bioprinting of skin. The material could maintain a moist microenvironment and barrier function, recreating a natural skin microenvironment to promote cell viability and proliferation
Jorgensen et al. 2020, Russia and United States	Bioprinted skin recapitulates normal collagen remodeling in full-thickness wounds	Test the ability of bioprinted human skin to integrate, form an epidermal barrier, and recapitulate normal collagen re-modeling in full-thickness wounds in mice	Extrusion (ITOP system, Kang et al. 2016)	Fibrinogen, gelatin, glycerol, and hyaluronic acid	This study demonstrated that bioprinted skin is easy to apply onto full-thickness wounds to integrate, form epidermal barrier, and recapitulate normal collagen remodeling. Bioprinted skin was superior in accelerating closure and epithelialization with

					mature and healthy epidermal coverage
Jorgensen et al. 2023, United States	Multicellular bioprinted skin facilitates human-like skin architecture *in vivo*	Fabricate a complex multilayered skin construct consisting of six primary human cell types to promote epidermal and dermal formation pigmentation, hair follicle formation, and neovascularization	Extrusion (ITOP system, Kang et al. 2016)	Fibrinogen, gelatin, glycerol, and hyaluronic acid	Bioprinted skin constructs with multiple cell types successfully engrafted and integrated into full-thickness excisional wounds in athymic mice. The construct accelerated the closure of the wounds without increasing contraction while also forming a human-like epidermis with rete ridges
Lee et al. 2021, Korea	3D microextrusion-inkjet hybrid printing of structured human skin equivalents	Introduce piezoelectric inkjet printing to generate an epidermal layer on top of a dermal layer formed by pneumatic microextrusion printing, and subsequently employe drop-on-demand piezoelectric type inkjet printing to distribute keratinocytes on the dermal mass in order to establish a simple and reproducible method for multilayer-structured skin model	Inkjet and pneumatic microextrusion (NR)	Type 1 porcine collagen	The inkjet printing method used in this study was able to provide higher accuracy and uniformity in the distribution of epidermal keratinocytes on the dermal layer. By combining pneumatic microextrusion and piezoelectric inkjet with the reported culture process, the protocol guaranteed high cell viability during the skin substitute fabrication
Li et al. 2023, China	3D bioprinting of heterogeneous tissue-engineered skin containing human dermal fibroblasts and keratinocytes	Employ a GelMA/BNC composite bioinks to print full-thickness heterogeneous tissue-engineered skin including layers of human dermal fibroblast networks with larger pores, basal layers with smaller pores, and multilayered human keratinocytes to provide a valid skin substitute for future clinical applications	Extrusion (3D-Bioplotter, Envision-TEC)	GelMA, bacterial nano-cellulose (BNC), lithium phenyl(2,4,6-trimethylbenzoyl) phosphinate (LAP)	The use of heterogeneous GelMA/BNC bioinks was useful to develop a tissue-engineered skin that includes layers of human dermal fibroblasts networks with larger pores, basal layers with smaller pores, and multilayered keratinocytes. The crosslinked 10%GelMA/1.5% BNC bioink supported the

					establishment of keratinocyte confluent monolayers, prerequisite for the formation of a stratified epidermis
Liu et al. 2022, China	Simple and robust 3D bioprinting of full-thickness human skin tissue	Present an approach that enables extrusion-based 3D bioprinting of a fullthickness human skin model in a simple, economic and robust way	Extrusion (3D Discovery bioprinter, Regenhu)	Gelatin, alginate, and fibrinogen	The bio-printed skin was not only morphologically representative of the human skin, but expressed the specific markers related to epidermal differentiation and stratum corneum formation. this study provides a simple, robust and cost-saving fabrication of full-thickness skin constructs, which can be applied for drug screening, cosmetic testing and clinical treatment
Seol et al. 2018, United States and Korea	3D Bioprinted BioMask for Facial Skin Reconstruction	Develop a novel strategy, called “BioMask”, which is a customized bioengineered skin substitute combined with a wound dressing layer that snugly fits onto the facial wounds	Extrusion (ITOP system, Kang et al. 2016)	Hyaluronic acid, glycerol, gelatin, and fibrinogen	The BioMask proved to be an excellent combination with the wound bed due to the high flexibility of PU. It Has a great potential to offer the effective and rapid restoration of aesthetic and functional facial skin. The bioengineered skin grafts composed of keratinocytes and fibroblasts were well applied to the wound region on the face-shaped structure, resulting in accelerating wound healing
Somasekharan et al. 2021, India	Biofabrication of skin tissue constructs using alginate, gelatin and	Explore an alginate, geleatine, diethylaminoethyl	Extrusion (Inkredible™, Cellink)	Alginate, gelatine, and diethylaminoethyl cellulose (DCEL)	The alginate-gelatin-DCEL bioink showed excellent shape fidelity

	diethylaminoethyl cellulose bioink	cellulose hydrogel bioink as a potential to cell adhesiveness, elasticity, and stability over a selective period of time and thereby to bioprint and biofabricate skin tissue equivalents			and shear thinning property after printing and crosslinking. Together with its mechanical stability, the bioink supported the cell viability of primary fibroblasts and keratinocytes in a co-culture medium over a period of 21 days of culture
Zhang et al. 2023, China	3D-Bioprinted Biomimetic Multilayer Implants Comprising Microfragmented Adipose Extracellular Matrix and Cells Improve Wound Healing in a Murine Model of Full-Thickness Skin Defects	Explore the use of microfragmented adipose ECM as the main component of the bioink, and different types of bioinks corresponding to dermis, epidermis, and vascularization functions to complete the 3D bioprinting of a biomimetic multilayer implant to be used as a skin substitute to repair full-thickness skin	Extrusion (3D printer, Envision TEC)	GelMA and hyaluronic acid methacryloyl	The results demonstrate that the microfragmented adipose ECM composite bioink has good biocompatibility, printability, and fidelity and can support cell adhesion. The biomimetic multilayer implants accelerated wound healing by promoting the contraction of new tissue inside the wound, collagen secretion and remodeling, and neovascularization. This study provides an efficient route to improve the timely fabrication of 3D bioprinted skin substitutes, which are critical for large-scale full-thickness skin defects

Abbreviations: 3D – Three-dimensional; ECM – Extracellular Matrix; GelMA – Gelatin Methacryloyl composites

Table 2. Detailed protocols for skin equivalents bioprinting (n=18).

Author, Year, and Country	Skin component	Matrix materials	Cell Cell density	Cell acquisition	Scaffold Design	Culture media	Bioprinter parameters
Albanna et al. 2019, United States	Epidermis	Fibrinogen NR, Collagen NR Crosslink: Thrombin 1:2 ratio cell suspension:ink	Keratinocyte $7.5x10^6$/mL 500 cell/drop	NR	1 layer	Not cultured after printing, the printouts were done directly on the animals	6.89 kPa of compressed air
	Dermis	Fibrinogen NR, Collagen NR Crosslink: Thrombin	Fibroblast $3.75x10^6$/mL 500 cell/drop	NR	1 layer		
Baltazar et al. 2023, United States	Epidermis	EpiLife medium (500µL) supplemented with HKSdaFREE and HKGE (AvantBio) and 5 ng/ml human recombinant animal-free KGF (Sigma-Aldrich), 1.64 mM CaCl2 and 50 µg/ml ascorbic acid	Keratinocyte $2x10^6$/mL	Cell isolation Discarded foreskin	1 layer (4 days after)	EpiLife™ HKSdaFREE + HKGE*, 5 ng/mL KGF, 1.64 mM $CaCl_2$, and 50µg/mL ascorbic acid	Extrusion pressure of 20 kPa
	Dermis	Human male AB serum (30µL); Human plasma fibronectin (60µL); 10X HAM-F12 medium (60µL); 6 mg/mL human collagen type 1 (330µL); 10x pH reconstitution buffer (60µL); VitroGel Hydrogel Matrix (Synthetic hydrogel) (60µL); Biodegradable polyglycolic acid (PGA) (60 mg/cm)	Fibroblast $4.5x10^6$/mL Endothelial $4.5x10^6$/mL Pericytes $5.5x10^5$/mL	Cell isolation Discarded foreskin, Umbilical cord blood, Discarded placentas	1 layer PGA mesh 1 layer		Extrusion pressure of 50 kPa
Cavallo et el. 2023, Italy	Epidermis	Bovin fibrinogen (30mg/mL) Alginate powder 8% (w/v) Crosslink: 100 mM $CaCl_2$ 25:9 (v/v)	Keratinocyte $2.5x10^6$/mL	Immortalized keratinocytes (HaCaT)	1 layer (1 day after) Air liquid interface after 3 days	DMEM 10% FBS, 1% Peinicilin-streptomicyn, 1% glutamine	Extrusion pressure of 12 kPa, with a printhead speed of 10 mm/s and a 22 G nozzle

	Dermis	Bovin fibrinogen (30mg/mL) Alginate powder 8% (w/v) Crosslink: 100 mM $CaCl_2$ 25:9 (v/v)	Fibroblast $4x10^6$/mL	Primary dermal fibroblasts from foreskin (PromoCell)	1 layer		
Choi et al. 2023, Korea	Acellular layer	15 % Gel-GMA 5% Silk-GMA (w/v) Crosslink: lithium phenyl-2,4,6 trimethyl benzoyl phosphonate 0.3% (w/v)	Acellular	-	1 layer to ensure easy air-liquid interface maturation	KGM2 (Keratinocytes Growth Medium-2, Promocell, Germany) and EGM2 (Endothelial Cell Growth Medium-2 BulletKitTM, Lonza, Switzerland) (1:1) for 1 day, then EGM2 reduced to the level of the junction of the epidermis and dermis	A UV Digital Micro-Mirror Device with a resolution of 30 μm A UV-LED (365 nm) A lens module with two UV-grade biconvex lenses UV power: 6.09 mW/cm³, Layer thickness: 50.00 μm, curing time for 3 skin layers: 4.00 s, curing time for 7 buffer layers: 3.70 s
	Epidermis	15 % Gel-GMA 5% Silk-GMA (w/v) Crosslink: lithium phenyl-2,4,6 trimethyl benzoyl phosphonate 0.3% (w/v)	Keratinocyte $2.x10^7$/mL	Normal human epidermal keratinocyte (NHEK)	1 layer Cultured in air-liquid interface after 1 day		
	Dermis	15 % Gel-GMA 5% Silk-GMA (w/v) Crosslink: lithium phenyl-2,4,6 trimethyl benzoyl phosphonate 0.3% (w/v)	Fibroblast $2x10^6$/mL Endothelial $1.8x10^7$/mL	Human dermal fibroblast (HDF), Human umbilical vein endothelial cell (HUVEC)	2 layers		
Dai et al. 2022 Taiwan	Epidermis	Polyurethane 10% (w/v), Gelatin 2.5% (w/v), DMEM medium 1% sodium bicarbonate Crosslink: 0.3N $CaCl_2$	Keratinocyte $2x10^6$/mL	Cell isolation from foreskin	2 layers	Low glucose DMEM	Nozzle temperature of 19 ◦C, printing velocity of 4 mm/s, and the air pressure of 0.16
	Dermis	Polyurethane 10% (w/v), Gelatin 2.5% (w/v), DMEM medium 1% sodium bicarbonate Crosslink: 0.3N $CaCl_2$	Fibroblast $2x10^6$/mL Endothelial $1x10^6$/mL	Cell isolation from foreskin, isolation from blood	5 layers		Nozzle temperature of 19 ◦C, printing velocity of 4 mm/s, and the air pressure of 0.128 (fibroblast-laden), 0.055 (EPC-laden) and 0.175 (fibroblast/EPC-laden)

Desanlis et al. 2020, France	Epidermis and dermis	Bovine gelatin 10% (w/v), very low viscosity alginate 0.5% (w/v), fibrinogen 2% (w/v)	Micro-explants 120 pieces of 4mm/8mL	Isolation from skin biopsies	NR	DMEM with glutamax-1, 10% CS, 20 ug/mL gentamicin, 100 UI/mL penicillin, and 1 ug/mL amphotericin B	0.9 mm diameter extrusion nozzle. 900 µm thick layers in a 1 x 1 x 0.5 cm (w x l x h)
Hafezi et al. 2020, United Kingdom	Epidermis and dermis	Chitosan 1.2% w/v, Polyethylene glycol 1.2% w/v, Genipin 1% w/v 2:5 cell suspension:ink	Keratinocyte $5x10^5$/mL Fibroblast $5x10^5$/mL	Primary epidermal keratinocytes and dermal fibroblasts (ATCC)	2 layers	DMEM	25 kPa pressure, 4 mm/s speed, 40 infill density, 22 G needle (0.41 mm inner diameter)
	Base	Alginate	Acellular		1 layer		10 kPa pressure, 6 mm/s speed, 40 infill density, 20 G needle (0.58 mm inner diameter)
Huyan et al. 2020, China	Epidermis	Sodium alginate 4% w/v, gelatin 10% w/v 1:1 cell suspension:ink	Keratinocyte $1x10^6$/mL	Primary epidermal keratinocytes (ATCC)	1 layer	DMEM, IMDM, RPMI (1:1:1) 10% FBS, 1% antibiotic antimycotic	A layers of 20 × 20 × 0.5 mm printed under the printing pressure 0.15 MPA and move speed 15 mm/s in 25°C
	Dermis	Sodium alginate 4% w/v, gelatin 10% w/v 1:1 cell suspension:ink	Fibroblast $1x10^6$/mL Endothelial $1x10^6$/mL	Primary dermal fibroblasts, dermal microvascular endothelial cells (ATCC)	1 layer		
Jiao et al. 2022, China and Portugal	Epidermis	Type 1 rat tail collagen 1% Sodium alginate not added to the skin construct	Keratinocytes $1x10^6$/mL	Primary epidermal keratinocytes (ATCC)	1 layer	NR	NR

	Dermis	Type 1 rat tail collagen 1% Sodium alginate not added to the skin construct	Fibroblast $1x10^6$/mL	Primary dermal fibroblasts (ATCC)	2 layers		
Jin et al. 2021, China	Epidermis	GelMA 20%	Keratinocyte $5x10^4/cm^2$	Immortalized keratinocytes (HaCaT)	Cells were seeded on the surface 1 layer	DMEM, 10% FBS, 1% penicillin/ streptomycin	The temperature of the nozzle and platform were controlled at around 20°C and 18°C, respectively. The printing speed was 5 mm/s and the pressure of the air compressor was set at 0.2 MPa.
	Dermis	Acellular dermal matrix from porcine skin 1.5% GelMA 10%	Fibroblast $1x10^6$/mL Endothelial $5x10^5$/mL	Fibroblast isolation from foreskin, primary umbilical vein endothelial cells (ATCC)	1 layer 1 layer		
Jorgensen et al. 2020, Russia and United States	Epidermis	Fibrinogen (30mg/mL), glycerol (100µL/mL), hyaluronic acid (3mg/mL), Cell suspension in aprotinin (40µg/mL) Crosslink: thrombin (20U/mL) in PBS	Keratinocyte $2x10^7$/mL Melanocyte $2x10^7$/mL	Isolation from skin sample, primary melanocytes (PromoCell)	1 layer	Keratinocyte serum-free medium with pituitary bovine serum and EGF, Melanocyte growth medium with SupplementMix, DMEM high glucose with FBS and P/S, Endothelial Growth Medium-2 with FBS,hydrocortisone, hFGF-b, VEGF, R3-IGF, ascorbic acid, hEGF, gentamycin, and heparin, Follicle Dermal Papilla Cell Growth Medium with SupplementMix (1:1:2:1:1:1)	In the printing process, the cell-laden hydrogel was printed through a 500-mm metal nozzle at 60–90 KPa of air pressure.
	Dermis	Fibrinogen (30mg/mL), glycerol (100µL/mL), hyaluronic acid (3mg/mL) Cell suspension in aprotinin (40µL/mL) Crosslink: thrombin (20U/mL) in PBS	Fibroblast $2x10^7$/mL Follicle papilla $2x10^7$/mL Endothelial $2x10^7$/mL	Isolation from skin sample, primary follicle dermal papilla cells (PromoCell), primary dermal microvascular endothelial cells (PromoCell)	1 layer		

	Hypodermis	Fibrinogen (30mg/mL), glycerol (100µL/mL), hyaluronic acid (3mg/mL), Cell suspension in aprotinin (40µg/mL) Crosslink: thrombin (20U/mL) in PBS	Pre-adipocyte $2x10^7$/mL	Isolation from skin sample	1 layer		
Jorgensen et al. 2023, United States	Epidermis	Fibrinogen (30mg/mL), glycerol (100µL/mL), hyaluronic acid (3mg/mL), Cell suspension in aprotinin (40µg/mL) Crosslink: thrombin (20U/mL) in PBS	Keratinocyte $2x10^7$/mL Melanocyte $2x10^7$/mL	Isolation from skin sample, primary melanocytes (PromoCell)	1 layer	Keratinocyte serum-free medium with pituitary bovine serum and EGF, Melanocyte growth medium with SupplementMix, DMEM high glucose with FBS and P/S, Endothelial Growth Medium-2 with FBS,hydrocortisone, hFGF-b, VEGF, R3-IGF, ascorbic acid, hEGF, gentamycin, and heparin, Follicle Dermal Papilla Cell Growth Medium with SupplementMix (1:1:2:1:1:1)	Cell-laden hydrogel was printed through a 500-µm metal nozzle at 70 to 130 kPa of air pressure.
	Dermis	Fibrinogen (30mg/mL), glycerol (100µL/mL), hyaluronic acid (3mg/mL), Cell suspension in aprotinin (40µL/mL) Crosslink: thrombin (20U/mL) in PBS	Fibroblast $2x10^7$/mL Follicle papilla $2x10^7$/mL Endothelial $2x10^7$/mL	Isolation from skin sample, primary follicle dermal papilla cells (PromoCell), primary dermal microvascular endothelial cells (PromoCell)	1 layer		
	Hypodermis	Fibrinogen (30mg/mL), glycerol (100µL/mL), hyaluronic acid (3mg/mL), Cell suspension in aprotinin (40µg/mL) Crosslink: thrombin (20U/mL) in PBS	Pre-adipocyte $2x10^7$/mL	Isolation from skin sample	1 layer		
Lee et al. 2021, Korea	Epidermis	EpiLife™	Keratinocyte $5x10^5$ total	Primary neonatal keratinocytes (Gibco)	Controlled drops after 2 weeks, cultured in air-liquid interface after 24h	Medium 106 with fetal bovine serum, hydrocortisone, EGF, FGF, heparin, and gentamicin/ amphotericin solution, after 2 weeks,	Piezoelectric inkjet printer with a nozzle with 80 µm diameter (MicroFab Inc.)

	Dermis	Porcine collagen type 1 0.6% w/v	Fibroblast $6.25x10^4$/mL	Primary neonatal fibroblasts (Gibco)	1 layer	EpiLife™ was added, then it was switched to keratinocytes differentiation media	A pneumatic extruder, equipped with a 4-gauge needle at a dispensing pressure of 10 kPa.
Li et al. 2023, China	Epidermis	GelMA 10% w/v, lithium phenyl-2,4,6-tri-methyl benzoyl phosphinate 1.5% w/v, bacterial nanocellulose 0.3 w/v Near-UV blue light crosslink (405nm)	Keratinocyte $5x10^5/cm^2$	Immortalized keratinocytes (HaCaT)	Cells were seeded on the basal layer Cultured in air-liquid interface after 4 days	DMEM, 10% FBS, 1% penicillin–streptomycin–amphotericin B	The disc-like basal layers with dimensions of 10 mm (diameter) × 0.5 mm (height) were printed on the dermal networks with 10Gel/1.5BNC at a pressure of 1.3 bar and a nozzle speed of 12 mm s^{-1}
	Dermis	GelMA 10% w/v, lithium phenyl-2,4,6-tri-methyl benzoyl phosphinate 0.3% w/v, bacterial nanocellulose 0.3 w/v Near-UV blue light crosslink (405nm)	Fibroblast $5x10^6$/mL	Dermal fibroblasts (Shanghai Bogoo Biotechnology Co., Ltd)	1 layer		The circular dermal networks (700 µm distance) with dimensions of 10 mm (diameter) × 1 mm (height) were printed with a pressure of 1 bar and a nozzle speed of 12 mm s^{-1} at 14 °C.
Liu et al. 2022, China	Epidermis	NR	NR	Immortalized keratinocytes (HaCaT)	Cells were seeded on the dermis layer Cultured in air-liquid interface	DMEM, 10% FBS, 1% penicillin–streptomycin	
	Dermis	Gelatin 5% w/v, Alginate 1%, 2mg/mL fibrinogen Crosslink: $CaCl_2$ 2% w/v Prepared with Laminin solution (50µg/mL) prior to epidermis print	Fibroblast $2x10^6$/mL	Isolation from foreskin	10 layers		The bio-ink was dispensed through a sterile metal needle (nozzle diameter 0.33mm), using pneumatic pressure of 1.8–2.5 Mpa and a deposition speed of 5 mm/s. The deposition speed and pressure were controlled by

							customized software developed by Regenhu.
Seol et al. 2018, USA and Korea	Epidermis	Hyaluronic acid (3mg/mL), glycerol 10% v/v, gelatin 30mg/mL, fibrinogen 20mg/mL Crosslink: Thrombin 20U/mL	Keratinocyte $1x10^7$/mL	Primary epidermal keratinocytes (Lonza)	1 layer	DMEM, 10% FBS, 1% penicillin–streptomycin	. The cell-laden hydrogels were printed on the face-shaped PU construct sequentially through a 300 um Teflon nozzle at 60 kPa of pneumatic pressure.
	Dermis	Hyaluronic acid (3mg/mL), glycerol 10% v/v, gelatin 30mg/mL, fibrinogen 20mg/mL Crosslink: Thrombin 20U/mL	Fibroblast $5x10^6$/mL	Primary dermal fibroblasts (Lonza)	1 layer		
	Base	Thermoplastic polyurethane (PU)	Acellular	-	1 layer		The PU material was heated to 160°C for printing and dispensed by 1500 kPa of pneumatic pressure
Somasekharan et al. 2021, India	Epidermis	Alginate 2% (w/v), gelatin 3.3% (w/v), Diethylaminoethyl cellulose (DCEL) 0.93% (w/v) Crosslink: $CaCl_2$ 0.1M	Keratinocyte $7x10^6$/mL	Primary epidermal keratinocytes (NR)	2 layers Cultured in air-liquid interface after 7 days for 3 weeks	Keratinocyte growth media, and fibroblasts growth media (3:1)	3 cc cartridge, fitted with a 410 µm diameter nozzle, at a pressure of about 120 KPa.

	Dermis	Alginate 2% (w/v), gelatin 3.3% (w/v), Diethylaminoethyl cellulose (DCEL) 0.93% (w/v) Crosslink: $CaCl_2$ 0.1M	Fibroblast $5x10^6$/mL	Primary fibroblasts (NR)	6 layers		
Zhang et al. 2023, China	Epidermis	GelMA 7.5% w/w, hyaluronic acid 1% w/w, 10% v/v LAP Crosslink: blue light (405nm)	Keratinocyte $1x10^6$/mL	Immortalized keratinocytes (HaCaT)	1 layer	DMEM high glucose and Endothelial cells media (3:1)	Extruding bioinks with three thermocontrolled chambers (bioinks A and B, 19−21 °C; bioink C, 20 °C) and a 23G nozzle with an inner diameter of 300 μm. The printing speed was 3.6−5.2 mm/s, and the pressure was 0.8−1.4 bar.
	Dermis	Microfragmented adipose extracellular matrix 20% v/v, GelMA 7.5% w/w, hyaluronic acid 1% w/w, 10% v/v LAP Crosslink: blue light (405nm)	Fibroblast $1x10^7$/mL Endothelial $1x10^7$/mL	Primary skin fibroblasts (OtwoBiotech) Primary umbilical vein endothelial cells (FuHeng Biology)	2 layers 1 layer		

Abbreviations: DMEM – Dulbecco's Modified Eagle's Medium; EGF – epidermal growth factor; FBS – Fetal Bovine Serum; FCS – Fetal Calf Serum; FGF – fibroblast growth factor; GelMA – gelatin methacryloyl; hFGFb – human fibroblast growth factor-basic; IMDM – Iscove's Modified Dulbecco's Medium; KGF – keratinocyte growth factor; LAP - Phenyl2,4,6-trimethylbenzoyl phosphinate; NR – Not Reported; R3-IGF – R3 insulin-like growth factor; RPMI – Roswell Park Memorial Institute; VEGF – vascularendothelial growth factor; w/v – weight/volume; w/w – weight/weight. * AvantBio - Model HKSdaFREE + HKGE - Animal Origin-Free Cell Culture Supplement System for Reconstructed Human Epidermis.

Table 3. Biomaterials properties and applications.

Biomaterials	Properties	Applications	Studies
Ab serum	Construct maturation, stable engraftment, mechanical stability	Vascularized skin construct	Baltazar et al. 2023
Alginate	Printability, biocompatibility, biodegradability, viscoelasticity, rapid crosslinking in the presence of calcium ions into a hydrogel, low cost, no bioactivity	Skin equivalent, wound healing, second degree thermal burns	Cavallo et al. 2023, Desanlis et al. 2020, Hafezi et al. 2020, Huyan et al. 2020, Jiao et al. 2022, Liu et al. 2022, Somasekharan et al. 2021
Bacterial nanocellulose (BNC)	High water-holding capacity, favorable rheological properties, good toughness and elasticity, printability, structural stability, and a similar morphology to collagen. Can be used as a mechanical enhancer to improve the mechanical strength of bioinks.	Full-thickness heterogeneous tissue-engineered skin, wound healing	Li et al. 2023
Biodegradable polyurethane (PU)	Printability, high resolution, biocompatibility, tunable degradation rate, elasticity, and proper shear thinning properties, tissue migration	Extensive full-thickness wounds	Dai et al. 2022
$CaCl_2$	Chemical crosslink to jellify the bioink after printing	Used with alginate and gelatin-based bioinks	Cavallo et al. 2023, Dai et al. 2022, Liu et al. 2022, Somasekharan et al. 2021
Chitosan	Biocompatible, biodegradable, bioactivity, mechanical strength (crosslinked), printability, pharmacological action action on various wound healing phases including haemostasias and antibacterial activity	*In vitro* skin regeneration and wound healing applications	Hafezi et al. 2020,
Collagen Type I	Construct maturation, stable engraftment, mechanical stability, cell attachment ligands	Full-thickness wounds, burns, ulcers, vascularized skin construct	Albanna et al. 2019, Baltazar et al. 2023, Jiao et al. 2022, Lee et al. 2021

Diethylaminoethyl cellulose (DCEL)	Non-cytotoxic nature, stable, elastic and porous hydrogel, improve cell adhesiveness, stability, porosity and homogenous distribution of cells across the printed construct	Skin tissue equivalents with biomimetic epidermal and dermal tissue histology	Somasekharan et al. 2021
Fibrinogen	Biocompatibility, biodegradability, physicochemical features, cell adhesion, rapid gelation to maintain the 3D shape	Full-thickness wounds, burns, ulcers, skin equivalent, wound healing, second degree thermal burns	Albanna et al. 2019, Cavallo et al. 2023, Desanlis et al. 2020, Jorgensen et al. 2020, Jorgensen et al. 2023, Liu et al. 2022, Seol et al. 2018
Fibronectin	Construct maturation, stable engraftment, mechanical stability, excellent bioactivity and degradation process	Vascularized skin construct	Baltazar et al. 2023
Gelatin	Biocompatibility, temperature-sensitive, few attachments ligands and lower bioactivity, good cell viability, cost-effectiveness, enhance cell binding, suitable for fibroblast growth, proliferation and extracellular matrix remodeling	*In vivo* and *In vitro* tissue-engineered skin graft with vascular endothelial cells, fresh wound model	Desanlis et al. 2020, Huyan et al. 2020, Jorgensen et al. 2020, Jorgensen et al. 2023, Liu et al. 2022, Seol et al. 2018, Somasekharan et al. 2021
GelMA composites	Biocompatibility, enzymatic cleavage, cell adhesion, and tailorable mechanical properties	Modification of gelatin hydrogels to improve printability, reproducibility and controllability for tissue engineering, drug delivery systems and 3D printing	Choi et al. 2023, Jin et al. 2021, Li et al. 2023, Zhang et al. 2023
Genipin	Bioactivity, mechanical strength, printability, anti-inflammatory, antibacterial, biocompatibility	In vitro skin regeneration and wound healing applications	Hafezi et al. 2020
Glycerol	Increase robustness to the gels and to prevent nozzle clogging	Multicelular bioprinting and wound healing applications	Jorgensen et al. 2020, Jorgensen et al. 2023, Seol et al. 2018
Hyaluronic acid	Enhance the dispensing uniformity	Full-thickness skin wounds	Jorgensen et al. 2020, Jorgensen et al. 2023, Seol et al. 2018, Zhang et al. 2023

Lithium phenyl-2,4,6 trimethyl benzoyl phosphonate (LAP)	Photo-initiator crosslink to initiate polymerization chain upon light exposure	Associated with GelMA	Choi et al. 2023, Li et al. 2023
Biodegradable polyglycolic acid (PGA)	Construct maturation, stable engraftment, mechanical stability	Vascularized skin construct	Baltazar et al. 2023
Silk Fibroin	Biocompatibility (high cell proliferation and adherence, low inflammation), biodegradability, non-cytotoxic and high tensile strength, stable porous 3D structures	*In vitro* full-thickness artificial skin model	Choi et al. 2023
Thermoplastic polyurethane (PU)	Wound dressing effect in skin regeneration. A supporting wound dressing material because it has high elastic property compared to other biocompatible thermoplastic polymers	Printed Biomask for patient-specific facial skin regeneration	Dai et al. 2022
Thrombin	Deposit thrombin on the fibrinogen matrix to produce fibrin	Full-thickness wounds, burns, ulcers	Albanna et al. 2019, Jorgensen et al. 2020, Jorgensen et al. 2023, Seol et al. 2018

APPENDICES

Appendix 1. Search strategies with appropriate key words and MeSH terms.

Database	Search strategy (Search date: January 12, 2024)	References
PubMed	("Three-Dimensional Printings" OR "3-Dimensional Printing" OR "3 Dimensional Printing" OR "3-Dimensional Printings" OR "3-D Printing" OR "3 D Printing" OR "3-D Printings" OR "Three-Dimensional Printing" OR "Three Dimensional Printing" OR "3D Printing" OR "3D Printings" OR "Organ-on-a-Chip Devices" OR "Organ on a Chip Devices" OR "Organ-on-a-Chip Device" OR "Organotypic Cell Cultures" OR "Organotypic Cell Culture" OR "Organ Chips" OR "Organ Chip" OR "Organ-on-a-Chip" OR "Organ on a Chip" OR "Organ-on-a-Chips" OR "Organotypic Cultures" OR "Organotypic Culture" OR "Organotypic Models" OR "Organotypic Model" OR "Organoids-on-a-Chip" OR "Organoids on a Chip" OR "Organoids-on-a-Chips" OR "Culture Technique, Organ" OR "Culture Techniques, Organ" OR "Organ Culture Technique" OR "Organ Culture" OR "Organ Cultures" OR "3D Cell Culture" OR "3D Cell Cultures" OR "3-Dimensional Cell Culture" OR "3 Dimensional Cell Culture" OR "3-Dimensional Cell Cultures" OR "Three-Dimensional Cell Culture" OR "Three Dimensional Cell Culture" OR "Three-Dimensional Cell Cultures" OR "3-D Cell Culture" OR "3 D Cell Culture" OR "3-D Cell Cultures" OR "Scaffold Cell Culture Techniques" OR "Scaffold 3D Cell Culture" OR "Scaffold Cell Culture" OR "Scaffold Cell Cultures" OR "tissue scaffolds"[MeSH Terms] OR tissue scaffold[Text Word] OR "Tissue Scaffolds" OR "Tissue Scaffold" OR "Tissue Scaffolding" OR "Tissue Scaffoldings" OR "bioprinting" OR "bioprinter" OR "bioprinters" OR "bioprintings" OR "3D bioprinting" OR "3D bioprinter" OR "3D bioprinters" OR "Three dimensional bioprinter" OR "Three dimensional bioprinting" OR "bioprinted" OR "Bilayer membrane scaffold" OR "multilayer membrane scaffold") AND ("skin"[MeSH Terms] OR skin[Text Word] OR "mouth mucosa"[MeSH Terms] OR oral mucosa[Text Word] OR "periodontium" OR "periodontal ligament" OR "PDL" OR "Periodontiums" OR "Oral Mucosa" OR "Buccal Mucosa" OR "gingiva" OR "gingival" OR "skin, artificial"[MeSH Terms] OR skin substitute[Text Word] OR "Artificial Skin" OR "Artificial Skins" OR "Skin Substitutes" OR "Skin Substitute" OR "Full-thickness skin") AND ("in vitro techniques"[MeSH Terms] OR in vitro[Text Word] OR "In Vitro Technique" OR "In Vitro" OR "In Vitro Testing" OR "In Vitro Testings" OR "In Vitro Tests" OR "In Vitro Test") AND ("wound healing"[MeSH Terms] OR wound healing[Text Word] OR "Wound Healings" OR "guided tissue regeneration"[MeSH Terms] OR guided tissue regeneration[Text Word])	907
Embase	('three-dimensional printings' OR '3-dimensional printing'/exp OR '3-dimensional printing' OR '3 dimensional printing'/exp OR '3 dimensional printing' OR '3-dimensional printings' OR '3-d printing'/exp OR '3-d printing' OR '3 d printing'/exp OR '3 d printing' OR '3-d printings' OR 'three-dimensional printing'/exp OR 'three-dimensional printing' OR 'three dimensional printing'/exp OR 'three dimensional printing' OR '3d printing'/exp OR '3d printing' OR '3d printings' OR 'organ-on-a-chip devices' OR 'organ on a chip devices' OR 'organ-on-a-chip device' OR 'organotypic cell cultures' OR 'organotypic cell culture'/exp OR 'organotypic cell culture' OR 'organ chips' OR 'organ chip'/exp OR 'organ chip' OR 'organ-on-a-chip'/exp OR 'organ-on-a-chip' OR 'organ on a chip'/exp OR 'organ on a chip' OR 'organ-on-a-chips' OR 'organotypic cultures' OR 'organotypic culture'/exp OR 'organotypic culture' OR 'organotypic models' OR 'organotypic model'/exp OR 'organotypic model' OR 'organoids-on-a-chip'/exp OR 'organoids-on-a-chip' OR 'organoids on a chip'/exp OR 'organoids on a chip' OR 'organoids-on-a-chips' OR 'culture technique, organ' OR 'culture techniques, organ' OR 'organ culture technique'/exp OR	1501

	'organ culture technique' OR 'organ culture'/exp OR 'organ culture' OR 'organ cultures' OR '3d cell culture'/exp OR '3d cell culture' OR '3d cell cultures' OR '3-dimensional cell culture'/exp OR '3-dimensional cell culture' OR '3 dimensional cell culture'/exp OR '3 dimensional cell culture' OR '3-dimensional cell cultures' OR 'three-dimensional cell culture'/exp OR 'three-dimensional cell culture' OR 'three dimensional cell culture'/exp OR 'three dimensional cell culture' OR 'three-dimensional cell cultures' OR '3-d cell culture'/exp OR '3-d cell culture' OR '3 d cell culture'/exp OR '3 d cell culture' OR '3-d cell cultures' OR 'scaffold cell culture techniques' OR 'scaffold 3d cell culture' OR 'scaffold cell culture' OR 'scaffold cell cultures' OR 'tissue scaffolds'/exp OR 'tissue scaffolds' OR 'tissue scaffold'/exp OR 'tissue scaffold' OR 'tissue scaffolding'/exp OR 'tissue scaffolding' OR 'tissue scaffoldings' OR 'bioprinting'/exp OR 'bioprinting' OR 'bioprinter'/exp OR 'bioprinter' OR 'bioprinters' OR 'bioprintings' OR '3d bioprinting'/exp OR '3d bioprinting' OR '3d bioprinter' OR '3d bioprinters' OR 'three dimensional bioprinter'/exp OR 'three dimensional bioprinter' OR 'three dimensional bioprinting' OR 'bioprinted' OR 'bilayer membrane scaffold' OR 'multilayer membrane scaffold') AND ('skin'/exp OR 'skin' OR 'mouth mucosa'/exp OR 'mouth mucosa' OR 'periodontium'/exp OR 'periodontium' OR 'periodontal ligament'/exp OR 'periodontal ligament' OR 'pdl' OR 'periodontiums' OR 'oral mucosa'/exp OR 'oral mucosa' OR 'buccal mucosa'/exp OR 'buccal mucosa' OR 'gingiva'/exp OR 'gingiva' OR 'gingival' OR 'skin artificial'/exp OR 'skin artificial' OR 'skin substitute'/exp OR 'skin substitute' OR 'artificial skin'/exp OR 'artificial skin' OR 'artificial skins' OR 'skin substitutes' OR 'full-thickness skin') AND ('wound healing'/exp OR 'wound healing' OR 'wound healings' OR 'guided tissue regeneration'/exp OR 'guided tissue regeneration') AND ('in vitro techniques'/exp OR 'in vitro techniques' OR 'in vitro technique'/exp OR 'in vitro technique' OR 'in vitro'/exp OR 'in vitro' OR 'in vitro testing' OR 'in vitro testings' OR 'in vitro tests' OR 'in vitro test')	
Web of Science	TS=("Three-Dimensional Printings" OR "3-Dimensional Printing" OR "3 Dimensional Printing" OR "3-Dimensional Printings" OR "3-D Printing" OR "3 D Printing" OR "3-D Printings" OR "Three-Dimensional Printing" OR "Three Dimensional Printing" OR "3D Printing" OR "3D Printings" OR "Organ-on-a-Chip Devices" OR "Organ on a Chip Devices" OR "Organ-on-a-Chip Device" OR "Organotypic Cell Cultures" OR "Organotypic Cell Culture" OR "Organ Chips" OR "Organ Chip" OR "Organ-on-a-Chip" OR "Organ on a Chip" OR "Organ-on-a-Chips" OR "Organotypic Cultures" OR "Organotypic Culture" OR "Organotypic Models" OR "Organotypic Model" OR "Organoids-on-a-Chip" OR "Organoids on a Chip" OR "Organoids-on-a-Chips" OR "Culture Technique, Organ" OR "Culture Techniques, Organ" OR "Organ Culture Technique" OR "Organ Culture" OR "Organ Cultures" OR "3D Cell Culture" OR "3D Cell Cultures" OR "3-Dimensional Cell Culture" OR "3 Dimensional Cell Culture" OR "3-Dimensional Cell Cultures" OR "Three-Dimensional Cell Culture" OR "Three Dimensional Cell Culture" OR "Three-Dimensional Cell Cultures" OR "3-D Cell Culture" OR "3 D Cell Culture" OR "3-D Cell Cultures" OR "Scaffold Cell Culture Techniques" OR "Scaffold 3D Cell Culture" OR "Scaffold Cell Culture" OR "Scaffold Cell Cultures" OR "Tissue Scaffolds" OR "Tissue Scaffold" OR "Tissue Scaffolding" OR "Tissue Scaffoldings" OR "bioprinting" OR "bioprinter" OR "bioprinters" OR "bioprintings" OR "3D bioprinting" OR "3D bioprinter" OR "3D bioprinters" OR "Three dimensional bioprinter" OR "Three dimensional bioprinting" OR "bioprinted" OR "Bilayer membrane scaffold" OR "multilayer membrane scaffold") AND TS=("skin" OR "mouth mucosa" OR "periodontium" OR "periodontal ligament" OR "pdl" OR "periodontiums" OR "oral mucosa" OR "buccal mucosa" OR "gingiva" OR "gingival" OR "skin artificial" OR "skin substitute" OR "artificial skin" OR "artificial skins" OR "skin substitutes" OR "full-thickness skin") AND TS=("in vitro techniques" OR "in vitro technique" OR "in vitro" OR "in vitro testing" OR "in vitro testings" OR "in vitro tests" OR "in vitro test") AND TS=("wound healing" OR "wound healings" OR "guided tissue regeneration")	218
Google scholar	("3D model") AND ("skin" OR "oral mucosa") AND ("in vitro")	70

Appendix 2. Excluded articles and reasons for exclusion (n=184).

Author/Year	Reasons for exclusion*
1) Aden et al. 2010	2
2) Akturk et al. 20216	2
3) Alamo et al. 2022	2
4) Alexaki et al. 2012	2
5) Artist et al. 2020	1
6) Artuc et al. 2002	2
7) Attiogbe et al. 2022	1
8) Augustine et al. 2019	2
9) Azari et al. 202	2
10) Bachmann et al. 2023	1
11) Barker et al. 2020	2
12) Basso et al. 2017	2
13) Basso et al. 2018	2
14) Bastidas et al. 2023	2
15) Benny et al. 2016	2
16) Bhowmick et al. 2016	2
17) Bilbo et al. 1993	2
18) Blanquer et al. 2024	2
19) Bolla et al. 2016	1
20) Bonvallet et al. 2014	2
21) Breetveld et al. 2006	2
22) Bucchieri et al. 2012	2

23) Buskermolen et al. 2016	2
24) Capon et al. 1998	2
25) Cavallo et al. 2022	1
26) Chandika et al. 2021	2
27) Chau et al. 2013	2
28) Chen et al. 2023	4
29) Chermnykh et al. 2018	2
30) Chhabra et al. 2020	2
31) Dai et al. 2018	2
32) Dearman et al. 2022	2
33) Dongari-Bagtzoglou et al. 2006	2
34) Ejiugwo et al. 2021	1
35) Farias dos Santos et al. 2023	2
36) Fedeli et al. 2022	2
37) Fernández-Martos et al. 2021	2
38) Ferreira et al. 2019	2
39) Gibot et al. 2012	2
40) Golinski et al. 2015	2
41) Gonzalez et al. 2021	2
42) Grøn et al. 1999	2
43) Grøn et al. 2002	2
44) Gu et al. 2010	2
45) Hada et al. 2005	2
46) Han et al. 2001	2
47) Hao et al. 2023	4
48) Hautier et al. 2007	2
49) Hensler et al. 2019	2

50) Hewitt et al. 2019	2
51) Hodge et al. 2023	2
52) Hodgkinson et al. 2013	1
53) Hodgkinson et al. 2016	2
54) Hollander et al. 2004	2
55) Holm et al. 2022	2
56) Hosseinzadeh et al. 2017	2
57) Huang et al. 2003	6
58) Huang et al. 2023	2
59) Hummelink et al. 2021	5
60) Hunt et al. 2009	2
61) Huth et al. 2018	2
62) Idrees et al. 2021	1
63) Intini et al. 2018	3
64) Johnson et al. 2023	1
65) Jumper et al. 2014	1
66) Kang et al. 2022	4
67) Kerzel et al. 2011	1
68) Killat et al. 2013	2
69) Kim et al. 2017	2
70) Kleszczynski et al. 2012	2
71) Kljenak et al. 2016	2
72) Konstantinova et al. 2017	2
73) Kristiansen et al. 2008	2
74) Lee et a. 2003	2
75) Lee et al. 2014	2
76) Lee et al. 2017	2

77) Limat et al. 1994	2
78) Lin et al. 2014	2
79) Liu et al. 2023	2
80) Llames et al. 2014	2
81) Lönnqvist et al. 2015	2
82) Lou et al. 2014	6
83) Luo et al. 2011	2
84) Maas-Szabowski et al. 2000	2
85) Madhyastha et al. 2012	2
86) Malheiro et al. 2022	2
87) Marjanovic et al. 2021	1
88) Marquardt et al. 2015	2
89) Mathes et al. 2010	2
90) Mauroux et al. 2023	6
91) McGuire et al. 2008	2
92) Meek et al. 2013	1
93) Mendoza et al. 2013 94) (2 abstracts)	1
95) Mendoza Garcia et al. 2015	2
96) Michael et al. 2013	4
97) Michael et al. 2013	1
98) Miranda-Alarcón et al. 2016	2
99) Miyazaki et al. 2019	2
100) Moharamzadeh et al. 2008	2
101) Moharamzadeh et al. 2015	2
102) Morelli et al. 2019	2
103) Morgun et al. 2023	2

104)	Nasir et al. 2018	2
105)	Nevins, 2010	6
106)	O'Leary et al. 1997	2
107)	O'Leary et al. 2002	2
108)	Okazaki et al. 2003	2
109)	Okazaki et al. 2019	1
110)	Ozdogan et al. 2020	2
111)	Pabst et al. 2014	2
112)	Paganelli et al. 2022	2
113)	Pal et al. 2016	2
114)	Pankow et al. 2006	2
115)	Paquet-Fifield et al. 2009	2
116)	Parenteau et al. 1996	2
117)	Patel et al. 2021	4
118)	Pedro et al. 2021	2
119)	Pelipenko et al. 2014	2
120)	Pellicena et al. 2016	1
121)	Peramo et al. 2008	2
122)	Peramo et al. 2011	2
123)	Pillet et al. 2017	2
124)	Pollok et al. 2011	2
125)	Post et al. 2021	2
126)	Protschka et al. 2019	1
127)	Purpura et al. 2016	2
128)	Ramphul et al. 2020	2
129)	Rasmussen et al. 2010	2
130)	Rayner et al. 2000	2

131)	Reijnders et al. 2015	2
132)	Riccio et al. 2023	2
133)	Roffel et al. 2019	2
134)	Roy et al. 2013	1
135)	Sahle et al. 2023	2
136)	Sarkar et al. 2013	2
137)	Scheyer et al. 2014	2
138)	Schmitt et al. 2017	2
139)	Schmitt et al. 2018	2
140)	Scuderi et al. 2009	2
141)	Sebastian et al. 2012	6
142)	Shamasha et al. 2023	1
143)	Shannon et al. 2022	2
144)	Shen et al. 2021	2
145)	Shevchenko et al. 2014	2
146)	Smith et al. 2022	2
147)	Sohutskay et al. 2021	2
148)	Solovieva et al. 2020	2
149)	Song et al. 2023	2
150)	Steiglitz et al. 2023	2
151)	Stoll et al. 1997	2
152)	Straseski et al. 2009	2
153)	Strid Orrhult et al. 2017	1
154)	Sutterby et al. 2022	1
155)	Swindell et al. 2020	2
156)	Szymanski et al. 2020	2
157)	Thelu et al. 2020	2

158)	Tomakidi et al. 1999	2
159)	Tutuianu et al. 2021	2
160)	Uberti et al. 2010	2
161)	Valente et al. 2018	2
162)	Varkey et al. 2014	2
163)	Wang et al. 2013	2
164)	Wang et al. 2016	2
165)	Wei et al. 2019	2
166)	Wills et al. 2015	2
167)	Willson et al. 2023	1
168)	Wright et al. 2009	2
169)	Wright et al. 2014	1
170)	Xiao et al. 2020	2
171)	Xu et al. 2011	1
172)	Xu et al. 2012	2
173)	Yanez et al. 2015	2
174)	Yang et al. 2023	2
175)	Yoon et al. 2018	2
176)	Zhang and Wen et al. 2020	2
177)	Zhang and Zhang et al. 2023	4
178)	Zhang et al. 2008	6
179)	Zhang et al. 2021	4
180)	Zhao et al. 2022	4
181)	Zhuang et al. 2018	2
182)	Zoller et al. 2014	2
183)	Zonari et al. 2014	2
184)	Zwicker et al. 2023	2

***Reason for exclusion:**

1. Reviews, conference abstracts, and book chapter (n=25).
2. Protocols not involving printing with a bioprinter (n=139).
3. Dressing-scaffolds testing not involving tissue substitute (n=1).
4. Wrong cells or acellular-scaffold component (n=8).
5. Wrong outcome (n=5).
6. Not available (n=6).

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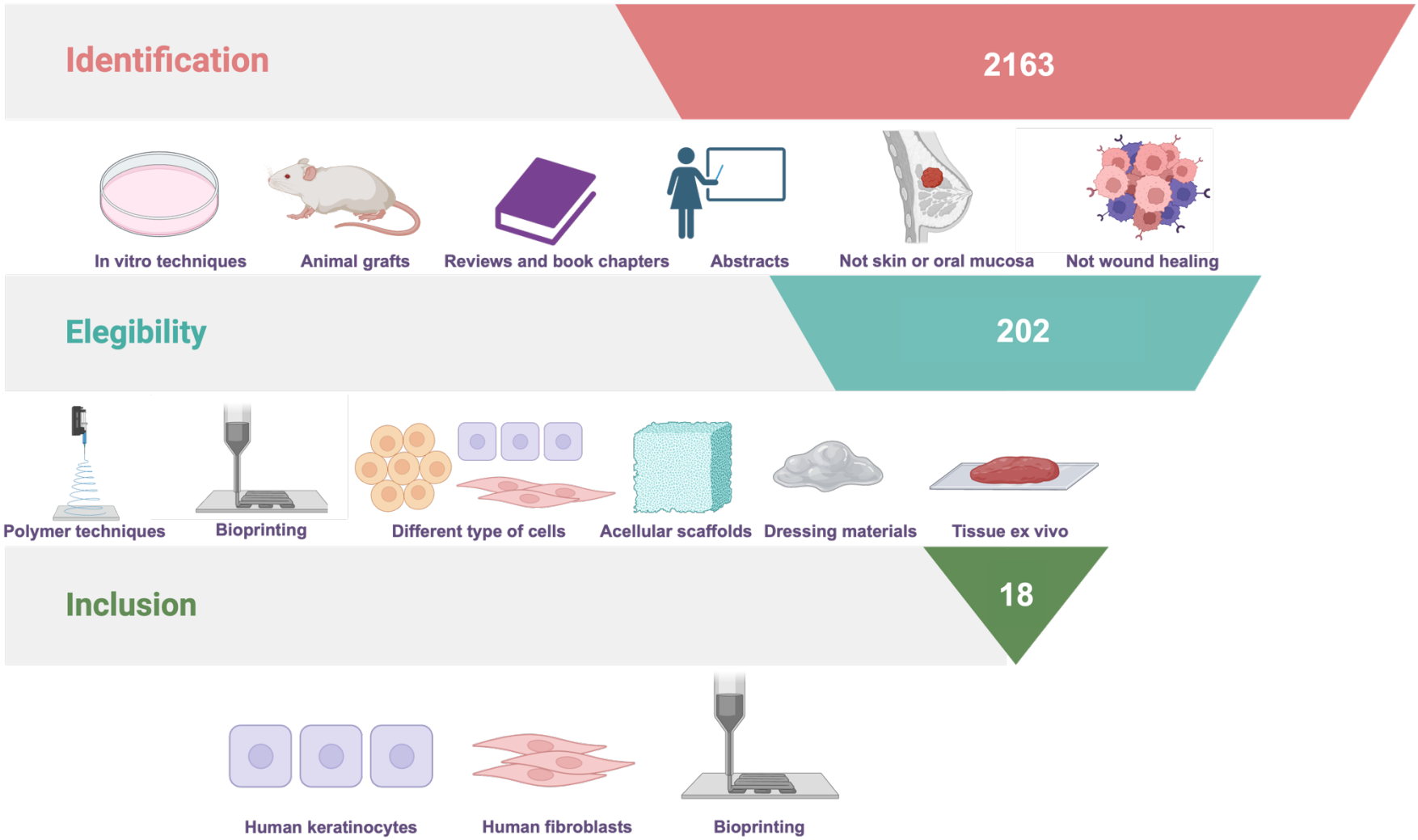
Appendix 3. Illustrative representation of the selection process funnel leading to final inclusion in the review.



Appendix 4. General data extraction table.

Author, Year and country	Journal	Journals impact factor	Keywords	Title	Aim	Type of tissue and focus	Bioprinter technique	Matrix components	Applied assays and analysis
Albanna et al. 2019, United States	Scientific Reports	3.8	NR	In situ bioprinting of autologous skin cells accelerates wound healing of extensive excisional full-thickness wounds	Describe the design and a proof-of-concept validation of a novel mobile skin bioprinting system	Skin Thickness wound	Inkjet	Fibrinogen and Collagen type I	Murine wound closure and porcine wound closure H&E, IHC
Baltazar et al. 2023, United States	Bioengineering & Translational Medicine	6.1	3D bioprinted, skin, vascularized, xeno-free	3D bioprinting of an implantable xeno-free vascularized human skin graft	Demonstrate the fabrication of a bioprinted vascularized skin graft generated under strict xenogeneic-free	Skin Thickness wound	Extrusion BioX™, Cellink	Human male AB serum, human plasma fibronectin, human Collagen type I, and VitroGel Hydrogel Matrix	Perfused vessels assessment in mice Skin equivalent: morphological characterization
Cavallo et el. 2023, Italy	Journal of functional biomaterials	5.0	Fibrinogen, alginate, bioink, 3D bioprinting, skin equivalent	Fibrinogen-based bioink for application in skin equivalent 3D bioprinting	Propose a bioink based on a fibrinogen and alginate blend to form a hydrogel by the enzymatic polymerization of fibrinogen with thrombin and by the ionic crosslinking of alginate with divalent calcium ions	Skin Thickness wound	Extrusion BioX™, Cellink	Fibrinogen and alginate	Bioink: Rheological properties, printability, mechanical properties, degradation, swelling, water uptake, morphology, biocompatibility Skin equivalent: cell viability, H&E
Choi et al. 2023, Korea	Acta Biomaterialia	9.4	3D printing, silk, fibroins hydrogels, skin	A digital light processing 3D-printed artificial skin model and full-thickness	Print a 3D artificial skin model using silk and fibroin to mimic the	Skin Thickness wound	Digital Light Processing 3D printer (NBR Tech. Ltd)	Gel-GMA, Silk-GMA dissolved in lithium phenyl-2,4,6 trimethyl	Bioink: Printability and printing resolution, microstructure,

				wound models using silk fibroin bioink	structural and cellular compositions of the human skin			benzoyl phosphonate	mechanical, and degradation analyzes, cell proliferation and viability Skin equivalent: H&E, IF, RT-PCR
Dai et al. 2022 Taiwan	Bioprinting	6.8	3D bioprinting, skin bioprinting, wound healing, polyurethane, tissue engineering	A bioprinted vascularized skin substitute with fibroblasts, keratinocytes, and endothelial progenitor cells for skin wound healing	3D bioprint a bilayer dermo-epidermal skin substitute using polyurethane gelatin multimaterial bioink with proper mechanical stability	Skin Thickness wound	Extrusion, Regenovo Bio-Printer-WS	Polyurethane gelatin, poly (ε-caprolactone) diol (PCL), poly (D, L-lactide) diol (PDLLA)	Bioink: Rheological properties Skin equivalent: Cell viability, proliferation, mice wound closure, H&E, IF
Desanlis et al. 2020, France	Tissue Engineering and Regenerative Medicine	4.4	3D bioprinting, non-cultured skin cells, on-site therapy, skin cells therapy, tissue engineering, wound healing	Validation of an implantable bioink using mechanical extraction of human skin cells: First steps to a 3D bioprinting treatment of deep second degree burn	Describe different steps and landmarks on experimental data obtained from mechanically extracted human skin cells and clinical grade implantable formulated bioink	Skin Second degree burn	Extrusion, LabSkin Creations/ TOBECA	Gelatin, very low viscosity alginate, and fibrinogen	Skin equivalent: Cell proliferation, H&E, IHC, WB, Mice: bioink toxicity, wound closure, perfusion, H&E
Hafezi et al. 2020, United Kingdom	Pharmaceutics	4.9	3D bioprinting, bioink, chitosan, genipin, human dermal fibroblasts, primary epidermal keratinocytes, skin regeneration	Bioprinting and preliminary testing of highly reproducible novel bioink for potential skin regeneration	Print keratinocytes and human dermal fibroblasts cells embedded in chitosan-Genipin-based inks by applying an extrusion bioprinting technique without	Skin Thickness wound	Extrusion BioX™, Cellink	Sodium alginate and chitosan–polyethylene glycol	Bioink: Rheological properties, and cross-link characterization Skin equivalent: Cell viability, morphology, and cell distribution

					damaging the cells				
Huyan et al. 2020, China	International Journal of Bioprinting	6.8	Three-dimensional printing, bilayer skin graft, gelatin-alginate complex hydrogel, vascularization	Pilot study of the biological properties and vascularization of 3D printed bilayer skin grafts	Fabricate a 3D-printed artificial skin including vascular features and hydrogel hybrid materials printed by an extrusion printing process	Skin Thickness wound	Extrusion, Custom-built	Sodium alginate and gelatin	Bioink: Cell viability Skin equivalent: Cell viability (fibroblasts), and cell tracking, mice wound contraction, H&E, and IHC
Jiao et al. 2022, China and Portugal	Journal of Bionic Engineering	4.9	Bioink, bioprinting, collagen/sodium alginate hydrogel, skin, wound healing, bionic	Properties of collagen/sodium alginate hydrogels for bioprinting of skin models	Propose blending collagen and sodium alginate to obtain hydrogels with excellent mechanical and biological properties for a bilayer skin construct	Skin Thickness wound	Extrusion, NR	Collagen type 1 and sodium alginate	Bioink: Rheological analysis, swelling, degradation, compression and tensile, morphology, number, proliferation, and distribution of cells Skin equivalent: Morphology and cell distribution
Jin et al. 2021, China	Acta Biomaterialia	9.7	3D bioprinting, wound healing, acellular dermal matrix, gelatin methacrylamide, full-thickness functional skin model	Three-dimensional bioprinting of a full-thickness functional skin model using a cellular dermal matrix and gelatin methacryla-mide bioink	Investigate a combination of acellular dermal matrix and GelMA for 3D bioprinting of skin, in which GelMA is used as a structural bioink, and acellular dermal matrix is printed as the matrix bioink, to satisfy the biological requirements of the cells, as well as the structural	Skin Thickness wound	Extrusion, Regenovo, Bio-Architect PRO	Porcine acellular dermal matrix and GelMA	Bioink: Rheological analysis, dsDNA, collagen, and glycosaminoglycan assessment, scanning electron microscopy observation, swelling ration, degradation, water retention capacity, permeability, single molecule force spectroscopy, cell viability, cell

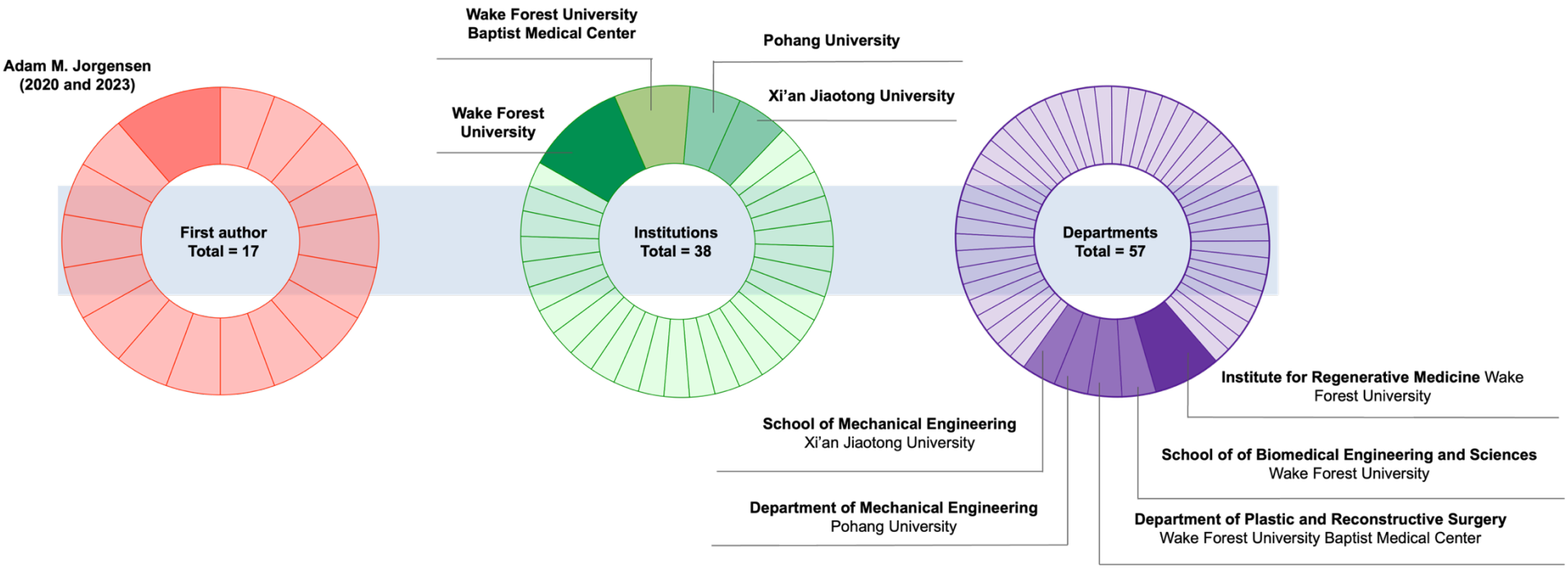
					requirements of the skin tissues				proliferation, cell adhesion Skin equivalent: Cell tracking, mice wound closure, H&E, WB
Jorgensen et al. 2020, Russia and United States	Tissue engineering Part: A	3.5	Skin, bioprinting, wound healing, animal models, extracellular matrix, tissue engineering	Bioprinted skin recapitulates normal collagen remodeling in full-thickness wounds	Test the ability of bioprinted human skin to integrate, form an epidermal barrier, and recapitulate normal collagen re-modeling in full-thickness wounds in mice	Skin Thickness wound	Extrusion, ITOP system (Kang et al. 2016)	Fibrin, gelatin, glycerol, and hyaluronic acid	Mice wound closure, epithelialization, H&E, collagen fiber quantification, IHC, structure and morphology
Jorgensen et al. 2023, United States	Science Translational Medicine	15.8	NR	Multicellular bioprinted skin facilitates human-like skin architecture in vivo	Fabricate a complex multilayered skin construct consisting of six primary human cell types to promote epidermal and dermal formation pigmentation, hair follicle formation, and neovascularization	Skin Thickness wound	Extrusion, ITOP system (Kang et al. 2016)	Fibrin, gelatin, glycerol, and hyaluronic acid	Cell viability, cell tracking, mice and porcine wound closure, epithelialization, contraction, H&E, collagen fiber quantification, IHC, RT-PCR
Lee et al. 2021, Korea	Bioprinting	6.8	Skin equivalent, microextrusion, inkjet printing	3D microextrusion-inkjet hybrid printing of structured human skin equivalents	Introduce piezoelectric inkjet printing to generate an epidermal layer on top of a dermal layer formed by pneumatic microextrusion printing, and subsequently	Skin Thickness wound	Inkjet and pneumatic microextrusion	Type 1 porcine collagen	Bioink: Jetting properties, viability, comparison between inkjet deposition and manual pipetting Skin substitute: H&E and IHC

					employe drop-on-demand piezoelectric type inkjet printing to distribute keratinocytes on the dermal mass in order to establish a simple and reproducible method for multilayer-structured skin model				
Li et al. 2023, China	Biomaterials Science	5.8	3D bioprinting, gelatin methacrylate, bacterial cellulose, tissue-engineered skin, wound healing	3D bioprinting of heterogeneous tissue-engineered skin containing human dermal fibroblasts and keratinocytes	Employ a GelMA/BNC composite bioinks to print full-thickness heterogeneous tissue-engineered skin including layers of human dermal fibroblast networks with larger pores, basal layers with smaller pores, and multilayered human keratinocytes to provide a valid skin substitute for future clinical applications	Skin Thickness wound	Extrusion 3D-Bioplotter (envision-TEC, Germany).	Gelatin methacrylate (GelMA), bacteriala nano-cellulose (BNC), m phenyl-2,4,6-trimethyl benzoyl phosphinate (LAP)	Bioink: Swelling test, rheological and mechanical properties, morphology characterization, printability, structural stability and fidelity, cell viability, proliferation, morphology and adhesion and mechanical strength Skin equivalent: trans epidermal electrical resistance, IF, mice wound closure and H&E
Liu et al. 2022, China	Bioengineered	4.2	Full-thickness skin, 3D bioprinting, bio-inks, stratum corneum formation	Simple and robust 3D bioprinting of full-thickness human skin tissue	Presented an approach that enables extrusion-based 3D bioprinting of a full-thickness	Skin Thickness wound	Extrusion 3D Discovery bioprinter (Regenhu, Switzerland).	Gelatin, alginate, and fibrinogen	Skin equivalent: Cell viability, H&E and IHC

					human skin model in a simple, economic and robust way				
Seol et al. 2018, USA and Korea	Bioprinting	6.8	Bioprinting, CAD/CAM, burn, skin regeneration, skin wound, tissue engineering	3D Bioprinted BioMask for Facial Skin Reconstruction	Develop a novel strategy, called “BioMask”, which is a customized bioengineered skin substitute combined with a wound dressing layer that snugly fits onto the facial wounds	Skin Facial reconstructio n	Extrusion, ITOP system (Kang et al. 2016)	Hyaluronic acid, glycerol, gelatin, and fibrinogen	Skin substitute: cell viability, animal wound closure, wound contraction, H&E
Somasekhara n et al. 2021, India	International Journal of Biological Macromolecul es	7.7	Hydrogels, printability, cocultures, tissue equivalents, tissue engineering, wound healing	Biofabrication of skin tissue constructs using alginate, gelatin and diethylaminoeth yl cellulose bioink	Explore an alginate, geleatine, diethylaminoethy l cellulose hydrogel bioink as a potential to cell adhesiveness, elasticity, and stability over a selective period of time and thereby to bioprint and biofabricate skin tissue equivalents	Skin Thickness wound	Extrusion Inkredible^{TM} (Cellink, Sweden)	Alginate, gelatine, and diethylaminoeth yl cellulose	Bioink: Printability, morphological, physical, chemical properties, rheological properties, biodegradation, mechanical properties, biocompatibility, cell viability Skin equivalent**:** IHC, H&E
Zhang et al. 2023, China	ACS Applied Materials & Interfaces	8.3	Microfragment ed adipose extracellular matrix, 3D bioprinting, wound healing, full-thickness skin defects	3D-Bioprinted Biomimetic Multilayer Implants Comprising Microfragmente d Adipose Extracellular Matrix and Cells Improve	Explore the use of microfragmented adipose ECM as the main component of the bioink, and different types of bioinks corresponding to	Skin Thickness wound	Extrusion 3D printer (Envision TEC, Germany)	GelMA and hyaluronic acid methacryloyl	Bioink: Rheological properties, cell viability, DNA quantification Skin substitute: mice wound closure, H&E and IHC

		Wound Healing in a Murine Model of Full-Thickness Skin Defects	dermis, epidermis, and vascularization functions to complete the 3D bioprinting of a biomimetic multilayer implant to be used as a skin substitute to repair full-thickness skin	

Appendix 5. Leading first authors and institutions contributing to the bioprinting of 3D skin substitutes loaded with human keratinocytes and fibroblasts (N=18 studies).



Appendix 6. Reported affiliations and mean area.

Author, Year and country	Reported Affiliations	Principal Institutions	Mean Area	Institution type
Albanna et al. 2019, United States and China	Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC, 27157, USA.	Wake Forest University	Regenerative Medicine	University
	Department of Pathology, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC, 27157, USA.	Wake Forest University	Pathology and Physiology	University
	Jiangsu Key Laboratory of Neuroregeneration, Nantong University, Nantong, China.	Nantong University	Regenerative Medicine	University
	Department of Surgery, Wake Forest School of Medicine, Medical Center Boulevard, WinstonSalem, NC, 27157, USA.	Wake Forest University	Surgery	University
	Virginia Tech-Wake Forest School of Biomedical Engineering and Sciences, Wake Forest School of Medicine, Winston-Salem, NC, 27157, USA	Wake Forest University	Biomaterials, Engineering and Technology	University
Baltazar et al. 2023, United States	Department of Immunobiology, Yale School of Medicine, New Haven, Connecticut, USA	Yale University	Immunobiology	University
	Department of Surgery, Yale University School of Medicine, New Haven, Connecticut, USA	Yale University	Surgery	University
	Department of Vascular Surgery, The First Hospital of China Medical University, Shenyang, China	The First Hospital of China Medical	Surgery	University Hospital
	Department of Chronic Disease Epidemiology, Yale University School of Public Health, New Haven, Connecticut, USA	Yale University	Chronic diseases	University
	College of Medicine, SUNY Downstate Health Sciences University, Brooklyn, New York, USA	SUNY Downstate Health Sciences University	Health Sciences	University
	Department of Surgery, Columbia University Medical Center, New York, New York, USA	Columbia University Medical Center	Surgery	University Hospital
	Humabiologics Inc, Phoenix, Arizona, USA	Humabiologics Inc	Biomaterials, Engineering and Technology	Company

	Department of General Surgery, Atrium Health Wake Forest Baptist, Winston-Salem, North Carolina, USA	Wake Forest Baptist Medical Center	Surgery	University Hospital
	Department of Biomedical Engineering, Yale University, New Haven, Connecticut, USA	Yale University	Biomaterials, Engineering and Technology	University
Cavallo et el. 2023, Italy	Institute of Life Sciences, Scuola Superiore Sant'Anna, 56127 Pisa, Italy	Scuola Superiore Sant'Anna	Health Sciences	University
	Institute of Clinical Physiology, National Research Council, 54100 Massa, Italy	National Research Council	Pathology and Physiology	National Research Center/Institute
	Department of Pharmacy, University of Pisa, 56126 Pisa, Italy	University of Pisa	Pharmacy	University
Choi et al. 2023, Korea	Department of Otorhinolaryngology -Head and Neck Surgery, Hallym University Kangnam Sacred Heart Hospital, Seoul 07441, Republic of Korea	Hallym University Kangnam Sacred Heart Hospital	Surgery	Hospital University
	Nano-Bio Regenerative Medical Institute, College of Medicine, Hallym University, 1 Hallymdaehak-gil, Chuncheon, Gangwon-do 24252, Republic of Korea	Hallym University	Regenerative Medicine	University
	Department of Otorhinolaryngology-Head and Neck Surgery, Chuncheon Sacred Heart Hospital, School of Medicine, Hallym University, Chuncheon 24252, Republic of Korea	Hallym University Kangnam Sacred Heart Hospital	Surgery	Hospital University
Dai et al. 2022 Taiwan	Department of Orthopedics, Tri-Service General Hospital, National Defense Medical Center, Taipei, 11490, Taiwan	Tri-Service General Hospital, National Defense Medical Center	Orthopedics	Hospital
	Division of Plastic and Reconstructive Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan	Tri-Service General Hospital, National Defense Medical Center	Surgery	Hospital
	Institute of Polymer Science and Engineering, National Taiwan University, Taipei, Taiwan, ROC	National Taiwan University	Biomaterials, Engineering and Technology	University
Desanlis et al. 2020, France	Hospices Civils de Lyon, Banque de Tissus et Cellules, Groupement Hospitalier Edouard Herriot, Lyon, France	Edouard Herriot Hospital	Health Sciences	Hospital

	LabSkin Creations, Edouard Herriot Hospital, Lyon, France	Edouard Herriot Hospital	Biomaterials, Engineering and Technology	Hospital
	Laboratoire de Biologie Tissulaire et Ingénierie Thérapeutique, UMR 5305, CNRS - Université Lyon 1, Institut de Biologie et Chimie des Protéines, SFR BioSciences Gerland-Lyon Sud, Lyon, France	Université Lyon	Biomaterials, Engineering and Technology	University
	3d.FAB, Univ Lyon, Université Lyon 1, CNRS, INSA, CPE-Lyon, ICBMS, UMR 5246, Villeurbanne, France	Université Lyon	Biomaterials, Engineering and Technology	University
Hafezi et al. 2020, United Kingdom	School of Science, Faculty of Engineering and Science, University of Greenwich, Chatham Maritime, Kent ME4 4TB, UK	University of Greenwich	Biomaterials, Engineering and Technology	University
	Centre for Innovation and Process Engineering Research, University of Greenwich, Chatham Maritime, Kent ME4 4TB, UK	University of Greenwich	Biomaterials, Engineering and Technology	University
Huyan et al. 2020, China	State Key Laboratory for Manufacturing System Engineering, School of Mechanical Engineering, Xi'an Jiaotong University, Xi'an, China'	Xi'an Jiaotong University	Biomaterials, Engineering and Technology	University
Jiao et al. 2022, China and Portugal	State Key Laboratory for Manufacturing System Engineering, Xi'an Jiaotong University, Xi'an 710049, China	Xi'an Jiaotong University	Biomaterials, Engineering and Technology	University
	Shaanxi Ketao-AM Technology Co., Ltd., Xi'an 710100, China	Shaanxi Ketao-AM Technology Co	Biomaterials, Engineering and Technology	Company
	3B's Research Group, I3Bs—Research Institute on Biomaterials, Biodegradables and Biomimetics, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, University of Minho, Barco, 4805-017 Guimarães, Portugal	University of Minho	Biomaterials, Engineering and Technology	University
	ICVS/3B's–PT Government Associate Laboratory, Barco, 4805-017 Guimarães, Portugal	Government Associate Laboratory	Biomaterials, Engineering and Technology	National Research Center/Institute
Jin et al. 2021, China	Department of Burns & Wound Care Center, The Second Affiliated Hospital of Zhejiang University College of Medicine, Hangzhou 310009, China	The Second Affiliated Hospital of Zhejiang University	Burns	University Hospital

	MOE Key Laboratory of Macromolecular Synthesis and Functionalization, Department of Polymer Science and Engineering, Zhejiang University, Hangzhou, China	Zhejiang University	Biomaterials, Engineering and Technology	University
	Department of Burns, Shaoxing Second Hospital, Shaoxing, China	Shaoxing Second Hospital	Burns	Hospital
	First People's Hospital of Hangzhou Xiaoshan District, Hangzhou, China	Hospital of Hangzhou Xiaoshan District	Health Sciences	Hospital
	Clinical Research Center, The Second Affiliated Hospital of Zhejiang University College of Medicine, Hangzhou, China	The Second Affiliated Hospital of Zhejiang University	Health Sciences	University Hospital
Jorgensen et al. 2020, Russia and United States	Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA	Wake Forest University	Regenerative Medicine	University
	FSBSI Institute of General Pathology and Pathophysiology, Moscow, Russia	FSBSI Institute of General Pathology and Pathophysiology	Pathology and Physiology	National Research Center/Institute
	Institute for Regenerative Medicine, Sechenov First Moscow State Medical University, Moscow, Russia	First Moscow State Medical University	Regenerative Medicine	University
	Department of Plastic and Reconstructive Surgery, Wake Forest University Baptist Medical Center, Winston-Salem, North Carolina, USA	Wake Forest Baptist Medical Center	Surgery	University Hospital
Jorgensen et al. 2023, United States	Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA.	Wake Forest University	Regenerative Medicine	University
	Department of Plastic and Reconstructive Surgery, Atrium Health Wake Forest Baptist Hospital, Medical Center Boulevard, Winston-Salem, NC 27157, USA	Wake Forest Baptist Medical Center	Surgery	University Hospital

Lee et al. 2021, Korea	Department of Materials Science & Engineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, 37673, Republic of Korea	Pohang University of Science and Technology	Biomaterials, Engineering and Technology	University
	Department of Mechanical Engineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, 37673, Republic of Korea	Pohang University of Science and Technology	Biomaterials, Engineering and Technology	University
	Department of Convergence IT Engineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, 37673, Republic of Korea	Pohang University of Science and Technology	Biomaterials, Engineering and Technology	University
	School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-Ro, Nam-Gu, Pohang, 37673, Republic of Korea	Pohang University of Science and Technology	Biomaterials, Engineering and Technology	University
Li et al. 2023, China	Shanxi Key Laboratory of Micro Nano Sensors & Artificial Intelligence Perception, College of Information and Computer, Taiyuan University of Technology, Taiyuan, 030024, PR China.	Taiyuan University of Technology	Biomaterials, Engineering and Technology	University
	Key Lab of Advanced Transducers and Intelligent Control System of the Ministry of Education, Taiyuan University of Technology, Taiyuan, 030024, PR China	Taiyuan University of Technology	Biomaterials, Engineering and Technology	University
	Shanxi Research Institute of 6D Artificial Intelligence Biomedical Science, Taiyuan, 030031, PR China	Shanxi Research Institute of 6D Artificial Intelligence Biomedical	Biomaterials, Engineering and Technology	National Research Center/Institute
	College of Information Science and Engineering, Hebei North University, Zhangjiakou, 075000, PR China	Hebei North University	Biomaterials, Engineering and Technology	University
	General Hospital of TISCO, North Street, Xinghualing District, Taiyuan 030809, PR China	General Hospital of TISCO	Health Sciences	Hospital

Liu et al. 2022, China	School of Biology, Food and Environment, Hefei University, Hefei, China	Hefei University	Biology, Biophysics, and Chemistry	University
	Department of Vascular Surgery, Xuanwu Hospital and Institute of Vascular Surgery, Capital Medical University, Beijing, China	Capital Medical University	Surgery	University
	Shandong Key Laboratory of Biophysics, Institute of Biophysics, Dezhou University, Dezhou, Shandong, China	Dezhou University	Biology, Biophysics, and Chemistry	University
	Department of Cardiovascular Surgery, Xi'an Children's Hospital, Xi'an, China	Xi'an Children's Hospital	Surgery	Hospital
Seol et al. 2018, USA and Korea	Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, WinstonSalem, North Carolina, USA	Wake Forest University	Regenerative Medicine	University
	School of Biomedical Engineering and Sciences, Wake Forest University-Virginia Tech, WinstonSalem, North Carolina, USA	Wake Forest University	Biomaterials, Engineering and Technology	University
	Department of Mechanical Engineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Gyungbuk, South Korea	Pohang University of Science and Technology	Biomaterials, Engineering and Technology	University
Somasekharan et al. 2021, India	Division of Thrombosis Research, Department of Applied Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala 695012, India	Sree Chitra Tirunal Institute for Medical Sciences and Technology	Biomaterials, Engineering and Technology	National Research Center/Institute
	Division of Tissue Culture, Department of Applied Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala 695012, India	Sree Chitra Tirunal Institute for Medical Sciences and Technology	Biomaterials, Engineering and Technology	National Research Center/Institute
Zhang et al. 2023, China	Department of Burns and Plastic Surgery, Fourth Medical Center of PLA General Hospital	Fourth Medical Center of PLA General Hospital	Burn	Hospital

CAPÍTULO 3 (Intended submission: Acta Biomaterialia; IF = 9.7)

https://www.sciencedirect.com/journal/acta-biomaterialia

Biomimetic cell delivery systems with 3D bioprinting technology

ABSTRACT

Three-dimensional (3D) bioprinting technology enables the fabrication of living scaffolds by integrating hydrogels with viable cells. This innovative approach holds great promise for creating tissue-engineered constructs capable of repairing and replacing damaged tissues. Despite its potential, cell-delivery systems for cell-based therapies using bioprinter remains under exploration. This study aimed to investigate the regenerative potential of bioprinting collagen-based scaffolds loaded with keratinocytes. The scaffolds were biofabricated using a robotic 3D bioprinter (Bio Assembly Bot 200, Advanced Solutions) and a 70mg/mL neutral bovine collagen type 1 bioink (Lifeink® 220, Cellink) loaded with immortalized human keratinocytes. Fluid diffusion and dimensional stability of the bioink were assessed over a period of 7 days. Additionally, the printability and shape fidelity of the scaffolds were evaluated. Cellular responses, including covering area, number of cells, and areas of cellular expansion within the scaffold, were analyzed using the QuPath software. Statistical analyses were performed using GraphPad Prism. The Lifeink® 220 bioink demonstrated significant fluid diffusion immediately upon immersion in culture media while maintaining dimensional stability with less than a 0.5% variation over a 7-day period. Printability tests showed minor variances between the computer-aided design (CAD) and the computer-aided manufacturing (CAM), indicating high overall shape fidelity. The parallel interconnected design (PID) significantly outperformed a solid design in promoting cellular migration. Specifically, the PID showed an initial covering area of 51 mm² (51%) on day 0, which increased to 99.1 mm² (99.1%) by day 3, whereas the solid design demonstrated a modest increase from 31 mm² (31%) to 39.6 mm² (39.6%) over the same period. Cell migration was notably higher in the PID, with 42.4% of cells migrating by day 3 compared to only 10.5% in the solid design. Additionally, the total number of remaining cells after 3 days was significantly higher for the PID group compared to the solid one (85.6% vs 77.2%, respectively). Moreover, increasing the initial cell density in the PID further enhanced cell proliferation and expansion within the scaffold, with a significant rise in cell numbers reaching 237.9% by day 3. This study demonstrates that collagen-based scaffolds loaded with keratinocytes hold significant potential for translational application. Of note, initial cellular density and scaffold design impact wound healing responses. Future research should explore combining optimized scaffolds with mechanical supports and therapeutic compounds to improve clinical effectiveness and broaden regenerative therapy outcomes. This study contributes to the development of personalized biomimetic cell delivery systems in tissue engineering and regenerative medicine.

KEYWORDS: 3D Bioprinter; Cell delivery; Biomimetic; Cell culture; Wound healing.

Este artigo contém resultados iniciais ainda não publicados. Para garantir a confidencialidade na parceria entre a Universidade de Brasília e a Universidade de Michigan, o conteúdo completo do artigo foi mantido em sigilo.

CONSIDERAÇÕES FINAIS

Os três artigos apresentados nesta tese contribuem de forma significativa para o avanço das pesquisas sobre reparo tecidual, utilizando modelos de cultura celular com diferentes níveis de complexidade. No Capítulo 1, foram exploradas monoculturas e co-culturas de queratinócitos e fibroblastos expostos à radiação ionizante e a extratos bacterianos, além dos efeitos reparadores da curcumina. Observou-se que os modelos de co-cultura influenciaram significativamente o fechamento de feridas *in vitro*, quando comparados às monoculturas. Além disso, a curcumina modulou as respostas celulares, promovendo aumento na viabilidade celular e alterações na morfologia das células em monocultura. Embora as análises dos genes da via PI3K-PTEN-AKT-mTOR tenham sido de caráter exploratório, os resultados sugerem padrões distintos de expressão gênica em queratinócitos e fibroblastos após o tratamento com curcumina. No contexto clínico da mucosite oral, a curcumina demonstra efeitos promissores, como a redução da dor, da intensidade do eritema, do tamanho das áreas ulceradas e do grau de mucosite (Normando et al. 2019; Wu et al. 2024). Esses efeitos são amplamente atribuídos às suas propriedades anti-inflamatórias, antioxidantes e antimicrobianas. Contudo, os mecanismos de ação responsáveis pelo reparo tecidual, tanto em nível celular quanto molecular, ainda não estão completamente esclarecidos. A utilização de modelos 3D com bioprinter, por exemplo, poderia avançar significativamente as evidências científicas, oferecendo respostas mais próximas ao comportamento fisiológico. Esses modelos 3D poderiam não apenas melhorar a compreensão dos mecanismos subjacentes ao reparo tecidual, mas também servir como ferramentas para testar e otimizar tratamentos, como o uso da curcumina, em condições que imitam melhor o ambiente clínico. Essa abordagem integrativa tem o potencial de acelerar a translação dos achados laboratoriais para aplicações clínicas, proporcionando tratamentos mais eficazes e personalizados para condições como a mucosite oral e outras lesões teciduais.

O segundo artigo destaca a importância da bioprinter 3D na engenharia de substitutos de pele, com ênfase no uso de queratinócitos e fibroblastos. A criação dos modelos 3D baseia-se em técnicas de cultura celular, em que as células são inicialmente cultivadas ou dissociadas de diferentes tecidos e órgãos (Shyam et al. 2023). A abordagem 3D permite a personalização de modelos de estudo e substitutos para transplante tecidual com o uso de células autólogas, favorecendo estudos sobre padrões de resposta individual e reduzindo o risco de rejeição em transplantes (Dixit et al. 2017). Os modelos 3D discutidos na revisão metodológica são ferramentas importantes para investigar medicamentos de efeitos duais, como a curcumina, facilitando análise mais detalhada das vias de sinalização envolvidas nos processos de reparo tecidual. Essa análise é particularmente relevante em condições como mucosite oral ou radiodermatite, em que o medicamento deve promover o reparo das células não neoplásicas lesionadas pelos efeitos adversos da terapia oncológica, sem causar resistência ou crescimento de células tumorais. Portanto, a padronização de protocolos e a colaboração multidisciplinar são fundamentais para melhorar a replicabilidade e a aplicabilidade dessas tecnologias. Pesquisas futuras podem se beneficiar de protocolos padronizados, permitindo sua personalização e aprimoramento conforme as necessidades clínicas.

Por fim, o terceiro artigo explora a otimização da bioimpressão 3D com matriz à base de colágeno para a entrega de células, destacando como o design do scaffold e a densidade celular inicial impactam diretamente a migração e proliferação celular. Esta pesquisa estabelece uma base para o desenvolvimento de sistemas biomiméticos de entrega celular, que podem ser integrados em terapias regenerativas personalizadas. A combinação de suportes mecânicos e compostos terapêuticos com designs de scaffolds otimizados é sugerida como uma estratégia promissora para potencializar a resposta celular e expandir o escopo das terapias baseadas em scaffolds. A literatura aponta que o uso de scaffolds inteligentes, capazes de responder a estímulos internos e externos, representa uma fronteira emergente na engenharia de tecidos, com o potencial de revolucionar o tratamento de feridas complexas e crônicas (Ahadian & Khademhosseini, 2018). Além disso, os sistemas de entrega de células, baseados em técnicas de cultura celular, oferecem benefícios significativos não apenas para lesões que requerem recobrimento epitelial, mas também para situações em que modificações genéticas são necessárias, como em condições autoimunes (Yanaga et al. 2001; Hirsch et al. 2017). Como as células são derivadas de técnicas de cultura, é possível realizar modificações genéticas para substituir células com respostas anormais por células transgênicas. Essa abordagem pode contribuir para o tratamento de condições dermatológicas diversas, promovendo resposta terapêutica mais eficaz e personalizada.

Em conclusão, a integração das evidências apresentadas nestes três artigos aponta direções promissoras na pesquisa de reparo tecidual, combinando abordagens tradicionais com inovações emergentes. O uso de compostos naturais, como a curcumina, em conjunto com tecnologias de bioprinter 3D e scaffolds otimizados, oferece não apenas a possibilidade de melhorar os resultados em saúde, mas também de personalizar tratamentos, adaptando-os às necessidades específicas de cada paciente e promovendo avanços significativos na medicina regenerativa. A continuidade das pesquisas e a colaboração interdisciplinar serão essenciais para transformar essas promessas em práticas clínicas eficazes e acessíveis, ampliando o impacto dessas tecnologias no tratamento de lesões e na engenharia de tecidos.

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APÊNDICE. Parecer consubstanciado do Conselho de Ética em Pesquisa, Faculdade de Ciências da Saúde da Universidade de Brasília – UnB

FACULDADE DE CIÊNCIAS DA SAÚDE DA UNIVERSIDADE DE BRASÍLIA - UNB

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Modelo in vitro de lesão radioinduzida estabelecido com cultura primária de Fibroblastos Gengivais Humanos

Pesquisador: MYLENE MARTINS MONTEIRO

Área Temática:

Versão: 3

CAAE: 52278221.6.0000.0030

Instituição Proponente:Faculdade de Ciências da Saúde da Universidade de Brasília

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.212.117

Apresentação do Projeto:

Conforme o documento 'PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1809826.pdf' postado em 17/01/2022:

"Desenho:

Para o estudo, será utilizada cultura primária de fibroblastos gengivais humanos. Serão coletados fragmentos de gengiva íntegra de 20 participantes de 18 a 25 anos. Os fragmentos deverão ser íntegros, sem inflamação e serem removidos durante procedimento odontológico a serem realizados indicados por um profissional, não estando vinculado ao projeto. Os procedimentos podem ser extração de dentes terceiros molares, inclusos ou não, extração de pré-molares, indicado pela ortodontia, ou cirurgia para instalação de implantes dentários osseointegrados. Após a coleta do explante, eles serão transportados ao laboratório em isopor contendo gelo, onde será lavado com solução salina de fosfato tamponada, fragmentado em partes de aproximadamente 1mm. Os fragmentos serão dispostos em placas e mantidos em condições ideais, em incubadora úmida, com troca regular do meio de cultura. Ao atingirem a confluência ideal, as células serão desprendidas das placas com solução de tripsina acrescida de EDTA e centrifugadas. As células poderão ser armazenadas em congelador a -80oC, sem identificação do doador, ou serem distribuídas em novas placas para realização dos experimentos ou expansão da cultura.Todas as células serão tratadas com a associação de três estímulos - lipopolissacarídeos (LPS – 10g/ml) de

Escherichia coli 0111:B4 (Sigma-Aldrich, San Luis, Missouri, EUA), extrato proteico de Porphyromonas gingivalis (Pg – 5g/ml), e 8 Gray (Gy) de radiação ionizante (RI) para a obtenção de lesão celular radioinduzida. Como tratamento, as células serão divididas em 4 grupos. O primeiro grupo será o grupo controle. O segundo grupo será tratado com 2,5 M de curcumina (referência 08511), adquirida da empresa Sigma- Aldrich (San Luis, Missouri, EUA), em forma liofilizada e com grau de pureza padrão analítico. O terceiro grupo será tratado com fotobiomodulação, utilizando um laser de baixa potência. O quarto grupo será associada a culmina ao laser de baixa potência."

Resumo:

A curcumina e a fotobiomodulação (PBM) são terapias de interesse em estudos, pelas propriedades anti-inflamatórias e de reparação tecidual. Como a sinalização da via PI3K-AKT-mTOR promove proliferação celular, estimulando regeneração, remodelação e reepitelização, a modulação dela pela aplicação de curcumina e PBM podem ser alternativas promissoras para reparo de lesões radioinduzidas. Objetiva-se analisar efeitos da curcumina e da fotobiomodulação nos processos de regeneração e reparo, em modelos in vitro, de lesões radioinduzidas. Serão utilizados queratinócitos humanos imortalizados e cultura primária de fibroblastos gengivais humanos. A lesão celular será induzida com três estímulos associados, lipopolisscarídeo de E.coli, extrato proteico de P.gingivalis e radiação ionizante. Como tratamento modulador, serão aplicados 2,5M de curcumina (definida em estudo anterior - referência 08511, Sigma Aldrich) e PBM (a definir pelo teste de viabilidade celular), de maneira isolada e/ou associada. Para analisar os efeitos, foram propostos os grupos experimentais: Queratinócito: [1] Veículo; [2] Curcumina (2,5M); [3] PBM (dose previamente definida); [3] Curcumina (2,5M) + PBM (dose previamente definida); Fibroblasto: [1] Veículo; [2] Curcumina (2,5M); [3] PBM (dose previamente definida); [3] Curcumina (2,5M) + PBM (dose previamente definida). Serão aplicados testes de viabilidade celular, cicatrização e migração celular, e avaliada expressão gênica de citocinas pró inflamatórias (TNF, IL1, IL6, NFkB) e sinalizadores da via PI3K-PTEN-AKT-mTOR. Espera-se contribuir no desenvolvimento de protocolos de tratamento para lesões radioinduzidas pela modulação da via PI3K-PTEN-AKT-mTOR, incentivando o aperfeiçoamento de fármacos associados com a terapia por PBM, como alternativa clínica viável para o SUS.

Introdução:

A curcumina, polifenol isolado da cúrcuma longa, vem sendo estudada como uma terapia

promissora, tendo em vista seus efeitos biológicos com propriedades antitumorais, antioxidantes, antimicrobianas e anti-inflamatórias. Autores revisaram uma série de ensaios clínicos mostrando os efeitos da curcumina no tratamento de diferentes doenças inflamatórias humanas, com poucos efeitos adversos. No que diz respeito ao reparo tecidual, estudos in vitro e in vivo demonstram que a aplicação tópica de preparos à base desse polifenol contribui para a redução da resposta inflamatória e acelera o fechamento de feridas. Com isso, a identificação de vias sinalizadoras que respondem à ação da curcumina é um tema de constante interesse, com o objetivo de desenvolver terapias específicas. Estudos recentes sugerem a ação da curcumina como reguladora da expressão gênica e proteica da via PI3K-AKT-mTOR em células tumorais, quando usada em altas doses. Entretanto, a sinalização dessa via, modulada por doses mais baixas de curcumina, para a obtenção de reparo tecidual, ainda não é muito explorada pela literatura.

De forma similar, a fotobiomodulação (PBM) é outra opção terapêutica em recorrente foco de análise, por influenciar de forma positiva o metabolismo celular. Essa terapia consiste na emissão de fóton de luz por meio da irradiação de laser de baixa potência, com resultados promissores em termos de analgesia, imunomodulação, reparo tecidual e regeneração de lesões teciduais. A terapia por PBM a nível celular, promove ação local, regional e sistêmica, de modo que sua resposta intracelular é percebida no aumento da ação da mitocôndria, resultando em uma maior produção de adenosina trifosfato (ATP) e espécies reativas de oxigênio. Em uma revisão crítica com mais de 50 estudos avaliando a PBM na viabilidade celular, proliferação, migração e produção de citocinas e fatores de crescimento em cultura de queratinócitos, observaram que a emissão de luz nos espectros vermelho e infravermelho apresentam melhor efeito estimulatório, sendo os mais favoráveis obtidos com a aplicação de intervalos de densidade de energia entre 0,1 a 5,0 J/cm2. Embora os mecanismos de ação dessa terapia ainda não estejam totalmente elucidados, assim como a curcumina, a PBM pode apresentar efeito modulador da via PI3K-AKT-mTOR.

O interesse na modulação da via PI3K-AKT-mTOR como alvo para o reparo tecidual pode ser explicado pois sua sinalização promove aumento da proliferação celular, estimulando também a regeneração, remodelação e reepitelização tecidual. Considerando a lesão na molécula de DNA causada por radiação ionizante, e o consequente aumento de fatores de transcrição e de citocinas inflamatórias decorrentes da agressão celular, a modulação da resposta por meio de terapias capazes de interagir com as fases de inflamação e reparação, pode ser promissora para o tratamento de leões radioinduzidas, como por exemplo, a mucosite oral. Dessa forma, avaliar a interação entre os efeitos da PBM e da curcumina, de maneira isolada e associada, tanto com mediadores inflamatórios, quanto como sinalizadores da via PI3K-AKT-mTOR poderá auxiliar na

identificação de novas terapias e na explanação dos mecanismos de ação a elas atribuídos. Portanto, o presente projeto tem como objetivo analisar os efeitos da curcumina e da fotobiomodulação, tanto como terapias isoladas, quanto associadas, para os processos de regeneração e reparo em modelo in vitro de lesão radioinduzida.

Hipótese:

Espera-se identificar os efeitos celulares e moleculares da fotobiomodulação e da curcumina para o tratamento de lesões induzidas por radiação ionizante. Considerando os efeitos conhecidos dessas terapias, é esperado que a associação das duas opções terapêuticas apresente resultado superior às aplicações isoladas.

"Metodologia Proposta:

Cultura celular: Serão utilizados queratinócitos humanos imortalizados (HaCaT) e cultura primária de fibroblastos gengivais humanos (FG). Para a cultura primária, serão coletados fragmentos de gen- giva íntegra de 20 participantes de 18 a 25 anos. Os fragmentos deverão ser íntegros, sem in- flamação e serão removidos durante procedimento odontológico a serem realizados indicados por um profissional, não estando vinculado ao projeto. Após a coleta do explante, eles serão transportados ao laboratório imerso e DMEM suplementado com 20% de soro fetal bovino e 1% de antibiótico e antifúngico, em isopor contendo gelo, onde será lavado com solução sali- na de fosfato tamponada e fragmentado em partes de aproximadamente 1mm. Os fragmentos serão dispostos em placas e mantidos em condições ideais, em incubadora úmida, com troca regular do meio de cultura. Ao atingirem a confluência ideal, as células serão desprendidas das placas com solução de tripsina acrescida de EDTA e centrifugadas para o estabelecimento da cultura. Um vez estabelecida, as células poderão ser armazenadas em congelador a -80oC, sem identificação do doador, ou serem distribuídas em novas placas para realização dos experimentos ou expansão da cultura. Modelo de Lesão Radio-induzida: Inicialmente, todas as células serão tratadas com a associação de três estímulos - lipopolissacarídeos (LPS – 10g/ml) de Escherichia coli 0111:B4 (Sigma-Aldrich, San Luis, Missouri, EUA), extrato proteico de Porphyromonas gingivalis (Pg – 5g/ml), e 8 Gray (Gy) de radiação ionizante (RI) para a obtenção de lesão celular radioinduzida. Esse modelo foi proposto em estudo anterior do Laboratório de Histopatologia Bucal da UnB e será replicada para novas análises. Curcumina: Como tratamento modulador da via PI3K-PTEN-AKT-mTOR, serão utilizados 2,5 M de curcumina (referência 08511), adquirida da empresa Sigma-Aldrich (San Luis, Missouri, EUA), em forma liofilizada e com grau de pureza padrão analítico. Essa

concentração foi definida de acordo com a curva dose-resposta, previamente desenvolvida e publicada por Borges et al. 2020, optando-se pelo valor da tabela IC50, que corresponde à concentração imediatamente anterior ao início da citotoxicidade de cada fármaco.Fotobiomodulação – curva dose -resposta: Para definir a dose ideal da terapia por fotobiomodulação, quatro densidades de energia (2 J/cm2, 3 J/cm2, 4 J/cm2 e 5 J/cm2) serão avaliadas por teste de viabilidade celular. Para isso, após o estímulo de lesão radio-induzida, as células serão irradiadas com laser de baixa potência, em comprimentos de onda nos espectros vermelho (660nm) e infravermelho (808nm), isolados e/ou associados. Após 24h, serão adicionados 10L de solução de MTT (Sigma-Aldrich, San Luis, Missouri, EUA) na concentração de 0,5 mg/mL em cada poço. Decorridas 4 horas, o meio de cultura será aspirado e 100L de isopropanol acidificado (25mL de isopropanol + 104L de HCl 100%) serão adicionados em cada poço. As placas serão então agitadas em velocidade baixa e, em seguida, a absorbância será aferida em espectrofotômetro Thermo Plate TP Reader (Thermo Fisher Scientific, Waltham, Massachusetts, EUA). A dose que apresentar melhor resultado para a viabilidade celular será selecionada para a continuação dos experimentos.

Critério de Inclusão:
Pacientes de ambos os gêneros com idade entre 18 e 25 anos; Fragmento de gengiva íntegra, sem inflamação, removida durante extração de dentes terceiros molares inclusos ou não com indicação de exodontia; Fragmento de gengiva sem inflamação removida durante extração de dentes pré-molares com indicação ortodôntica de exodontia; Fragmento de gengiva sem inflamação removida durante cirurgia para instalação de implantes dentários osseointegrados; Estar de acordo e assinar o Termo de Consentimento Livre e Esclarecido

Critério de Exclusão:
Gengiva com aspecto clínico de inflamação"

"Metodologia de Análise de Dados:
Teste de Viabilidade Celular: Após 24h da aplicação dos tratamentos, serão adicionados 10L de solução de MTT (Sigma-Aldrich, San Luis, Missouri, EUA) na concentração de 0,5 mg/mL em cada poço. Após 4 horas, o meio de cultura será aspirado e 100L de isopropanol acidificado (25mL de isopropanol + 104L de HCl 100%) serão adicionados em cada poço. As placas serão então agitadas em velocidade baixa e, em seguida, a absorbância será aferida em espectrofotômetro

Thermo Plate TP Reader (Thermo Fisher Scientific, Waltham, Massachusetts, EUA).Imunofluorescência (morfologia celular): Após 24h da aplicação dos tratamentos, as células serão fixadas com 2mL de paraformaldeído 3%, lavadas e reagidas com solução de bloqueio. Para avaliar a distribuição de F actina, as lamínulas serão preparadas com coloração por faloídina; para analisar os filamentos intermediários, as células serão reagidas com anticorpos desenvolvidos para Citoqueratina 10 e 14 nos queratinócitos, e Vimentina nos fibroblastos, seguido dos anticorpos secundários. Os núcleos celulares serão corados (Hoechst 33342 - Thermo Fisher Scientific) e as lamínulas serão fixadas com Fluroshield. A análise será realizada em microscópio de imunofluorescência (Nikon Eclipse 80i), as imagens registradas através de uma câmera digital monocromática acoplada (QImaging ExiAqua) e a quantificação realizada com o software ImageJ (Institutos Nacionais de Saúde, Bethesda, MD).Teste de Cicatrização e Migração Celular (Scratch assay)As monocamadas celulares 100% confluentes serão arranhadas manualmente com uma ponta de pipeta (200 L), lavadas com PBS e tratadas de acordo com os grupo experimentais. Um microscópio invertido (Zeiss Primo Vert, Göttingen, Alemanha) equipado com câmera digital (Zeiss ERc 5s, Göttingen, Alemanha) será usado para obter 12 imagens de grupo a cada 12h, a partir do tempo 0h até o fechamento completo da ferida. Expressão gênica (citocinas inflamatórias e PTEN, AKT, mTOR e PIK3CA)Após 24h da aplicação dos tratamentos, a expressão gênica de citocinas pró inflamatórias (TNF, IL1, IL6, NFkB) e de sinalizadores da via PI3K-PTEN-AKT-mTOR será avaliada por meio da reação em cadeia da polimerase em tempo real/quantitativa (RT-qPCR). A extração do RNA total será feita de acordo com o método estabelecido pelo TRizol® (Invitrogen), seguida da síntese de DNA complementar (cDNA) por transcrição reversa, utilizando-se o kit High Capacity cDNA Reverse Transcription (Applied Biosystems), e amplificação por qPCR com o kit PowerUp® SYBR® Green Master Mix (Applied Biosystems). Análise estatística: As análises serão realizadas com o GraphPad Prism versão 9.0.2 (GraphPad Software, La Jolla, CA). Para comparações múltiplas, serão utilizados os métodos one-way ou two-way ANOVA, seguido de pós-teste de Tukey. Para análise entre dois grupos, será realizado o teste de normalidade, sendo aplicado o Teste T para dados paramétricos e Mann-Whitney para dados não paramétricos. O critério de significância será o valor $p<0,05$.

Desfecho Primário:

A pesquisa básica visa ao primeiro passo para o desenvolvimento de protocolos de tratamento de lesões radioinduzidas por meio da modulação da via PI3K-PTEN-AKT-mTOR. A associação da terapia à laser com a curcumina, poderá incentivar protocolos duais, não apenas com foco na fase

profissional-dependente, mas também com a utilização domiciliar de fármacos de fácil acesso, sendo uma alternativa clínica viável para Sistema Único de Saúde (SUS). A criação de protocolos que podem ser utilizados na prevenção ou tratamento de lesões radioinduzidas poderá refletir em uma melhor qualidade de vida de pacientes oncológicos que devem ser submetidos a tratamentos de radioterapia ou quimioterapia. A promoção do reparo dessas lesões pode contribuir para uma melhor alimentação, comunicação e deglutição, assim como sua negligência pode levar a inviabilização da continuidade do tratamento proposto para o câncer. Além disso, a identificação dos alvos terapêuticos melhora a saúde do paciente, não apenas no âmbito da odontologia, mas também em outras áreas da saúde, uma vez que essas lesões podem levar à necessidade de hospitalizações para controle da infecção, manejo da dor e nutrição parenteral, aumentando os custos do tratamento.

Tamanho da Amostra no Brasil: 20.

Data do Primeiro Recrutamento: 10/02/2022"

"Haverá retenção de amostras para armazenamento em banco?
Sim

Justificativa:
O Projeto de Pesquisa será realizado com fragmentos gengivais obtidos em cirurgias orais tais como extrações dentárias e instalação de implantes e cedidos pelos pacientes ou pelos responsáveis. Durante a realização dos experimentos e após sua conclusão, as células serão armazenadas em congelador à -80°C, sem identificação do doador. A autorização do participante para o armazenamento das células encontra-se em Termo de Consentimento Livre e Esclarecido. Dependendo dos resultados obtidos no presente estudo, o material armazenado poderá ser utilizado em pesquisas futuras. Por isso, há a necessidade de armazenamento dos tecidos coletados, para não precisar de uma nova coleta em outros indivíduos."

Objetivo da Pesquisa:

Conforme o documento 'PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1809826.pdf' postado em 17/01/2022:

"Objetivo Primário:

O presente estudo tem como objetivo analisar os efeitos da curcumina e da fotobiomodulação, tanto como terapias isoladas, quanto associadas, nos processos de regeneração e reparo em modelo in vitro de lesão radioinduzida estabelecido com cultura primária de fibroblastos gengivais.

Objetivo Secundário:

a) Avaliar os efeitos isolados e associados das terapias por fotobiomodulação e o uso da curcumina na modulação da via PI3K-PTEN-AKT-mTOR, com foco no reparo tecidual após estímulo radio-induzido; b) Avaliar a morfologia, a viabilidade, a produção de células tronco e o potencial de proliferação e migração celular dos tratamentos com fotobiomodulação e curcumina, isolados e associados, em células previamente estimuladas por radiação ionizante; c) Analisar a expressão gênica e proteica de citocinas pró-inflamatórias, e de sinalizadores da via PI3K-PTEN-AKT-mTOR, após aplicar os tratamentos com fotobiomodulação e curcumina, isolados e associados, em células previamente estimuladas por radiação ionizante."

Avaliação dos Riscos e Benefícios:

Conforme o documento 'PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1809826.pdf' postado em 17/01/2022:

"Riscos:

Os fragmentos gengivais utilizados na pesquisa serão aqueles obtidos quando da extração de dentes por razões terapêuticas não estando, portanto, o procedimento cirúrgico vinculado ao projeto. Para os pacientes que aceitarem cederem amostras de gengiva para utilização no projeto, o risco poderia ser vincular a amostra a sua identidade. Nesse sentido, a confidencialidade do participante será garantida por meio da codificação das células com números.

Benefícios:

A pesquisa não oferece benefícios diretos e imediatos aos participantes, mas como benefícios futuros, espera-se que a pesquisa permita uma melhor compreensão da expressão gênica de citocinas proinflamatórias de tecidos gengivais tratados com produtos naturais tais como curcumina, resveratrol e EGCG, no intuito de estabelecer novas medidas terapêuticas para o uso baseado em evidências científicas, proporcionando melhora na qualidade de vida dos pacientes

oncológicos"

Comentários e Considerações sobre a Pesquisa:

Trata-se de pesquisa de mestrado da PPG-ENF que será executado pela mestranda Mylene Martins Monteiro sob a supervisão da Profa. Dra. Eliete Neves da Silva Guerra.

O cronograma indica que o primeiro contato com os participantes ocorrerá em 2022.

O orçamento, de financiamento próprio, no total de R$6.452,00 indica gastos com informática, ressarcimento de transporte e alimentação dos participantes e itens de consumo necessários à execução da pesquisa.

Considerações sobre os Termos de apresentação obrigatória:

Documentos acrescentados ao processo e analisados para emissão deste parecer:

1. Informações Básicas do Projeto: "PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1809826.pdf" postado em 17/01/2022.
2. Projeto Detalhado ATUALIZADO: "8Projeto_Jan_16_22_revisao.pdf e 8Projeto_Jan_16_22_revisao.docx" postado em 17/01/2022.
3. Carta ao CEP-FS: "CARTA_DE_RESPOSTAS_Jan_16_22.docx e CARTA_DE_RESPOSTAS_Jan_16_22.pdf" postada em 17/01/2022.
4. Modelo de TCLE ATUALIZADO: "4TCLE_Jan_16_22.pdf e 4TCLE_Jan_16_22.docx" postado em 17/01/2022.

Recomendações:

Não se aplicam.

Conclusões ou Pendências e Lista de Inadequações:

Análise das respostas às pendências apontadas no Parecer Consubstanciado No. 5.116.251 e 5.174.226:

1. Uniformizar os objetivos do projeto de pesquisa em todos documentos e no formulário online da Plataforma Brasil.

RESPOSTA: Os objetivos foram uniformizados e descritos como: "O presente estudo tem como objetivo analisar os efeitos da curcumina e da fotobiomodulação, tanto como terapias isoladas, quanto associadas, nos processos de regeneração e reparo em modelo in vitro de lesão radioinduzida estabelecido com cultura primária de fibroblastos gengivais." Foram modificados no formulário online (objetivo primário, página 4 “Detalhamento do estudo”), e nos arquivos anexados: 4TCLE_Nov_29_21 (Página 1, parágrafo 5) e no 8Projeto_Nov_29_21 (Página 4, objetivos gerais, parágrafo 1).

ANÁLISE: PENDÊNCIA ATENDIDA.

2. No formulário online da Plataforma Brasil, na seção 'Outras informações, justificativas ou considerações a critério do pesquisado' consta que o projeto "Apresenta uma equipe de 5 pesquisadores, além da discente, composta por professores pesquisadores e alunos de pós- graduação e graduação.", mas somente 3 currículos foram apresentados. Solicita-se que sejam enviados os 3 currículos dos outros membros da equipe de pesquisa, e que seja explicado o papel de cada um dos membros da equipe de pesquisa.

RESPOSTA: Foi alterado o número de participantes do grupo de pesquisa. São 4 pesquisadores, além da discente, composta por alunos da graduação e pós graduação. Também foi anexado os 2 currículos dos membros que estavam faltando nos documentos “Lattes_ Bruna_Bastos_Silveira_da_Silva" e “Lattes_Victor_Paiva_Barbosa”. Todos os integrates possuem produção científica própria e contribuem com o projeto auxiliando nas pesquisas e experimentos, quando necessário, sob supervisão da pesquisadora principal.

ANÁLISE: PENDÊNCIA ATENDIDA.

3. No formulário online da Plataforma Brasil, na seção 'Haverá retenção de amostras para armazenamento em banco?' as pesquisadoras indicam interesse em armazenar o material biológico colhido por tempo indeterminado e informam que 'Dependendo dos resultados obtidos no presente estudo, o material armazenado poderá ser utilizado em pesquisas futuras. Por isso, há a necessidade de armazenamento dos tecidos coletados, para não precisar de uma nova coleta em outros indivíduos.".

Esta modalidade de armazenamento de material biológico, que inclui a utilização do material para projetos futuros e ainda não delineados caracteriza a constituição de um BIOBANCO conforme a PORTARIA No 2.201, DE 14 DE SETEMBRO DE 2011 MS/CNS (https://bvsms.saude.gov.br/bvs/saudelegis/gm/2011/prt2201_14_09_2011.html). Caso seja de interesse da equipe de

pesquisa a constituição de um BIOBANCO, solicita-se que seja apresentada uma solicitação formal

conforme consta na portaria supracitada. Caso contrário, solicita-se que sejam feitas as adequações necessárias em todos documentos, incluindo Projeto Detalhado, TCLE e formulário online da PB, indicando nitidamente que o material coletado será utilizado exclusivamente para os objetivos delineados neste projeto de pesquisa, e que o material biológico coletado será destruído ao término do projeto de pesquisa, ou após 05 anos, o que ocorrer primeiro (CNS/MS 466/2012 XI.2.f).

RESPOSTA: A modalidade que estamos buscando é a de biorrepositório, onde a colecao de material biologico humano, será coletado e armazenado ao longo da execucao desse projeto de pesquisa especifico, conforme regulamento ou normas tecnicas, eticas e operacionais pre-definidas, sob responsabilidade institucional e sob gerenciamento do pesquisador, sem fins comerciais.

Com isso, os documentos para adequação à Resolução CNS 441/2011, Portaria MS 2.201/11 e Norma Operacional CNS 001/2013, em relação ao armazenamento de material biológico foram anexados: "Justificativa_Armazenamento_Nov_29_21" e "Declaracao" . Tal informação também foi adicionada ao TCLE (Página 1, parágrafo 3 e Página 1, parágrafo 6). Foi também incluído ao projeto o item sobre armazenamento e uso futuro de material biológico armazenado (Página 6, item 6).

ANÁLISE: De fato, as pesquisadoras têm razão na solicitação, e a proposta é plenamente compatível com o estabelecimento de um biorrepositório. Em adição à documentação enviada, solicita-se que o TCLE seja adequado, e que conste no TCLE do participante que a amostra será armazenada respeitando-se a confidencialidade e a autonomia do participante da pesquisa, e que a amostra biológica coletada ficará armazenada (Laboratorio, faculdade, universidade), e sob a responsabilidade de qual pesquisador(a). Também deve constar que o/a participante da pesquisa pode a qualquer momento retirar o seu consentimento para o armazenamento da amostra biológica, e que será comunicado sobre a perda, alteração ou destruição de suas amostras biológicas ou da decisão de interrupção da pesquisa, como também sobre o fechamento ou transferência do biorrepositório. Qualquer alteração nos objetivos da pesquisa proposta, assim como na eventual utilização do material biológico em pesquisas futuras, o participante de pesquisa será contatado para reconsentimento, ou novo consentimento no caso de novo projeto de pesquisa, e que para toda modificação será apreciada pelo CEP antes de ser executada. Diante da necessidade de reconsentimento, ou novos consentimentos futuros, sugerimos fortemente que o contato telefônico e/ou e-mail dos participantes seja armazenado pelas pesquisadoras.

PENDÊNCIA PARCIALMENTE ATENDIDA.

RESPOSTA: O TCLE foi adequado constando as seguintes alterações sugeridas: A amostra será

armazenada, respeitando a confidencialidade, no laboratório de Histopatologia Bucal, na faculdade de Ciências da Saúde, na Universidade de Brasília, sobre a responsabilidade da pesquisadora Mylene Martins Monteiro." (página 1, parágrafo 6) e "O(a) senhor(a) ainda será comunicado sobre perda, alteração ou destruição de suas amostras biológicas, da decisão de interromper a pesquisa ou do fechamento ou transferência do biorrepositório." (página 2, parágrafo 7). Ainda foi adicionado um local para que seja possível armazenar o contato dos participantes (página 2).
ANÁLISE: PENDÊNCIA ATENDIDA.

4. Solicita-se que o TCLE seja apresentado em linguagem acessível e compreensível ao público leigo. Não é necessário que os participantes conheçam em detalhe toda a metodologia do projeto de pesquisa, mas sim quais são os objetivos da pesquisa e como será feita a coleta de dados e como o material biológico colhido será armazenado e utilizado.

RESPOSTA: No TCLE as alterações referentes ao detalhamento da metodologia foi alterada, assim como a linguagem compreensível ao público leigo. Essas alterações se encontram na página 1, parágrafo 1.
ANÁLISE: PENDÊNCIA ATENDIDA.

Todas as Pendências foram atendidas. Não foram observados óbices éticos.
Protocolo de pesquisa em conformidade com as Resolução CNS 466/2012, 510/2016 e complementares.

Considerações Finais a critério do CEP:
Conforme a Resolução CNS 466/2012, itens X.1.- 3.b. e XI.2.d, os pesquisadores responsáveis devem apresentar relatórios parciais semestrais, contados a partir da data de aprovação do protocolo de pesquisa; e um relatório final do projeto de pesquisa, após a conclusão da pesquisa.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:				
Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1809826.pdf	17/01/2022 20:20:56		
Projeto Detalhado / Brochura Aceito	8Projeto_Jan_16_22_revisao.pdf	17/01/2022 20:19:38	MYLENE MARTINS MONTEIRO	

Continuação do Parecer: 5.212.117

Investigador	8Projeto_Jan_16_22_revisao.pdf	17/01/2022 20:19:38	MYLENE MARTINS MONTEIRO	Aceito
Outros	CARTA_DE_RESPOSTAS_Jan_16_22.docx	17/01/2022 20:18:44	MYLENE MARTINS MONTEIRO	Aceito
Outros	CARTA_DE_RESPOSTAS_Jan_16_22.pdf	17/01/2022 20:18:08	MYLENE MARTINS MONTEIRO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	4TCLE_Jan_16_22.pdf	17/01/2022 20:16:58	MYLENE MARTINS MONTEIRO	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	4TCLE_Jan_16_22.docx	17/01/2022 20:16:50	MYLENE MARTINS MONTEIRO	Aceito
Projeto Detalhado / Brochura Investigador	8Projeto_Jan_16_22_revisao.docx	17/01/2022 18:40:04	MYLENE MARTINS MONTEIRO	Aceito
Outros	Justificativa_Armazenamento_Nov_29_21.pdf	01/12/2021 11:48:56	MYLENE MARTINS MONTEIRO	
Outros	Declaracao.pdf	01/12/2021 11:48:21	MYLENE MARTINS MONTEIRO	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Declaracao.docx	30/11/2021 16:23:41	MYLENE MARTINS MONTEIRO	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	Justificativa_Armazenamento_Nov_29_21.docx	30/11/2021 16:13:23	MYLENE MARTINS MONTEIRO	
Outros	Lattes_Victor_Paiva_Barbosa.pdf	30/11/2021 16:04:44	MYLENE MARTINS MONTEIRO	Aceito
Outros	Lattes_Bruna_Bastos_Silveira_da_Silva.pdf	30/11/2021 16:03:34	MYLENE MARTINS MONTEIRO	Aceito
Outros	2Termo_concordancia_Sept_22_21.docx	28/09/2021 20:27:25	MYLENE MARTINS MONTEIRO	Aceito
Declaração de concordância	2Termo_concordancia_Sept_22_21.pdf	22/09/2021 12:15:58	MYLENE MARTINS MONTEIRO	Aceito
Outros	1Carta_encaminhamento_Sept_22_21.pdf	22/09/2021 12:15:02	MYLENE MARTINS MONTEIRO	Aceito
Outros	1Carta_encaminhamento_Sept_22_21.docx	22/09/2021 12:13:49	MYLENE MARTINS MONTEIRO	Aceito
Outros	3Termo_responsabilidade_Sept_22_21.docx	22/09/2021 12:13:21	MYLENE MARTINS MONTEIRO	Aceito
Outros	3Termo_responsabilidade_Sept_22_21.pdf	22/09/2021 12:13:03	MYLENE MARTINS MONTEIRO	Aceito

Continuação do Parecer: 5.212.117

Outros	Lattes_Mylene_Martins_Monteiro.pdf	22/09/2021 12:12:37	MYLENE MARTINS MONTEIRO	Aceito
Outros	Lattes_Juliana_Amorim_dos_Santos.pdf	22/09/2021 12:12:18	MYLENE MARTINS MONTEIRO	Aceito
Outros	Lattes_Eliete_Neves_da_Silva_Guerra.pdf	22/09/2021 12:12:05	MYLENE MARTINS MONTEIRO	Aceito
Cronograma	6Cronograma_Sept_22_21.pdf	22/09/2021 12:03:20	MYLENE MARTINS MONTEIRO	Aceito
Orçamento	5planilha_de_orcamento_Sept_22_21.pdf	22/09/2021 12:03:00	MYLENE MARTINS MONTEIRO	Aceito
Folha de Rosto	7folhaDeRosto_Sept_22_21.pdf	22/09/2021 12:00:02	MYLENE MARTINS MONTEIRO	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BRASILIA, 26 de Janeiro de 2022

Assinado por:
Fabio Viegas Caixeta
(Coordenador(a))