UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS - PPGCB

LIDIANE CRISTINA PINHO NASCIMENTO PORTELA

CARACTERIZAÇÃO E AVALIAÇÃO DO POTENCIAL CICATRIZANTE DE FILMES À BASE DE POLÍMEROS OBTIDOS DE RESÍDUOS DO PROCESSAMENTO DO PESCADO

Recife
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutora em Ciências Biológicas.

Área de concentração: Bioquímica de Organismos Aquáticos

Orientador: Prof º. Dr. Ranilson de Souza Bezerra

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Recife
2020

Catalogação na fonte:
Bibliotecária Claudina Queiroz, CRB4/1752

Portela, Lidiane Cristina Pinho Nascimento
Caracterização e avaliação do potencial cicatrizante de filmes à base de polímeros obtidos de resíduos do processamento do pescado / Lidiane Cristina Pinho Nascimento Portela - 2020.
113 folhas: il., fig., tab.

Orientador: Ranilson de Souza Bezerra
Coorientador: Thiago Barboza Cahú
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Ciências Biológicas. Recife, 2020.

Inclui referências e anexos.

1. Biopolímeros 2. Subprodutos 3. Curativos tópicos
I. Bezerra, Ranilson de Souza (Orientador) II. Cahú, Thiago Barboza (Coorientador) III. Título

660.6 CDD (22.ed.) UFPE/CB-2021-200

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Aprovada em: 14/02/2020

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Dedico esta tese à minha família e a meu esposo.

AGRADECIMENTOS

Agradeço primeiramente a Deus pelo dom da vida e por todas as bênçãos que alcancei por Suas mãos. Sem Ele e sem a intercessão da Virgem Maria eu não teria chegado tão longe.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa.

Ao Prof. Dr. Ranilson de Souza Bezerra que aceitou orientar-me, sempre acreditando no meu trabalho e me incentivando na pesquisa.

Ao meu co-orientador Dr. Thiago Cahú, por ter trabalhado comigo desde a elaboração do projeto, execução de muitos experimentos até os ajustes finais na tese, bem como o esclarecimento de tantas dúvidas que surgiram. A este que foi mais do que um co-orientador, mas um grande amigo que guardarei para toda a vida, meu muito obrigada.

À Thaís Santos, que me acompanhou durante sua iniciação científica e, que, com certeza, sem sua ajuda eu não teria conseguido fazer tudo que fiz. Obrigada pela sua dedicação, empenho e amizade.

À minha família, que é tudo para mim e que faz a minha vida mais feliz. Agradeço por todo apoio e força que me dão em minhas escolhas. Em especial, agradeço à minha mãe, que é um presente de Deus na minha vida, que me inspira a ser alguém melhor a cada dia e que está sempre ao meu lado, com muito carinho e amor.

Ao meu esposo, que esteve em todos os momentos ao meu lado, me apoiando e me dando forças para continuar na caminhada.

Aos meus amigos que compartilharam comigo todas as alegrias e tristezas ao longo desta jornada. Em especial aos amigos que fiz no Laboratório de Enzimologia que sempre me ajudaram e me apoiaram na realização dos meus experimentos, além dos momentos de descontração nas horas vagas.

RESUMO

Os subprodutos do processamento do pescado apresentam-se como uma fonte alternativa para obtenção de biopolímeros naturais com propriedades favoráveis a aplicações biotecnológicas e biomédicas. Este trabalho objetivou extrair o polissacarídeo quitina para obtenção da quitosana a partir da carapaça do camarão marinho, e as proteínas colágeno e tripsina da pele e do intestino da tilápia do Nilo (respectivamente), e avaliar o potencial cicatrizante de filmes e soluções filmogênicas destes biopolímeros. Após obtenção e purificação dos biopolímeros, foram preparados filmes contendo quitosana e colágeno (chi/col) e filmes contendo quitosana, colágeno e tripsina (chi/col/try), bem como soluções filmogênicas, contendo quitosana e colágeno diluídos em ácido acético. O grau de desacetilação da quitosana foi de 79,6 % e o rendimento do colágeno foi de 22,82 ± 1,5%. Os testes de teor de umidade e de intumescimento foram feitos em diferentes intervalos de tempo, e para tais testes, os filmes chit/coll demonstraram capacidade de absorção e de manutenção de líquidos (575 ± 35,35 % e 32,55 ± 0,76 %, respectivamente). A microscopia eletrônica de varredura e os testes de área superficial e porosidade dos filmes mostraram que a superfície dos filmes chit/coll possuía ondulações que podem favorecer a agregação e adesão celular. As análises de Termogavimetria e Calorimetria exploratória diferencial (TG/DSC) indicaram que o filme apresenta boa resistência à perda de massa mesmo em temperaturas elevadas. O teste mecânico mostrou que os filmes chi/col apresentavam boa resistência à tração de alongamento. No ensaio de citotoxicidade, as soluções filmogênicas e os filmes chi/col apresentaram efeitos imunomoduladores em células mononucleares do sangue periférico (PBMCs), com baixa taxa de nerose celular. A capacidade antimicrobiana das soluções filmogênicas foi testada utilizando-se as cepas bacterianas *Staphylococcus aureus* e *Staphylococcus epidermidis* onde houve inibição do crescimento bacteriano na presença das soluções. Ratos tratados com filmes chi/col e chi/col/try apresentaram maior contração da ferida (73,04 ± 7,8 % e 77,43 ± 2,37 %, respectivamente) em comparação com o controle usando apenas tampão salina (54,5 %). Esses resultados demonstraram que os biopolímeros extraídos dos subprodutos do processamento do pescado podem ser explorados como uma nova fonte para obtenção de produtos no mercado farmacêutico e na área médica.

Palavras-chave: subprodutos; biomoléculas; membranas poliméricas; curativos tópicos.

ABSTRACT

The by-products of fish processing are an alternative source for obtaining natural biopolymers with properties favorable to biotechnological and biomedical applications. This work aimed to extract the polysaccharide chitin to obtain chitosan from the shell of the marine shrimp, and the proteins collagen and trypsin of the skin and intestine of the Nile tilapia (respectively), and to evaluate the healing potential of films and filmogenic solutions of these biopolymers. After obtaining and purifying the biopolymers, films containing chitosan and collagen (chi/col) and films containing chitosan, collagen and trypsin (chi/col/try) were prepared, as well as filmogenic solutions containing chitosan and collagen diluted in acetic acid. Chitosan degree of deacetylation was 79.6 % and the yield of collagen was 22.82 ± 1.5 %. The moisture content and swelling tests were performed at different time intervals, and for such tests, the chi/col films demonstrated the ability to absorb and maintain liquids (575 ± 35.35 % and 32.55 ± 0, 76 %, respectively). Scanning electron microscopy and tests of the surface area and porosity of the films showed that the surface of the chit/coll films had undulations that can favor cell aggregation and adhesion. The Thermogavimetry and Differential Exploratory Calorimetry (TG/DSC) analyzes indicated that the film has good resistance to mass loss even at high temperatures. The mechanical test showed that the chit/coll films had good resistance to elongation traction. In the cytotoxicity assay, filmogenic solutions and chi/col films showed immunomodulatory effects on peripheral blood mononuclear cells (PBMCs), with a low rate of cell nerosis. The antimicrobial capacity of filmogenic solutions was tested using the bacterial strains *Staphylococcus aureus* and *Staphylococcus epidermidis* where there was inhibition of bacterial growth in the presence of the solutions. Rats treated with chi/col and chi/col/try films showed greater wound contraction (73.04 ± 7.8 % and 77.43 ± 2.37 %, respectively) compared to saline buffer control (54.5 %). These results demonstrated that the biopolymers extracted from the by-products of fish processing can be explored as a new source for obtaining products in the pharmaceutical market and in the medical field.

Keywords: by-products; biomolecules; polymeric membranes; topical dressings.

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LISTA DE ABREVIATURAS E SIGLAS

ΔΨm	Potencial de membrana
$[Ca^{2+}]$ cyt	Concentração de cálcio citosólico
ASC	Acid Soluble Collagen
BApNA	Bezoil-arginil p-nitro-anilida
BET	Brunauer, Emmet, Teller - Medida da área superficial
BJH	Barret, Joyer e Halenda - Medida do tamanho de poro
CFSE	carboxyfluorescein diacetate 5-ester N-succinimidyl ester
CBA	Cytometric bead array
DD%	Degree of deacetylation
DSC	Differential scanning calorimetry
DPPH	α,α-diphenyl-β-picrylhydrazyl
ECAU	Ethics Committee on Animal Use
ERO	Espécie Reativa de Oxigênio
FAO	Food and Agriculture Organization of the United Nations
FITC	Fluorescein Isothiocyanate
FNT α	Fator de Necrose Tumoral α
FT-IR	Fourier-transform infrared spectroscopy
FvW	Fator de von Willebrand
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
IBGE	Instituto Brasileiro de Geografia e Estatística
IC50	Half maximal Inhibitory Concentration
IFN-γ	Interferon-gamma
IGF	Insulin-like Growth Factor
IL-1	Interleucina 1
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-8	Interleucina 8
IL-10	Interleucina 10
IL-17	Interleucina 17
MEC	Matriz extracelular
MEV	Microscopia Eletrônica de Varredura

MPA	Ministério de Pesca e Aquicultura
NO	Nitric oxide
OMS	Organização Mundial de Saúde
PARs	Proteases acopladas à proteína G
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PIB	Produto Interno Bruto
PSTI	Pancreatic Secretory Trypsin Inhibitor (Inibidor Pancreático de Tripsina)
RBC	Red blood cells
SDS-PAGE	Eletroforese em Gel de Poliacrilamida com SDS
SEM	Scanning electron microscope
TG	Termogavimetry
TGF-α	Transforming Growth Factor α
TGF-β	Transforming growth factor β
TNF-α	Tumor Necrosis Factor-α

SUMÁRIO

1 INTRODUÇÃO

Subprodutos do processamento do pescado, como cabeças, vísceras, peles, ossos e escamas, têm ganhado considerável atenção nos últimos anos por seu potencial como fonte de moléculas bioativas. Por serem considerados de baixo valor e um problema às indústrias pesqueiras para descartá-los, esses materiais ainda encontram resistência por parte dos produtores em serem contabilizados como co-produtos, visto que podem representar até 70% do pescado produzido e serem em grande parte não comestíveis. Há duas décadas tem havido um aumento da conscientização em relação aos aspectos econômicos, sociais e ambientais, com a finalidade de otimizar o uso destes subprodutos (FAO, 2014).

Biopolímeros naturais, como proteínas e polissacarídeos, com atividade biológica, podem ser usados em diversos dispositivos biomédicos, uma vez que normalmente esses compostos apresentam características de biocompatibilidade, além de baixa toxicidade e biodegradabilidade, propriedades desejáveis para promover a regeneração tecidual, por exemplo. Por isso, biomateriais têm sido cada vez mais empregados para auxiliar a cicatrização de feridas. Além disso, algumas moléculas têm também um potencial confirmado para engenharia de tecidos, como um agente terapêutico promissor que favoreça a ligação entre células em cultivo e *in vivo*, e como polímeros bioativos que propiciam o microambiente adequado para substituição de pele em feridas extensas e queimaduras (GUATAM et al., 2014; NWE; FURUIKE; TAMURA, 2010). Dentre os biopolímeros naturais mais utilizados com este propósito, destacam-se os polissacarídeos, como a quitina e a quitosana, e diferentes tipos de proteínas, como o colágeno e a gelatina (CHANDIKA; KO; JUNG, 2015).

Os biopolímeros de pescado podem representar interessantes substitutos para aqueles normalmente obtidos de mamíferos, permitindo que a exploração destes produtos seja mais sustentável e capaz de fornecer materiais mais seguros do ponto de vista sanitário. Questões religiosas e algumas práticas alimentares e de estilo de vida impedem o consumo de materiais oriundos de mamíferos, e os produtos à base de pescado podem servir ainda como uma alternativa viável para determinadas aplicações.

Os organismos aquáticos vivem num meio ambiente rico em micronutrientes e muitas vezes acumulam diversos tipos de moléculas bioativas, que variam grandemente em concentração e potencial de aplicação. O uso desses organismos como fonte de biomateriais,

principalmente pela utilização avançada de partes não comestíveis e co-produtos, alia a preservação do ambiente e a exploração de novas fontes de materiais biocompatíveis.

A utilização de subprodutos do processamento do pescado para a recuperação de biomoléculas como carotenóides (SANTOS, 2006), colágenos (HWANG et al., 2007) e enzimas (BEZERRA et al., 2005; ESPÓSITO, 2009 ASSIS et al., 2007) tem sido reportada, com várias aplicações. Diversas proteases foram extraídas de vísceras de peixes mostrando características interessantes para aplicações na biotecnologia e indústria. A tripsina, por exemplo, é uma serino protease que pode ser utilizada na cicatrização de lesões cutâneas, uma vez que é capaz de auxiliar na coagulação sanguínea, na lise do coágulo de fibrina e transporte de proteínas de membrana (RAO et al., 1998). Ademais, estudos mostram a participação da tripsina, juntamente com a trombina, na clivagem e ativação de proteases acopladas à proteína G (PARs), que contribuem para a defesa do corpo em resposta à inflamação e invasão de patógenos (VERGNOLLE et al., 2001).

A quitosana é um polissacarídeo obtido comercialmente de carapaças de crustáceos a partir da desacetilação alcalina da quitina, e possui características favoráveis à sua utilização em diferentes setores, por ser biodegradável, biocompatível, hidrofílica, possuir baixa toxicidade e ser mecanicamente estável. Sua aplicação estende-se a abordagens biotecnológicas e biomédicas, como por exemplo, na engenharia de tecidos e imobilização de proteínas (XI, 2005; DALLAN, 2005; KIM & RAJAPAKSE, 2005; MUZZARELLI, 2009; JAYAKUMAR et al., 2011; SAHOO, 2009).

O colágeno é também um biomaterial utilizado para obtenção de filmes, imobilização de proteínas e carreamento de fármacos (CHATTOPADHYAY & RAINES, 2014). Esta proteína faz parte da matriz celular e é um dos componentes mais importantes na estrutura do tecido, representando cerca de 50 % das proteínas presentes na pele. O colágeno possui estrutura composta por uma única configuração tripla-hélice de três subunidades polipeptídicas, conhecidas como cadeia-α, e está compreendida numa família que inclui 29 tipos de colágeno a partir de uma grande variedade de tecidos animais, sendo mais abundante, em vertebrados, o colágeno do tipo I (NELSON & COX, 2014).

Em virtude de sua ubiqüidade, baixa imunogenicidade e capacidade de ser moldado em estruturas fortes e biocompatíveis, desempenha um papel importante no tratamento de feridas. Filmes incluindo colágeno como componente são comumente usados, dada a importância desta proteína, na remodelação de tecidos, no auxílio para atração de novas células (CHATTOPADHYAY & RAINES, 2014; FONDER et al., 2008).

Estas características, somadas ao grande interesse da indústria farmacêutica em expandir a diversidade de novos medicamentos naturais, reduzindo os custos de obtenção e efeitos sobre o meio ambiente, além de agregar valor aos descartes da indústria pesqueira, permite uma abordagem relevante para o desenvolvimento de tratamentos alternativos de lesões cutâneas.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Caracterizar o potencial cicatrizante de filmes produzidos a partir de quitosana, colágeno e tripsina oriundos de resíduos do processamento do pescado.

1.1.2 Objetivos específicos

- Purificar uma tripsina das vísceras do peixe tilápia do nilo;- preparar filmes à base de quitosana de camarão, colágeno e tripsina de peixe;
- Avaliar as propriedades físicas e químicas dos filmes;
- Avaliar a citotoxicidade das células pbmc estimuladas pela solução filmogênica de quitosana e colágeno e filmes de quitosana/colágeno;
- Aplicar os filmes chi/col e filmes de quitosana/colágeno/tripsina no tratamento de lesões cutâneas em ratos;
- Avaliar a eficácia da cicatrização promovida pelos filmes sob o ponto de vista pré-clínico e histológico.

2 REVISÃO BIBLIOGRÁFICA

2.1 ESTRUTURA DA PELE E LESÕES DÉRMICAS

A pele representa o maior e mais externo órgão do corpo humano, totalizando, aproximadamente, 16% do peso corporal total (ROGER et al., 2019). Este órgão realiza diversas funções importantes, especialmente como barreira protetora ao corpo, protegendo-o da ação de agentes externos (como microrganismos patogênicos), além de impedir a perda excessiva de líquidos, manter a temperatura corpórea e sintetizar vitamina D com a exposição aos raios solares (BAJAY; JORGE; DANTAS, 2003; HAMEED et al., 2019).

A pele é um órgão complexo que apresenta três camadas distintas: epiderme, derme e hipoderme (figura 1) (AMBEKAR & KANDASUBRAMANIAN, 2019). A epiderme é um epitélio queratinizado estratificado, composto de queratinócitos, melanócitos, células de Langerhans e Merkel, que ajuda a manter a temperatura corporal e está subdividida em cinco camadas: basal, espinhosa, granular, lúcida e cornificada (ROGER et al., 2019; TSUTSUMI & DENDA, 2007). A função de barreira da pele é principalmente atribuída ao estrato córneo, que apresenta entre 10 e 20 camadas compostas de queratinócitos achatados, separados por camadas de lipídios densamente compactadas (MENON et al., 2012).

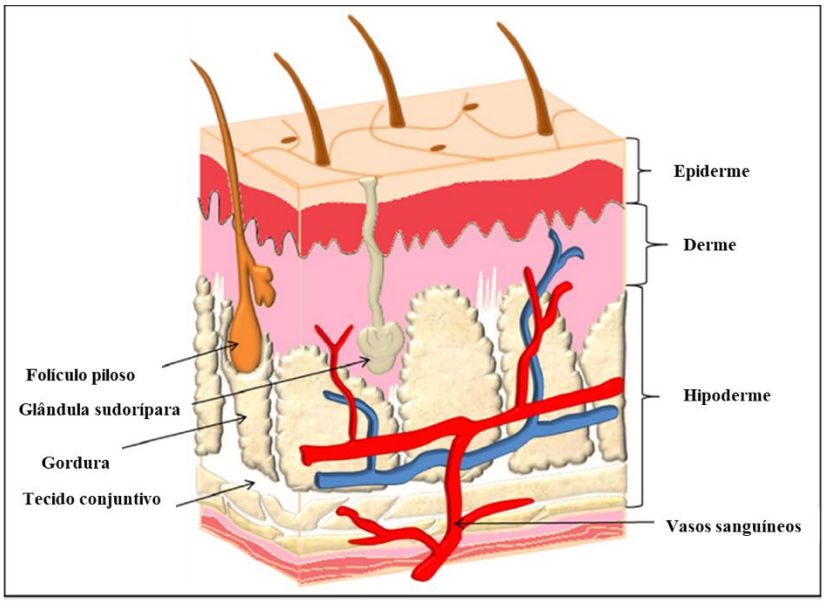
A derme fornece um suporte essencial à epiderme, provendo amortecimento ao corpo devido ao estresse e à tensão externos, e, ao contrário da epiderme, a derme apresenta sistemas vasculares e linfáticos que a permeiam junto aos apêndices, como folículos capilares, terminações nervosas e glândulas secretoras (ROGER et al., 2019). O principal tipo de célula encontrado na derme é o fibroblasto, responsável pela produção dos componentes da matriz extracelular (MEC), como colágeno, fibronectina e elastina, responsáveis pela elasticidade e resistência à tração que a pele possui (ROGER et al., 2019). A junção dermo-epidérmica separa a derme e a epiderme e facilita a troca de substâncias (MUROYAMA & LECHLER, 2012).

A hipoderme não é um segmento da pele, mas um tecido subcutâneo que se encontra abaixo da derme, constituída por fibroblastos, macrófagos e adipócitos. Sua principal função é o auxílio na vinculação da derme com ossos e músculos (AMBEKAR & KANDASUBRAMANIAN, 2019).

A integridade da pele é fundamental para a manutenção da homeostase fisiológica do corpo humano (GASPAR-PINTILIESCU; STANCIUC; CRACIUNESCU, 2019). Uma ferida na pele representa uma interrupção da continuidade do tecido e pode se apresentar em maior

ou em menor extensão, como resultado de qualquer tipo de trauma físico, químico, mecânico ou desencadeada por algum procedimento cirúrgico, que aciona as frentes de defesa do organismo para contra-atacar (BLANES et al, 2004).

Figura 1. Estrutura da pele humana com sua delimitação pelas camadas epiderme, derme e hipoderme, além dos anexos, vasos sanguíneos e tecido conjuntivo adjacente.



Fonte: AMBEKAR & KANDASUBRAMANIAN, 2019.

As lesões cutâneas podem ser classificadas em diferentes tipos, a depender do local de sua ocorrência, da sua aparência e da duração do processo de reparo (quadro 1) (GASPAR-PINTILIESCU; STANCIUC; CRACIUNESCU, 2019). Dentre as classificações das lesões, destacam-se os tipos relacionados à duração do processo de reparo: as lesões agudas e as lesões crônicas.

Feridas agudas são geralmente lesões teciduais que cicatrizam completamente, com cicatrizes mínimas e dentro de um prazo de 8 a 12 semanas. As principais causas de feridas agudas incluem lesões mecânicas devido a fatores externos, como escoriações e cortes. As lesões mecânicas também incluem feridas penetrantes e feridas cirúrgicas causadas por incisões (PERCIVAL, 2002). Outros exemplos de feridas agudas incluem queimaduras e lesões químicas, que surgem de uma variedade de fontes, como radiação, eletricidade e produtos químicos corrosivos (BOATENG & CATANZANO, 2015).

Feridas crônicas, no entanto, surgem de lesões teciduais que cicatrizam lentamente (normalmente não curam dentro de 12 semanas) e podem reaparecer (GURTNER et al., 2008). A lesão crônica muitas vezes é desencadeada pela falta de adaptação a respostas das

células imunes à cicatrização, o que pode estar relacionado a condições fisiológicas subjacentes, como o diabetes (MOORE et al., 2006; VIDMAR, CHINGWARU, CHINGWARU 2016).

As feridas crônicas podem ser facilmente contaminadas por agentes patogênicos e geralmente envolvem perda significativa de tecido, o que pode afetar estruturas vitais, como ossos, articulações e nervos. Além disso, a falha na cicatrização destas lesões pode levar a uma produção excessiva de exsudatos (fluido que contém vários componentes, como água, eletrólitos, nutrientes, mediadores inflamatórios, entre outros compostos, que permitem o processo natural de cicatrização), o que também pode comprometer o tecido saudável ao redor da ferida, elevando o nível da dor e o tempo de cicatrização (BOATENG & CATANZANO, 2015; LIMA et al., 2019).

Quadro 1. Classificação das lesões teciduais.

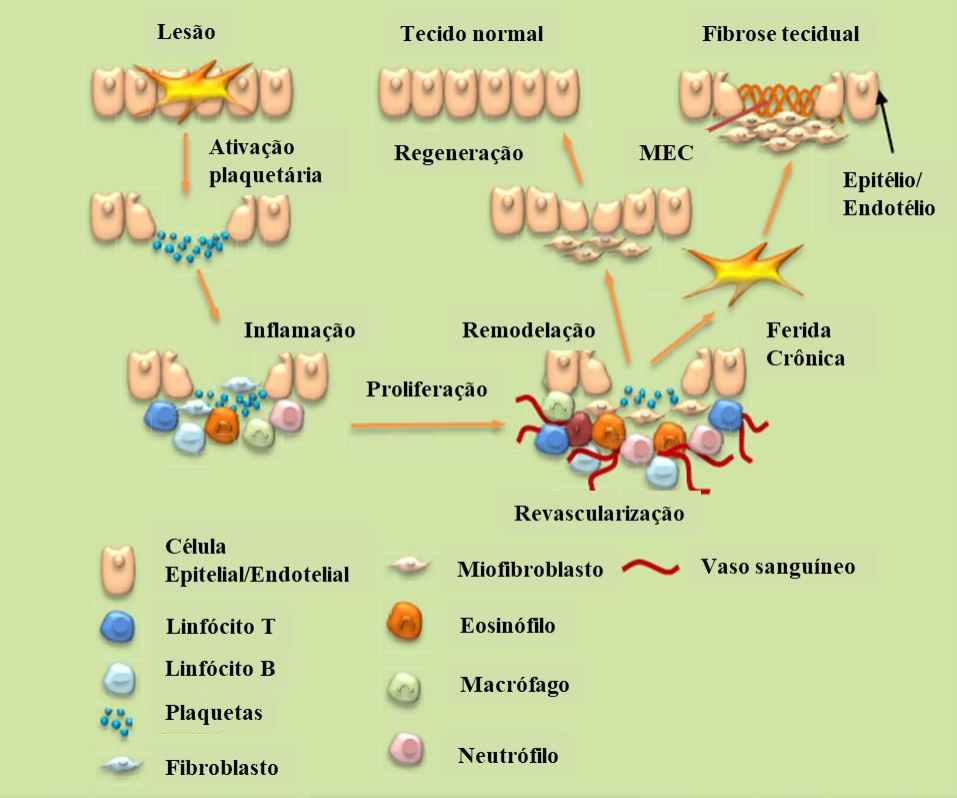
Critérios	Tipo de lesão	Características	Referências
Aparência	Necrótica	Acúmulo de tecido morto	BOATENG et al., 2008
	Escara	Tecido necrótico úmido e grosseiramente reidratado	
	Granulação	Tecido de granulação abundante com produção excessiva de exsudato.	
	Epitelização	Associada à formação da nova epiderme.	
	Infectada e fétida	Contaminação com microrganismos, inflamação e formação de pus.	
Processo de reparo	Aguda	Lesões teciduais que geralmente curam entre 8 a 12 semanas; causada por fatores mecânicos, químicos ou queimaduras.	PERCIVAL, 2002
	Crônica	Atraso na cicatrização devido a condições fisiológicas subjacentes (como o diabetes), com grande produção de exsudato, o que leva à maceração de tecido cutâneo saudável.	HAN & CEILLEY, 2017
Área afetada	Superficial	Afeta a superfície epidérmica da pele.	CROUZET et al., 2015
	Espessura parcial	Afeta a epiderme e as camadas dérmicas internas.	
	Espessura completa	Afeta a epiderme, a derme e o tecido subcutâneo subjacente.	

Fonte: GASPAR-PINTILIESCU, STANCIUC, CRACIUNESCU, 2019.

2.2 MECANISMOS BIOLÓGICOS DE CICATRIZAÇÃO DA PELE

O processo de cicatrização ocorre para restaurar a integridade anatômica e funcional do tecido. Trata-se de um mecanismo complexo baseado numa série de eventos bioquímicos e celulares (intra e intercelulares) sincronizados, que resulta da resposta tecidual à lesão com o propósito de regenerá-la (LIMA et al., 2019; XU et al., 2016; VIDMAR, CHINGWARU, CHINGWARU, 2016). Durante a cicatrização, uma sequência de eventos ocorre em cascata, levando a diferentes estágios do processo: hemostasia, inflamação, proliferação e remodelação (REINKE & SORG, 2012) (figura 2).

Figura 2. Modelo geral dos estágios do mecanismo de cicatrização tecidual com apresentação das principais células envolvidas neste processo.



Fonte: VIDMAR; CHINGWARU; CHINGWARU, 2016.

Hemostasia

A hemostasia é uma resposta instantânea à lesão para interromper a perda de sangue, através da coagulação sanguínea e ativação plaquetária, com formação de um tampão temporário (AMBEKAR & KANDASUBRAMANIAN, 2019). A hemostasia é um processo que envolve uma infinidade de receptores extravasculares que atuam em conjunto com os

componentes do sangue (VIDMAR; CHINGWARU; CHINGWARU, 2016). A primeira resposta à lesão da pele ocorre pelo acúmulo de trombócitos e células inflamatórias que se ligam ao colágeno na MEC (AMBEKAR & KANDASUBRAMANIAN, 2019).

O trombócito é capaz de secretar proteínas, como trombospondina, fator de von Willebrand (FvW) e fibronectina, além de fatores de crescimento como fator de crescimento da insulina (IGF), fator de crescimento derivado de plaquetas (PDGF), interleucina 1 (IL-1) e fatores de crescimento transformadores (TGF-α, TGF-β), que auxiliam nos estágios pós-hemostasia da cicatrização da ferida (BIELEFELD; AMINI-NIK; ALMAN, 2013).

A liberação de fatores de coagulação estimula a deposição da matriz de fibrina para garantir a estabilidade do tampão plaquetário (BIELEFELD, AMINI-NIK, ALMAN, 2013). Este tampão estabelece um ambiente adequado para a deposição de fibroblastos, leucócitos, queratinócitos e células endoteliais e atua como um fator de crescimento (AMBEKAR & KANDASUBRAMANIAN, 2019).

Inflamação

A inflamação auxilia na atração de leucócitos para a lesão, ativando vários mediadores e fatores quimiotáticos entre 24 e 48 horas pós-lesão, e são necessários três dias para completar a fase inflamatória. Durante esta fase, os mastócitos liberam fragmentos cheios de enzimas, como a histamina, que também atua como importante mediadora para a dilatação dos vasos sanguíneos. Um dos sinais externos da fase inflamatória é o inchaço (ou edema) da ferida causado pelo acúmulo de líquido no local da lesão (AMBEKAR & KANDASUBRAMANIAN, 2019).

O trombócito libera os sinais químicos que são responsáveis pela atração de neutrófilos ao local da ferida e sua adesão às células endoteliais (EMING; KRIEG; DAVIDSON, 2007). Os neutrófilos, por sua vez, participam do processo inflamatório eliminando, através da fagocitose, corpos estranhos, como patógenos, células mortas e componentes da matriz danificada (BIELEFELD; AMINI-NIK; ALMAN, 2013).

Na fase de inflamação, os macrófagos, o fator de necrose tumoral-α (TNF-α), o fator de transformação do crescimento-β (TGF-β) e o fator de crescimento derivado de plaquetas (PDGF), além de citocinas, como as IL-1 e IL-6, produzem fator de crescimento que ajuda, posteriormente, no estágio pós-inflamação na proliferação de fibroblastos e de células endoteliais (AMBEKAR & KANDASUBRAMANIAN, 2019). Por fim, os macrófagos fagocitam os neutrófilos e a depleção de células inflamatórias mostra o início do estágio de proliferação (BIELEFELD; AMINI; ALMAN, 2013).

Proliferação

A formação de tecido de granulação é o principal evento da fase proliferativa. O tecido de granulação é constituído de células inflamatórias, fibroblastos e neovascularização em uma matriz de fibronectina, colágeno, glicosaminoglicanos e proteoglicanos. A formação de tecido de granulação ocorre de 3 a 5 dias após a lesão e se sobrepõe à fase inflamatória anterior (SCHOUKENS, 2019).

Após sete dias, a MEC acumulada ainda estará servindo de apoio para a migração de células. O colágeno é a proteína estrutural essencial para todos os estágios de cicatrização de feridas, mas desempenha um papel dominante, especialmente, nos estágios de proliferação e remodelação (RAMASASTRY, 2005). A angiogênese, restauração de novas arteríolas no local da ferida pelas células endoteliais, começa com a ligação das células endoteliais dos vasos existentes a fatores de crescimento com a ajuda de seus receptores, e é uma etapa muito importante, pois através do desenvolvimento das arteríolas recém-formadas haverá fornecimento de nutrientes e oxigênio ao local da lesão e às novas células (KIWANUKA; JUNKER; ERIKSSON, 2012).

A proliferação de células endoteliais é facilitada quando a lâmina basal é desfeita pela ação de enzimas proteolíticas das células endoteliais ativadas. Esse processo é conhecido como brotação e esses brotos recém-formados formam canais interconectados. As células e pericitos do músculo liso auxiliam na estabilização das paredes dos vasos, e uma vez iniciado o fluxo sanguíneo, a angiogênese é concluída (SORG; KRUEGER; VOLLMAR, 2007).

As citocinas inflamatórias estimulam o processo de reepitelização, pois o fibroblasto produz fatores de crescimento, como o fator de crescimento de hepatócitos, o fator de crescimento de queratinócitos e o fator de crescimento epidérmico, que atrai os queratinócitos para migrar para o local da ferida e proliferar formando uma cobertura de células sobre o leito da lesão (AMBEKAR & KANDASUBRAMANIAN, 2019). Ademais, os fibroblastos se combinam com células musculares lisas (os miofibroblastos) para fechar a lesão, puxando as bordas da ferida para a sua contração (GIBRAN; BOYCE; GREENHALGH, 2007).

Remodelação

A remodelação consiste na formação de um novo epitélio sobre o local da lesão, onde as novas células começam a migrar para as bordas da ferida. Essa camada epitelial fornece uma vedação entre a ferida subjacente e o ambiente. As células epidérmicas nas bordas da ferida sofrem alterações estruturais, permitindo que elas se separem de suas conexões com outras células epidérmicas e sua membrana basal (SCHOUKENS, 2019).

A remodelação é o estágio final da cicatrização de feridas, que começa a partir de duas semanas após a lesão e pode durar até um ano. Todos os processos iniciados nos estágios de inflamação e proliferação terminarão no estágio de remodelação (SINGH; YOUNG; MCNAUGHT, 2017). As glândulas sudoríparas e os folículos capilares não têm capacidade de cicatrizar após sérios danos à ferida. Macrófagos, células endoteliais e miofibroblastos saem do local da ferida e os demais sofrem apoptose (AMBEKAR & KANDASUBRAMANIAN, 2019).

A atividade metabólica da cicatrização de feridas sofre um declínio à medida que as pequenas arteríolas se agregam em vasos sanguíneos maiores. A MEC também sofre alterações, como a substituição do colágeno reticular (tipo III) produzido no estágio de proliferação pelo colágeno fibrilar (tipo I) (AMBEKAR & KANDASUBRAMANIAN, 2019). Finalmente, a ferida é reparada via apoptose e ocorre a migração das células do local da ferida e degradação da MEC por metaloproteinases da matriz (CALEY; MARTINS; O'TOOLE, 2015).

Para que os processos cicatriciais ocorram corretamente é necessária a manutenção de um ambiente com condições reguladas, como teor de umidade e temperatura adequados, bem como a não contaminação por agentes externos e microrganismos (CHEN et al., 2017; NAPAVICHAYANUN et al., 2015). Além disso, o ambiente deve fornecer quantidades adequadas de oxigênio, nutrientes e outros compostos relevantes (WANG & WINDBERGS, 2017; SCALISE et al., 2015).

2.3 TERAPIA TÓPICA PARA O TRATAMENTO DE LESÕES DA PELE

O principal objetivo das terapias para lesões é inibir ou matar microrganismos patogênicos que possam causar infecções, e acelerar o processo de cicatrização da ferida com redução das cicatrizes e da dor para os pacientes (SHANMUGAPRIYA & KANG, 2019).

Dentre as formas farmacêuticas convencionais utilizadas para a veiculação de fármacos por via tópica estão as preparações semissólidas (pomadas, emulsões, géis, cremes) e as soluções. Para o tratamento de pequenas feridas causadas por queimaduras existe ainda a possibilidade de utilizar sistemas convencionais transdérmicos (adesivos), entretanto, desvantagens como dor intensa ao colocar ou retirar o adesivo, danos ao processo de cicatrização da ferida no momento da remoção do adesivo e irritação do local de aplicação fazem com que sejam pouco utilizados (OSHIRO JUNIOR; SHIOTA; CHIAVACCI, 2014).

As coberturas, materiais ou produtos utilizados para tratar ou ocluir a ferida, impregnados ou não com agentes tópicos, podem ser classificadas como primárias, quando colocadas diretamente sobre a lesão, ou secundárias, quando têm como função cobrir as coberturas primárias. Inúmeros estudos têm sido realizados com o intuito de desenvolver uma metodologia para obtenção de curativos que reduzam os efeitos da contaminação nas lesões, favoreçam o processo cicatricial e ofereçam melhores resultados estéticos (LIMA-JÚNIOR et al., 2017).

Materiais formadores de filmes representam uma alternativa aos sistemas convencionais e apresentam a vantagem de tornar o tratamento mais confortável e funcional, uma vez que são transparentes, permitindo a visualização da ferida, podem ser aplicados em feridas de maior extensão e podem ser removidos mais facilmente (OSHIRO JUNIOR; SHIOTA; CHIAVACCI, 2014).

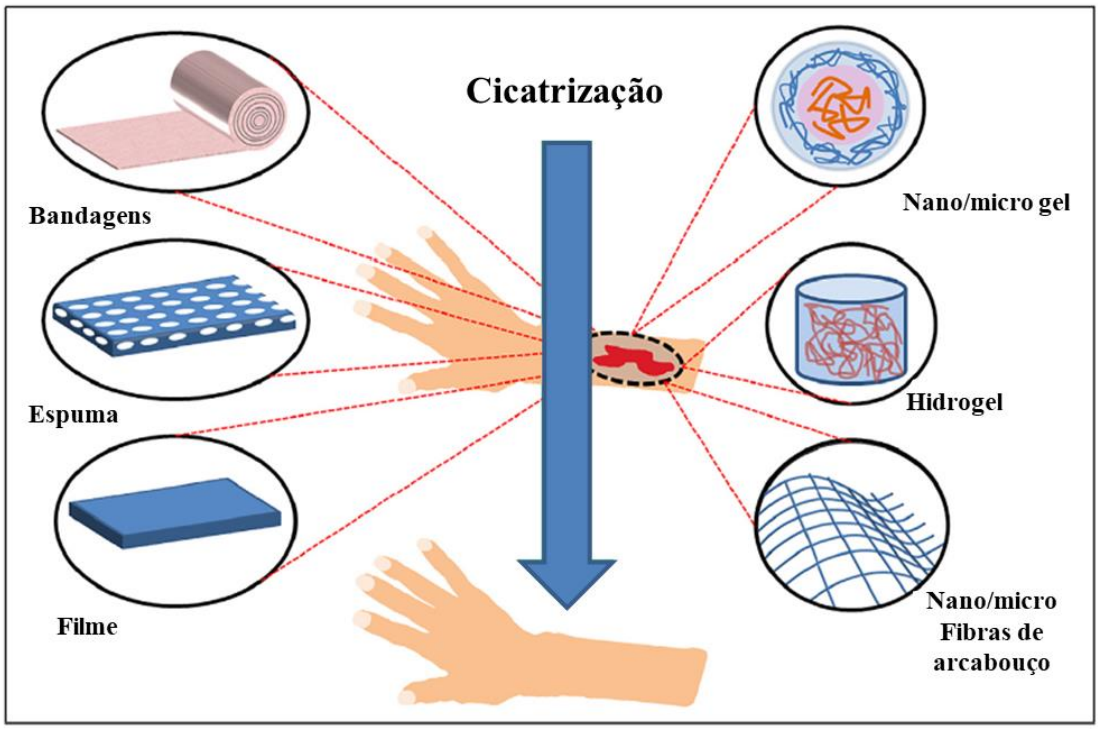
2.4 CURATIVOS

Os curativos são mais comumente utilizados no tratamento de feridas devido às limitações dos enxertos (doador, complicações cirúrgicas, rejeição, contração e instabilidade) e cultura de células autólogas (o longo tempo necessário para cultivar e preparar as células) (DREIFKE; JAYASURIYA; JAYASURIYA, 2015).

Um curativo ideal para feridas deve se aderir ao tecido lesionado, manter equilibradas as condições de umidade, permitir a troca de oxigênio e de fluidos da lesão e protegê-la contra infecções bacterianas, garantindo assim um microambiente ideal para acelerar o processo de cicatrização (DHIVYA; PADMA; SANTHINI, 2015). Atualmente, os curativos para pele são classificados como curativos temporários e substitutos de pele permanentes e subdivididos ainda de acordo com o material que os compõem (figura 3) (AMBEKAR & KANDASUBRAMANIAN, 2019; DHIVYA; PADMA; SANTHINI, 2015).

Os curativos existentes e comercializados podem se apresentar como diferentes materiais (sintéticos ou naturais) e com várias formas físicas (esponjas, hidrogéis, hidrocolóides, filmes, membranas). Estas diferentes formulações têm propriedades distintas que as tornam adequadas para o tratamento de um tipo particular de ferida. Por exemplo, esponjas exibem uma enorme porosidade, fornecem isolamento térmico e sustentam a umidade no local da ferida. No entanto, são mecanicamente fracas, podem provocar maceração da pele e são inadequadas para o tratamento de queimaduras de terceiro grau ou feridas com escara seca (DHIVYA; PADMA; SANTHINI, 2015).

Figura 3. Classificação dos principais tipos de curativos, utilizados para aceleração e eficácia do processo cicatricial, de acordo com o material componente.



Fonte: AMBEKAR & KANDASUBRAMANIAN, 2019.

Por outro lado, os hidrogéis são caracterizados por sua capacidade de armazenar grandes quantidades de água, o que lhes permite proporcionar um ambiente úmido à ferida. Porém, os hidrogéis exibem fracas propriedades mecânicas, exigindo um curativo secundário (CALÓ & KHUTORYANSKIY, 2015; AHMED, 2015). Ademais, alguns dos hidrocolóides podem ser citotóxicos, apresentar odor desagradável, baixa estabilidade mecânica e manter um pH ácido no local da ferida (KAMOUN; KENAWY; CHEN, 2017; POTT et al., 2014).

Por sua vez, sabe-se que as membranas agem como barreiras físicas e também reproduzem a arquitetura da MEC. Além disso, seus poros são cruciais para garantir a proliferação celular, as trocas gasosas, o suprimento de nutrientes e o controle da saída de fluidos. As principais desvantagens associadas ao uso da membrana são originadas pelos materiais e solventes utilizados em sua produção (BHATTARAI et al., 2004; ZAHEDI et al., 2010).

O curativo deve ser cuidadosamente escolhido de modo a evitar traumas secundários e danos quando mudanças no curativo são necessárias (HAYES & SU, 2011). Na clínica, o

método terapêutico comum para lesões dérmicas extensas é o curativo e o tratamento com antibióticos em altas doses (ANJANA et al., 2017; LIPSKY & HOEY, 2009).

No entanto, as pequenas lesões, como arranhões, cortes e queimaduras mais brandas, geralmente, não são consideradas graves pela maioria das pessoas, que acabam por não buscar ajuda médica para o auxílio da cicatrização destas feridas (KUHLMANN, 2019). Os pacientes deixam estas lesões sem qualquer atendimento médico ou as tratam com curativos de primeiros socorros (algodão, ataduras e gazes), que somente fornecem uma barreira contra sujeira, contaminação bacteriana e fatores mecânicos, mas não podem proporcionar uma troca gasosa da lesão com o ambiente nem fornecer um microambiente úmido, o que dá origem à desidratação da ferida e induz a um trauma após a remoção (DENG, 2019; RICHARDSON, 2004).

Um dos principais cuidados que deve ser levado em consideração para a cicatrização de lesões, é deixa-la livre da ação de microrganismos patogênicos, pois a infecção da ferida pode retardar o processo cicatricial. Em um ser humano saudável, a infecção é evitada por ativação do sistema imunológico para abolir os patógenos invasores. Nesse processo, os macrófagos iniciam a migração para o local da ferida e, em seguida, realizam a fagocitose dos patógenos. Em um estágio posterior da infecção, a resposta imune é realizada pela ativação dos linfócitos T auxiliares que secretam interferon-γ e ligante CD40 para coordenar a resposta imune adaptativa e humoral para matar e remover as bactérias invasoras (ANDONOVA & URUMOVA, 2013).

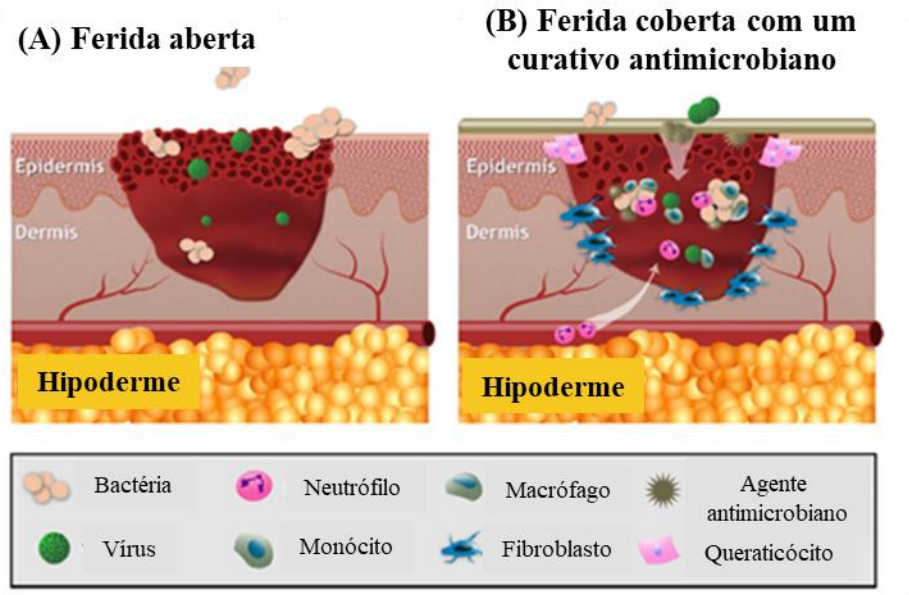
No entanto, uma vez que o sistema imunológico não esteja em condições de realizar a remoção de agentes patogênicos, a infecção pode ocorrer, causando a deterioração do tecido de granulação, de fatores de crescimento e componentes da MEC (colágeno, elastina e fibrina), comprometendo o processo normal de cicatrização da ferida (figura 4) (HAN & CEILLEY, 2017; SIDDIQUI & BERNSTEIN, 2010).

Para conseguir a inibição da atividade e/ou crescimento de microrganismos sobre a lesão, diferentes abordagens envolvendo materiais com atividade antimicrobiana intrínseca, superfície modificada ou incorporação de agentes antimicrobianos estão sendo usadas para produzir curativos (VIG et al., 2017).

Geralmente, a maioria das feridas infectadas é contaminada por patógenos encontrados nos arredores do ambiente, isto é, microrganismos endógenos que vivem nas membranas mucosas e pela microflora disponível na pele adjacente (SARHEED et al., 2016). Nos estágios iniciais da formação da ferida predominam os organismos gram-positivos, especificamente *Staphylococcus aureus*. Nos estágios posteriores, são observadas espécies

gram-negativas, como a *Escherichia coli*, que tendem a invadir camadas mais profundas da pele, causando danos significativos aos tecidos (CARDONA & WILSON, 2015).

Figura 4. Representação do processo de cicatrização em uma ferida aberta sem qualquer proteção (A) e uma ferida coberta por um curativo com propriedades ou agentes antimicrobianos (B).



Fonte: SIMÕES et al., 2018.

Alguns agentes antimicrobianos têm sido empregados em curativos, são eles: antibióticos (por exemplo, tetraciclina, ciprofloxacina, gentamicina e sulfadiazina), nanopartículas (por exemplo, nanopartículas de prata) e produtos naturais (por exemplo, mel, óleos essenciais e quitosana) (SARHEED et al., 2016).

Além da atividade antimicrobiana, também é importante que os curativos apresentem atividade biológica por si só ou liberem constituintes bioativos (medicamentos) incorporados a eles. Os medicamentos incorporados podem desempenhar um papel ativo no processo de cicatrização de feridas diretamente, como limpeza ou debridamento do tecido necrótico (BOATENG & CATANZANO et al., 2015).

O uso tópico de agentes bioativos na forma de soluções, cremes e pomadas, para a administração de drogas na ferida não é muito eficaz, pois eles absorvem rapidamente o fluido e, no processo, perdem sua capacidade reológica e tornam-se móveis (BOATENG et al., 2008). Por esse motivo, o uso de curativos sólidos é preferível, especialmente para feridas com grande produção de exsudato, pois proporcionam melhor controle deste líquido e se estabelecem melhor e por mais tempo no local da lesão, ao contrário dos curativos

tradicionais, como gaze e algodão, que não atuam ativamente (BOATENG & CATANZANO et al., 2015).

Os curativos temporários devem fornecer absorção do exsudato e proteção da ferida até o seu fechamento, enquanto a nova pele que está sendo formada se integre à pele antiga, acelerando o processo de regeneração (GASPAR-PINTILIESCU; STANCIUC; CRACIUNESCU, 2019).

2.5 POLÍMEROS NATURAIS PARA OBTENÇÃO DE CURATIVOS

Polímeros naturais, sintéticos ou bioativos podem ser obtidos de plantas, bactérias, fungos, ou fontes animais e são comumente usados devido à sua biocompatibilidade e biodegradabilidade (quadro 2). Um número crescente de materiais poliméricos com boa biocompatibilidade tem sido aplicado no preparo de curativos para feridas, como colágeno (FRANK et al., 2018; MUTHUKUMAR et al., 2014), quitosana (DEBONE et al., 2018; KHAN & MUJAHID, 2018), ácido hialurônico (YAMAMOTO et al., 2013), pectina (CHEN et al., 2017) e poliuretano (LIU et al., 2018). Esses curativos de biopolímero mantêm uma barreira protetora na superfície da ferida, beneficiam a remoção do excesso de exsudato e reduzem a dor, estabelecendo-se como curativos inovadores emergentes (ALJGHAMI et al., 2019; PATRULEA et al., 2015).

Alguns polímeros são utilizados como biomateriais podendo ser aplicados em componentes implantados no corpo humano, para a substituição de partes danificadas ou doentes. No entanto, para serem utilizados com este fim, os polímeros devem apresentar não toxicidade e compatibilidade com os tecidos do corpo, além de não promover reações biológicas adversas (ORÉFICE; PEREIRA; MANSUR, 2006).

Para uso no tratamento de feridas, os polímeros devem apresentar várias funções, como proteção contra infecções, diminuir o acúmulo de exsudatos e adsorção de tecido necrótico, manutenção do ambiente úmido, permeabilidade ao oxigênio e promoção do processo de cicatrização (OH & LEE, 2019).

Até agora, várias tecnologias têm sido aplicadas para preparar biomateriais com propriedades físicas e mecânicas equivalentes ou superiores em comparação com polímeros à base de petróleo, vendidos comercialmente (NETRAVALI; HUANG; MIZUTA, 2007). Membranas formadas de polissacarídeos e polímeros hidrofílicos naturais ou sintéticos vêm sendo constantemente estudadas para aplicações em engenharia de tecidos, como filmes para fins variados, plásticos biodegradáveis, biomateriais e hidrogéis (BATISTA, 2015).

Quadro 2. Resumo dos principais tipos de polímeros usados na produção de curativos.

Tipos de polímeros	Polímeros	Vantagens
Naturais inertes	Carboximetilcelulose	Os polímeros naturais podem ser obtidos de fontes vegetais, bacterianas, fúngicas ou animais e são comumente usados devido à sua biocompatibilidade e biodegradabilidade.
	Celulose bacteriana	
	Fibroína de seda	
	Pectina	
	Carragena	
Sintéticos	Óxido de polietileno	Sua natureza hidrofílica que auxilia na capacidade de absorção de umidade e transmissão de vapor d'água, manutendo o ambiente da ferida úmido, evitando a coleta de exsudato em excesso. São geralmente adesivos, o que permite residência prolongada além de serem biocompatíveis e possuírem maior resistência mecânica que as naturais descritas acima.
	Álcool polivinil	
	Ácido lático poli-L	
	Polietilenoglicol	
	Poliuretano	
Naturais bioativos	Colágeno	São comumente usados por causa de sua biocompatibilidade e biodegradabilidade, mas mais importante, eles têm um efeito terapêutico ativo em um ou mais estágios da cicatrização de feridas. A maioria deles faz parte da matriz natural do corpo ou contém componentes que possuem atividade fisiológica como parte do processo natural de cicatrização.
	Gelatina	
	Ácido hialurônico	
	Quitosana	
	Alginato de sódio	

Fonte: BOATENG & CATANZANO, 2015.

A escolha do polissacarídeo para a formação de membranas, usadas como curativos, é importante, pois deve ser levada em consideração sua afinidade com o composto ativo de interesse e as propriedades mecânicas deste (NAGAR; CHAUHAN; YASIR, 2011). Por isso,

destacam-se a quitosana e o colágeno, que tem as características necessárias e favoráveis para a produção de membranas, especialmente para uso médico.

2.5.1 Quitosana e Colágeno como polímeros naturais filmogênicos

2.5.1.1 Quitosana

A quitosana é um polissacarídeo derivado N-desacetilado da quitina, o segundo polissacarídeo mais abundante na natureza, sendo encontrada em exoesqueleto de crustáceos, insetos e também em algumas espécies de fungos (CAHÚ et al., 2017; SCHOUKENS, 2019). A quitosana é principalmente produzida a partir de crustáceos, como caranguejos e lagostins, devido à grande disponibilidade dos exoesqueletos de crustáceos como subprodutos do processamento de frutos do mar (CRINI & BADOT, 2008; HAMED; ÖZOGUL; REGENSTEIN, 2016).

Uma das características mais importantes da quitosana é a sua versatilidade estrutural devido à presença de um grupo amino reativo e hidroxil na sua cadeia molecular (AHMED et al., 2018). A quitosana é solúvel em meio aquoso de pH ácido devido à presença de seus grupos amino que apresentam um valor de pKa de 6,3 (SCHOUKENS, 2019).

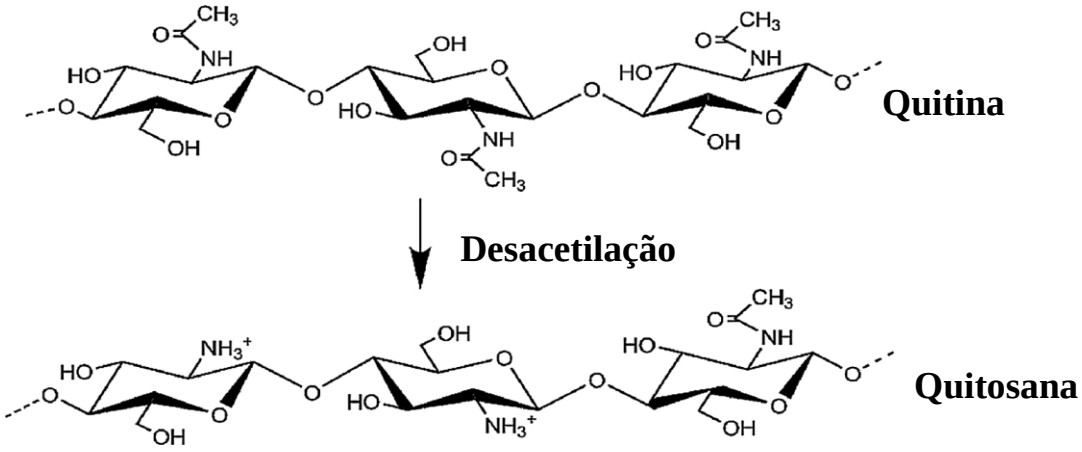
A estrutura da quitosana é composta por unidades de 2-amino-2-desoxi-β-D-glicose (D-glucosamina desacetilada) e N-acetil D-glucosamina β-1,4 (FAN et al., 2018). O mecanismo de desacetilação para transformar quitina em quitosana (figura 5), ocorre com eliminação de grupos acetis a partir do tratamento da quitina com hidróxido de sódio (NaOH) concentrado, deixando para trás apenas o grupo amina (NH_2) (AHMED; AHMAD; IKRAM, 2014; AHMED & IKRAM, 2016).

Diferentemente da fibra vegetal, a quitosana possui cargas iônicas, que lhe conferem a capacidade de se ligar quimicamente com gorduras, lipídios, colesterol, íons metálicos, proteínas e macromoléculas com carga negativa. Por isto, a quitosana e seus derivados têm alcançado um crescente interesse comercial como biomateriais devido às suas excelentes propriedades, incluindo biocompatibilidade, biodegradabilidade, adsorção, capacidade de formar filmes e quelar íons metálicos (SCHOUKENS, 2019).

As propriedades físicas, químicas e biológicas da quitosana, como solubilidade, cristalinidade, biodegradabilidade, viscosidade e biocompatibilidade dependem fortemente de seu grau de desacetilação e peso molecular (DASH et al., 2011; ISLAM; BHUIYAN; ISLAM, 2017). O grau de desacetilação da quitosana fornece a proporção de D-glicosamina em relação ao total de D-glicosamina e N-acetil-D glicosamina. A quitosana precisa ter um

grau de desacetilação maior que 60 %, o que também indica que a quitosana consiste em pelo menos 60 % de D-glicosamina (AVCU et al., 2018).

Figura 5. Estrutura da quitina e da quitosana e como elas se diferem após a desacetilação.



Fonte: HAMEDI et al., 2018.

A quitosana pode ser processada em diferentes formas, como membranas, nanogéis, filmes, micro/nanofibras, ataduras, micro/nanopartículas e hidrogéis, dependendo das propriedades e funções desejadas. Os filmes de quitosana têm sido utilizados principalmente para aplicações biomédicas e alimentares, como curativos/cicatrização de feridas, liberação de medicamentos e revestimento/embalagem de alimentos (AVCU et al., 2018).

A quitosana destaca-se como o polímero mais utilizado para fabricação de curativos para feridas à base de materiais poliméricos disponíveis no mercado, alguns exemplos são o HidroKi®, Axiostat®, Chitopack®, Tegasorb®, e KytoCel® (DREIFKE; JAYASURIYA; JAYASURIYA, 2015; SIMÕES et al., 2018). O amplo emprego da quitosana como cicatrizante é justificado pelo baixo custo de aquisição do polímero e, especialmente, porque a quitosana atua nas diferentes fases da cicatrização, como na fase de hemostasia, onde pode modular a ativação de plaquetas, promovendo a coagulação sanguínea *in vivo* (MUZZARELLI et al., 2007).

Na fase inflamatória, a quitosana pode regular a atividade das células inflamatórias e a liberação de fatores pró-inflamatórios, proporcionando um microambiente favorável à cicatrização da ferida, e no processo proliferativo a quitosana fornece um suporte não proteico de matriz para o crescimento do tecido (MIGUEL; MOREIRA; CORREIA, 2019).

Além disso, a quitosana se despolimeriza gradualmente liberando N-acetil-β-D-glicosamina, o que estimula a proliferação de fibroblastos, síntese de ácido hialurônico, angiogênese e deposição ordenada de colágeno no local da ferida. Tais eventos melhorarão o processo de cicatrização e impedirão a formação de cicatrizes no processo de remodelação (AHMED, IKRAM, 2016; PATRULEA et al., 2015). A quitosana apresenta ainda propriedades hemostáticas, auxilia na função dos macrófagos e neutrófilos que ajudam na cicatrização mais rápida das feridas, pode promover granulação tecidual e re-epitelização para iniciar o processo de cicatrização (CAHÚ, 2017; SCHOUKENS, 2019).

Ademais, a quitosana é capaz de evitar a ocorrência de infecções na pele, consideradas uma das principais complicações associadas ao processo de cicatrização de feridas (SIMÕES et al., 2018). Esse efeito antibacteriano resulta da interação dos grupos amina de carga positiva da quitosana com os peptidoglicanos de carga negativa da parede celular bacteriana, o que pode levar a desequilíbrios osmóticos internos e, portanto, inibir o crescimento dos microrganismos (ARKOUN et al., 2017).

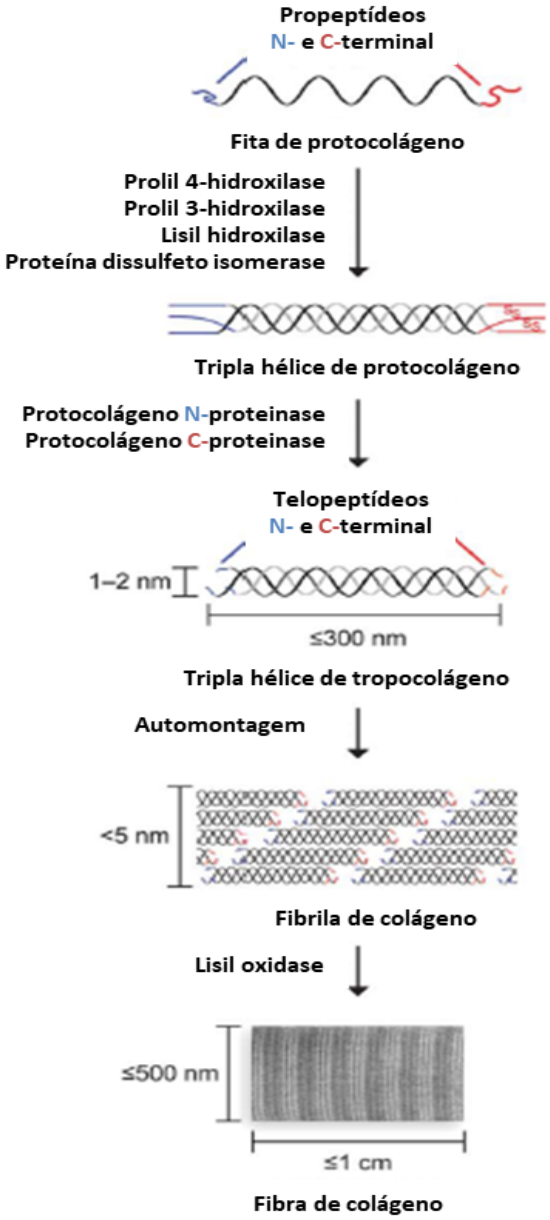
2.5.1.2 Colágeno

O colágeno é o principal componente do tecido conjuntivo. Consiste em três cadeias polipeptídicas torcidas no formato de uma corda de três fios e formando uma hélice tripla (figura 6). O colágeno contém uma sequência de aminoácidos repetida de Gly-X-Y, onde X e Y são principalmente prolina e hidroxiprolina. Atualmente, existem pelo menos 29 tipos de colágeno conhecidos (KACZMAREK et al., 2017). Os colágenos fibrilares apresentam um alto teor de resíduos carregados (~ 15 % a 20 %) e uma pequena porcentagem de resíduos hidrofóbicos (~ 6 %). Foi sugerido que os resíduos de Alanina e os iminoácidos contribuem para a estabilidade da tripla hélice do colágeno, enquanto os resíduos carregados e hidrofóbicos são importantes para interações com outras moléculas de colágeno, bem como com receptores e outros componentes da matriz (AN; LIN; BRODSKY, 2016).

O colágeno está distribuído de forma específica nos tecidos orgânicos: o tipo I está presente no osso, pele, tendão, córnea; o tipo II em cartilagem e corpo vítreo; e o tipo III, juntamente com o tipo I, na pele, além de vasos sanguíneos e tecidos mais flexíveis. O colágeno tipo I é um heterotrímero que consiste em duas cadeias α1 e uma cadeia α2, e esse tipo de colágeno constitui a base da maioria das aplicações de biomateriais. Os colágenos tipo II e III mostram homologia com a cadeia α1 do colágeno tipo I, mas são homotrímeros. Os colágenos não fibrilares incluem colágeno tipo IV, que forma estruturas semelhantes a redes

nas membranas basais, bem como colágenos associados à membrana (AN; LIN; BRODSKY, 2016).

Figura 6. Via biossintética para as fibras de colágeno apresentando a oxidação das cadeias laterais de lisina que leva à formação espontânea de ligações cruzadas de hidroxilisilpiridinolina e lisilpiridinolina.



Fonte: CHATTOPADHYAY & RAINES, 2014.

O colágeno, a proteína mais abundante no corpo humano, tem uma longa história como material natural utilizado em diversas aplicações biomédicas, incluindo a administração de medicamentos (RAMSHAW et al., 2009). Os resíduos marinhos, incluindo peles, escamas

e ossos, são cada vez mais considerados uma fonte promissora de materiais colágenos e podem ser uma alternativa econômica às fontes de mamíferos (ENNAAS et al., 2016).

As fibrilas de colágeno são reticuladas para fornecer resistência mecânica e integridade à MEC, e a distribuição dos diâmetros das fibrilas, bem como o grau de reticulação, influenciam fortemente a resistência à tração e a elasticidade dos tecidos. Moléculas ou fibras de colágeno extraídas podem formar hidrogéis, filmes ou esponjas que podem ser usadas como compressas hemostáticas, curativos, enxertos e arcabouços para cirurgia e engenharia de tecidos (FLECK & SIMMAN, 2010; RAMSHAW; WERKMEISTER; DUMSDAY, 2014).

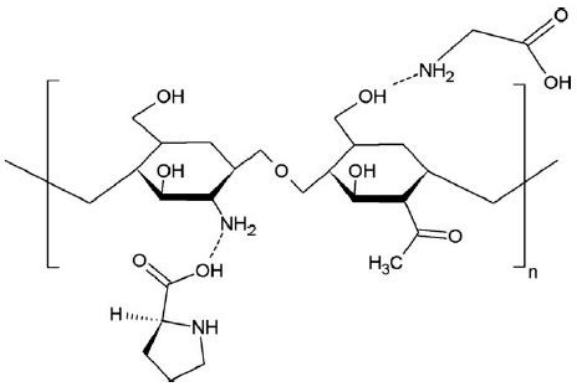
Entre os materiais bioativos e biocompatíveis para curativos, o colágeno é provavelmente o biomaterial mais promissor, devido à mínima resposta inflamatória, baixa citotoxicidade e sua propriedade de promover o crescimento celular. Além de fornecer força e integridade a matriz tecidual, o colágeno também desempenha papel na hemostasia (LOCKHART et al., 2001).

A tripla hélice do colágeno interage com um grande número de moléculas que desencadeiam eventos biológicos. As interações do colágeno com os receptores da superfície celular regulam muitos processos celulares, incluindo adesão, proliferação e migração (LEITINGER & HOHENESTER, 2007). A maioria dos substitutos da pele disponíveis no mercado é à base de colágeno. Desde os curativos acelulares como BioBrane (Smith & Nephew) ou Integra (Integra) aos equivalentes autólogos carregados com queratinócitos Apligraf (Novartis) ou OrCell (Ortec International Inc.) (SCHOUKENS, 2019).

Quitosana e colágeno podem ser combinados para criar novos materiais com propriedades únicas, apropriadas para a engenharia de tecidos. As interações entre quitosana e colágeno contribuem para a formação de complexos por interações eletrostáticas ou pontes de hidrogênio (figura 7) (SIONKOWSKA et al., 2004).

A quitosana revela natureza catiônica, que permite as interações com partes aniônicas das moléculas de colágeno anfifílico. A adição de colágeno à quitosana permite obter estruturas biomiméticas e composições semelhantes ao osso natural (CHEN et al., 2010). As principais formas aplicáveis de complexos de quitosana/colágeno são filmes, géis e esponjas (SKWARCZYŃSKA; BINIAŚ; MODRZEJEWSKA, 2016). Os materiais à base de quitosana e colágeno podem ser usados como substâncias bioativas, que influenciam o comportamento das células.

Figura 7. Ligação por ponte de hidrogênio entre a quitosana e o principal aminoácido do colágeno.



Fonte: KACZMAREK et al., 2017.

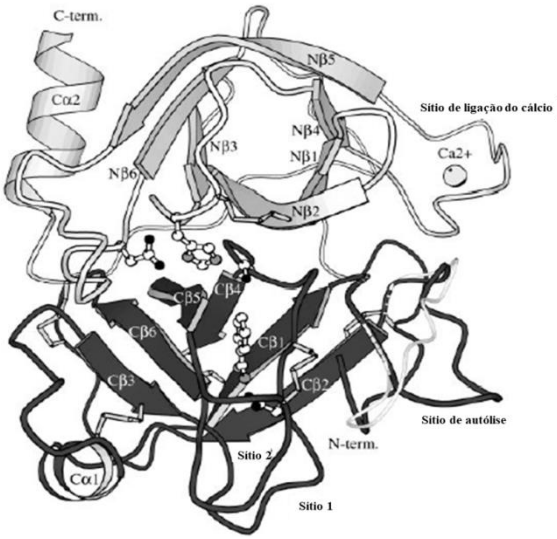
O uso da mistura de quitosana e colágeno melhora as propriedades mecânicas dos materiais (SANDHIYA; NANDHINI; PANDIMADEVI, 2016). As esponjas de quitosana/colágeno mostram maior resistência à degradação enzimática em comparação com o colágeno puro (CUY et al., 2003; KACZMAREK & SIONKOWSKA, 2018). A quitosana e o colágeno demonstraram excelente miscibilidade em diferentes proporções (RAFTERY et al., 2016).

A adição de colágeno à quitosana aumenta o diâmetro dos poros e a porosidade de arcabouços (ANANDHAKUMAR et al., 2017). Os arcabouços de quitosana/colágeno podem fornecer um ambiente pró-inflamatório e ativador para realizar estudos que objetivam analisar os efeitos de agentes exógenos, além de possibilitar interações celulares potenciais com outras células do sistema imunológico *in vitro* (FERNANDES et al., 2011; DANESHMANDI; DIBAZAR; FATEH, 2016).

2.5.2 Tripsina como molécula bioativa em biomateriais

A tripsina (EC 3.4.21.4) é uma importante enzima proteolítica, pertencente à família S1 do clã PA das serino protease, e é responsável pela clivagem de ligações peptídicas na porção C-terminal dos resíduos dos aminoácidos arginina e lisina (figura 8). As tripsinas são endopeptidases com peso molecular geralmente entre 22 e 28 kDa, encontradas em organismos eucariotos, procariotos e vírus (BOUGATEF, 2013; POLGÁR, 2005)

Figura 8. Estrutura geral de uma tripsina aniônica de salmão (2TBS) sobreposta com uma tripsina aniônica bovina (3PTB). (C-temr) porção carboxiterminal; (Cβ) β-folha do domínio C; (Cα) α-hélice do domínio C; (Nβ) β-folha do domínio.



Fonte: Leiros et al., 2000.

A tripsina é uma das proteases digestivas mais importantes para a maioria dos vertebrados, sendo sintetizada no pâncreas na forma de um precursor inativo, o pré-tripsinogênio, e então armazenada em grânulos de zimogênio, juntamente com o inibidor pancreático de tripsinas, o PSTI (Pancreatic Secretory Trypsin Inhibitor), que impede a sua ativação dentro do pâncreas. Ainda ligado ao retículo endoplasmático rugoso, o seu peptídeo sinal é retirado tornando-se o tripsinogênio que então é secretado no duodeno, onde será ativado pela enteropeptidase (enzima proteolítica secretada pelas células intestinais) ou por autocatálise. A ativação é feita pela liberação do propeptídeo pela hidrólise de Lys15-Ile16, resultando na β-tripsina de cadeia única (VANDERMARLIERE et al., 2013; HIROTA et al., 2006; VARALLYAY et al., 1998).

Em condições normais, a ativação da tripsina é altamente controlada, no entanto, quando a ativação do tripsinogênio é muito alta ou a capacidade do PSTI se ligar à tripsina seja muito baixa, então esta enzima pode acarretar a autodigestão do pâncreas, resultando numa doença conhecida como pancreatite (HIROTA et al., 2006).

A principal função da tripsina é a clivagem de proteínas, ingeridas durante a alimentação, em peptídeos, que serão digeridos por exopeptidases transformando-se em fragmentos ainda menores que podem então ser absorvidos pelo intestino, permitindo a ingestão de aminoácidos essenciais não sintetizados pelo organismo. Além disto, a tripsina também participa da ativação do receptor da insulina, pela clivagem de seu domínio extracelular e participam da clivagem e ativação de proteases acopladas à proteína G (PARs), que

contribuem para a defesa do organismo com respostas inflamatórias (VANDERMARLIERE et al., 2013).

Nos últimos anos o uso de proteases, como a tripsina, tem sido investigado quando aplicado em lesões cutâneas, com o objetivo de avaliar o potencial destas na cicatrização. Estes estudos demonstram a eficácia da tripsina na recuperação do tecido lesionado, uma vez que a mesma é capaz de auxiliar na coagulação sanguínea, na lise do coágulo de fibrina e transporte de proteínas de membrana (RAO et al., 1998). Estudos mostram a participação da tripsina, juntamente com a trombina, na regulação de células alvos, a partir da clivagem e ativação de proteases acopladas à proteína G (PARs), que contribuem para a defesa do corpo em resposta à inflamação e invasão de patógenos (VERGNOLLE et al., 2001).

Devido a estas potencialidades, além de sua clivagem altamente específica e estabilidade em diferentes condições biológicas, como altas temperaturas, ampla faixa de pH e presença de íons, agentes oxidantes e surfactantes, a tripsina é uma das proteases mais utilizadas comercialmente em diferentes setores, como associadas à biotecnologia, medicina e outros setores industriais (quadro 3) (VANDERMARLIERE et al., 2013; BOUGATEF, 2013).

Quadro 3. Exemplos de estudos clínicos do uso terapêutico da tripsina em humanos e animais.

Indicações clínicas	**Aplicações**	**Estudos clínicos**
Inflamação	Sistêmica	Humphries, 1971; Martin et al., 1955
Cicatrização	Tópica	Rodeheaver et al., 1974; Lecht e Stepheson, 1968
Queimadura	Tópica/Sistêmica	Stucke, 1949; RaviKumar et al., 2001
Complicações pós-operatórias	Sistêmica/Tópica	Krause, 1961
Artrites	Sistêmica	Mazourov et al., 1997; Rovenska et al., 1999[b]
Reparos entre cartilagem e osso	Implantes pré-tratados com tripsina	Chen et al., 2000[b]

Fonte: GUDMUNDSDÓTTIR & PÁLSDOTTIR, 2005.

A imobilização de uma tripsina comercial suína em filmes de quitosana apresentou bons resultados, aumentando a atividade catalítica da enzima bem como estendendo o tempo de estocagem à temperatura ambiente (28 dias a 25 °C), além de ter sido eficaz como curativo, auxiliando na cicatrização de lesões cutâneas em camundongos (MONTEIRO et al.,

2007). No entanto, existem poucos estudos que contemplem o uso de proteases em tratamentos tópicos de lesões, em especial proteases de peixe.

As tripsinas obtidas a partir do processamento de peixes tropicais são proteases com estabilidade em condições adversas, como a presença de íons e tenso-ativos, ampla faixa de pH e altas temperaturas (MARCUSCHI et al., 2010). Adicionalmente, estas enzimas possuem alta atividade catalítica, propriedades que as tornam mais atrativas para aplicação como curativo dérmico quando comparada aos seus homólogos obtidos de mamíferos.

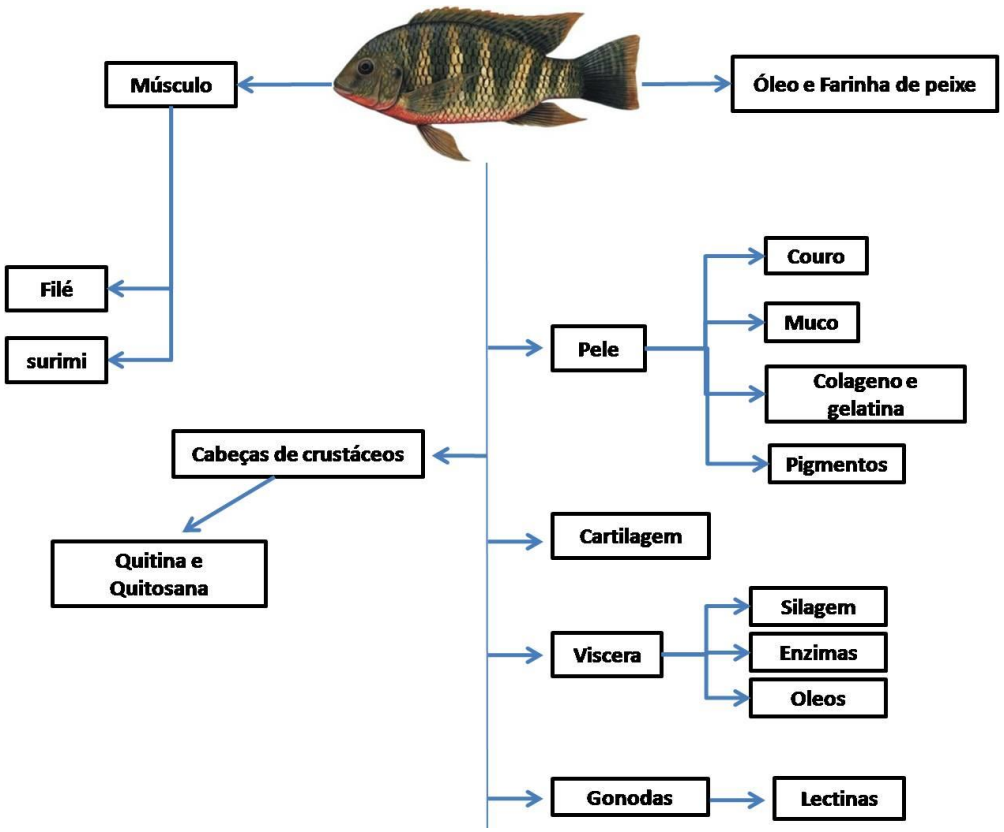
2.6 APROVEITAMENTO DE RESÍDUOS DA INDÚSTRIA PESQUEIRA

No passado, subprodutos do pescado, como cabeça, víscera, pele, ossos e escamas, eram considerados de baixo valor ou um problema às indústrias pesqueiras para descartá-los. Há duas décadas tem havido um aumento da conscientização em relação aos aspectos econômicos, sociais e ambientais desta atividade, com a finalidade de otimizar o uso dos subprodutos do pescado. Uma grande variedade de metodologias para o aproveitamento desses resíduos tem sido desenvolvida, como a produção de rações para alimentação em Aquicultura, criação de gado, uso como fertilizantes, etc. (FAO, 2014).

Ademais, importantes biomoléculas para a indústria farmacêutica, com aplicações biotecnológicas e biomédicas podem ser obtidas a partir dos insumos do pescado, como carotenóides (SANTOS, 2006), enzimas (peptidases, lipases e carboidrases) (SHAHIDI & JANAKKAMIL, 2001; KIM & MENDIS, 2006; BLANCO et al., 2007; BEZERRA et al., 2005; ESPÓSITO et al., 2009; ASSIS et al., 2007), quitina e quitosana (SHAHIDI e JANAKKAMIL, 2001; KIM e MENDIS, 2006), colágeno e gelatina (GONZÁLEZ et al., 2007; KIM & MENDIS, 2006; LEE et al., 2009; HWANG et al., 2007) (figura 9).

Alguns destes biomateriais têm sido cada vez mais empregados para promover a cicatrização de feridas, uma vez que apresentam propriedades favoráveis ao processo cicatricial. Além disso, algumas moléculas têm também um potencial confirmado como substituto na engenharia de tecidos, como um agente terapêutico promissor para favorecer a ligação entre células em cultivo e *in vivo*, e como polímeros bioativos que propiciam o microambiente adequado para substituição de pele em feridas extensas e queimaduras (GUATAM et al., 2014; NWE; FURUIKE; TAMURA, 2010). Dentre os biopolímeros naturais mais utilizados com este propósito, destacam-se os polissacarídeos, como a quitina e a quitosana, e diferentes tipos de proteínas, como o colágeno e a tripsina (CHANDIKA; KO; JUNG, 2015).

Figura 9. Apresentação de algumas das principais biomoléculas e produtos que podem ser extraídos a partir do processamento de peixes e crustáceos pela indústria pesqueira.



Fonte: BLANCO et al., 2007 (adaptado).

3 ARTIGO 1 - ASSESSMENT OF CHITOSAN/COLLAGEN FILMS AS A POTENTIAL WOUND DRESSING MATERIAL

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Abstract

Aquatic ecosystem has been identified as an ideal source of biomaterials because it contains limitless diversity. Studies showed that biopolymers from aquatic environment can be used for different applications, including skin regeneration, since they have good biocompatibility, biodegradability, low toxicity and low cost to produce them. Chitosan and collagen were isolated from marine shrimp heads and Nile tilapia fish skin, respectively, and their potential as wound dressing was evaluated as filmogenic solution and film. Thereby, the characterization was performed by FT-IR (Fourier Transform Infrared Spectroscopy), SDS-PAGE, scaning electron microscope (SEM), Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) analyzis and thermal analyzis such as Thermogravimetry (TG) and Differential Scanning Calorimetry (DSC). Mechanical properties, swelling capacity and moisture of films with chitosan and collagen were analyzed as well as the zeta potential of filmogenic solutions. FT-IR results showed characteristics peaks for chitosan, collagen and their mixture, and the measured of chitosan deacetylation degree was 79.6 %. SDS-PAGE confirmed the isolation of a type I collagen from fish skin with α-chains, β (dimmer) and γ (trimer) bands. Chitosan/collagen films (chi/col film) had the ability to absorb great amount of water and also stay hydrated for a wide time interval in moisture content and water absorption tests. SEM images showed a rough and wavy surface. TG analysis indicate that the two polymers interacted yielding a miscible blend with intermediate thermal degradation properties. DSC curve showed one endothermal peak for water evaporation which confirm that there is no phase separation between polymers. The superficial area (BET) and size poros (BJH) of the film were 3.6148 m^2/g and 0.000192 cm^3/g, respectively. Positive values of filmogenic solutions were presented in zeta potential test, and the mechanical experiment showed that chi/col film had a tensile strength of 28 MPa and an elongation at break of 97.37 %. The findings of the study indicate that the chi/col film is expected to have wide application in tissue engineering as a wound dressing for topical skin lesion.

Keywords: biomolecules; wound dressing; tissue engineering.

Introduction

Biopolymers are important molecules that could be produced by organisms, such as microorganisms, animals, and plants and, because they have bioactivity in many different

industrial areas, they are emerging in the market. Therefore, in recent years much focus have been drawn towards engineering biodegradable biomaterials (CHALEAWLERT-UMPON et al., 2011; MAZZARINO et al., 2012), specially in regenerative medicine and drug delivery systems (ALAGHA; NOURALLAH; HARIRI, 2019).

The continuous identification of new and useful natural biomaterials has led to the exploration of the aquatic ecosystem. As a result of it, this ecosystem has been identified as an ideal source of biomaterials especially due to its wide diversity. The development of natural products for skin tissue regeneration has been greatly advanced by exploration of the aquatic environment, since some of those biomaterials present good biocompatibility, biodegradability, low toxicity, low cost, and can be found abundantly (SELLIMI et al., 2018).

Among the biomaterials that can be chosen with potential for pharmaceutical and medical industries, it is important to highlight chitosan and collagen. Chitosan is a polysaccharide derived from deacetylated chitin (CROISIER & JÉRÔME, 2013) which is the second most abundant polymer in nature after cellulose (HEJAZI & AMIJI, 2003). It is a linear polysaccharide composed of N-acetyl-D-glucosamine and D-glucosamine units, whose units are linked by 1-4-β-glycosidic bonds. It has been given to chitosan a special interest in topical drugs delivery field due to its biocompatibility, biodegradable and nonimmunogenic properties (UENO; MORI; FUJINAGA, 2001). The cationic nature of chitosan allows ionic interactions with other ionic compounds, leading to new materials.

Collagen is an abundant fibrillar protein of animal tissues and constitutes approximately 30% of total animal protein (MUYONGA; COLE; DUODU, 2004). Type I collagen is a heterotrimer that consists of two α1 chains and α2 chain, and this type of collagen forms the basis of most biomaterial applications (AN; LIN; BRODSKY, 2015). The highest utilization of collagen is in pharmaceutical applications, including production of wound dressings, vitreous implants and as carriers for drug delivery. One of the interesting properties of collagen is its good moisturizing nature, biocompatibility, low toxicity and biodegradability. The collagen can be processed into a number of forms such as sheets, tubes, sponges, powders, injectable solution dispersions and films, all of which have been found use in medical systems (RAMASAMY & SHANMUGAM, 2015; CHEN et al., 2015).

Collagen and chitosan can be used to obtain films for biomedical applications because they present significant biocompatibility. Moreover, they are miscible in different ratios (LEWANDOWSKA et al., 2016). The aim of the study was to obtain polymeric blends based on chitosan and collagen, from a low cost source with specific properties to form films. Various analyses were made and several properties of the materials were characterized, such

as physical and morphological characterization, thermal analysis, porosity and mechanical properties. This study was performed mainly to predict if chitosan/collagen films have potential to be used as a wound dressing for dermal injuries.

Material and Methods

Extraction of chitin and purification of chitosan from Pacific white shrimp (*Litopennaeus vannamei*)

The heads of the Pacific white shrimp were provided by Noronha Pescado Ltda, and stored in the freezer (- 20 °C) for the following steps. Then, enzymatic hydrolysis was performed at 40 °C for 3 hours with subsequent inactivation of the enzymes at 100 °C for 10 minutes. The shrimp peels were washed with distilled water, oven dried and stored at room temperature. Subsequently, they were submitted to a purification process, according to the methodology of Cahú et al. (2012), with slight modifications. After purification, chitosan solution was prepared in a concentration of 1 % (10 mg/mL) by its resuspention in 1 % acetic acid. Chitosan sample was characterized by Fourier transform infrared spectroscopy (in KBr pellets, range 4000 – 400 cm^{-1}) in a FT-IR Bomem MB100 spectrophotometer. The degree of deacetylation (DD) was calculated by infrared spectra, according to Brugnerotto et al. (2001).

Obtaining collagen from Nile Tilapia skin (*Oreochromis niloticus*)

Nile tilapia fish skin were provided by Noronha Pescado Ltda and kept at 4 ºC until collagen extraction. Initially, the skin was cut into small pieces and stirred in 0.1 M NaOH solution for 1 h (three changes) at 4 °C, for removing soluble proteins from fish skin. After the last base change, 10 % butyl alcohol was added and stirred for 12 h at 4 °C to clean fat. Then, 1 L of 0.5 M acetic acid was added for 12 h (two changes) at 4 °C. A change in collagen pH (around 8.0) was induced until its preciptation and then all samples was centrifuged. Thereafter, dialysis, with distilled water, and lyophilization were performed. Collagen sample was characterized by Fourier transform infrared spectroscopy (in KBr pellets, range 4000 – 400 cm^{-1}) in a FT-IR Bomem MB100 spectrophotometer and by SDS-PAGE electrophoresis were performed according to Laemmli method (1970).

Chitosan/Collagen film obtainment

Collagen (1 % w/v) and chitosan (1% w/v) solutions were prepared separately by dissolving it in 1 % (v/v) aqueous acetic acid. Both solutions had 10 mg/mL for final

concentration and were stirred overnight using magnetic stirrer, in room temperature for chitosan and 4 °C for collagen. After solubilization, chitosan and collagen solutions were mixed and cast in molds (lids of microplates of 96 wells) and placed in dry oven at 37 °C until being dryed. To complete discs formation, the films were placed in the fridge overnight and were kept at 4 °C until being used. The disc area was 34.79 mm^2 and the film was named chi/col film.

Physical and morphological characterization of the chi/col film

Moisture content

The samples (triplicate) were weighed on an analytical balance and subsequently heating it in a dry oven at 105 °C for 1 h. The film was then cooled in a dissector until room temperature and the moisture content was calculated in relation to its weight loss according to the following formula:

Moisture (%) = (W1−W2/ W1) * 100

Where W1 = weight (g) of the sample before drying, W2 = weight (g) of the sample after drying.

Water absorption Test

The water absorption capacity of the film (triplicate) was determined by swelling these films in PBS buffer at room temperature according to Kaczmarek et al. (2018), with slights modifications. The weight of the chi/col films was noted after blotting it with filter paper to remove excess and was recorded for every 1 h, 2 h, 3 h and 24 h. Percentage of water absorption of the films at a given time was calculated from the formula:

Water Absorption = (Ws - Wo)/ Ws*100

Where Ws = the weight of the film (moist) at given time, Wo = the initial weight of the films.

Scanning Electron Microscopy (SEM)

The surface morphology of the films was observed using a digital Scanning Electron Microscope (SHIMADZU SS-550), under the conditions of current and electron beam power of 10 kV. Before testing, the samples were placed on an aluminium support and covered with

a thin gold layer (Sputter Coater, SCDO50) to improve the thermal conduction. Images were taken with 500 and 10.000 x magnification.

Zeta potential

Zeta potential measurement of the 10 mg/mL of filmogenic solutions (chitosan solution, 10 mg/mL collagen solution and 10 mg/mL chitosan/collagen mixture (v/v)) was done by using a Malvern nano zetasizer instrument (Zetasizer 3000 HAS; Malvern Instruments Ltd, Worcestershire, UK).

Thermogravimetry (TG) and differential scanning calorimetry (DSC)

The thermal properties of the chi/col films were determined using Thermogravimetry (TGA-50 Shimadzu) and differential scanning calorimetry (DSC-60 plus Shimadzu). The thermogravimetry analysis used 16 mg of film mass, temperature between 30 and 500 °C, heating rate of 10 °C/min, and nitrogen at 40 mL/min as carrier gas. The differential scanning calorimetry analysis used 16 mg of film mass, temperature between 30 and 300 °C, heating time of 10 °C/min, and nitrogen at 40 mL/min as carrier gas.

Surface area and Porosity analysis

The technique consists in promoting the adsorption of gas (nitrogen) to the material sprayed and previously treated in a vacuum station. Adsorption is then performed by insertion of the gas under controlled pressure into the analysis station at low temperature. Surface area and pore characteristics (size and distribution) were determined from the ratio of the amount of adsorbed/desorbed gas to the gas pressure (isotherm) using a Micromeritics ASAP 2420 Surface and Pore Size Analyzer. A multiple point Brunauer-Emmet-Teller (BET) method (nitrogen adsorption) and Barrett-Joyner-Halenda (BJH) analysis was employed to determine specific pore volume by nitrogen adsorption and desorption techniques (SANTANA et al., 2012).

Mechanical tests

The tensile tests were conducted with a texturometer using a universal testing machine (WDW 300E) equipped with a 0.5 kN load cell. The films were fixed to the grips of the equipment with initial distance of 30 mm and crosshead speed of 25 mm/min^{-1}. The properties determined were maximum tensile strength (MPa), elongation at break (%), and

modulus of elasticity (GPa) according to the methods and standards of the American Society for Testing and Material (ASTM D882-12, 2012).

Statistical analysis

Statistical analysis were performed to obtain means, standard deviation, experimental percentage calculations and significant differences using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) and OringPro 8SRO software program, from OriginLab Corporation using one-way analysis of variance (ANOVA), followed by Tukey's test ($p<0.05$).

Results and Discussion

Polymers yield

Chitosan extraction from sea shrimp heads and collagen from Nile tilapia fish skin were performed in different stages and satisfactory yields were obtained for both biomolecules. Collagen yield was 22.82 % ± 1.56 in 100 g of fish skin. The yield value presented was higher than several collagen extracted from fish, such as *Cichla ocellaris*, 2.9 % (OLIVEIRA, 2019), *Katsuwonus pelamis*, 2.47 % (DI et al., 2014) and *Brama australis*, 1.5 % (SIONKOWSKA et al., 2015). The total yield of collagen was known to vary with the type of extraction and fish species (RADHIKA et al., 2019). Chitosan yield was 17 ± 4 g after all processing, for 1 kg of shrimp head used in the process. The degree of deacetylation of chitosan was 79.61 %, similar to that obtained in the deacetylation of a chitosan also from sea shrimp (*L. vannamei*) with similar protocol (CAHÚ et al., 2012). The degree of deacetylation is one of the most important properties for chitosan, as it is a factor that influences its solubility and reactivity, and in the differentiation of chitin and chitosan (CAHÚ et al., 2012; KASAI et al., 2000).

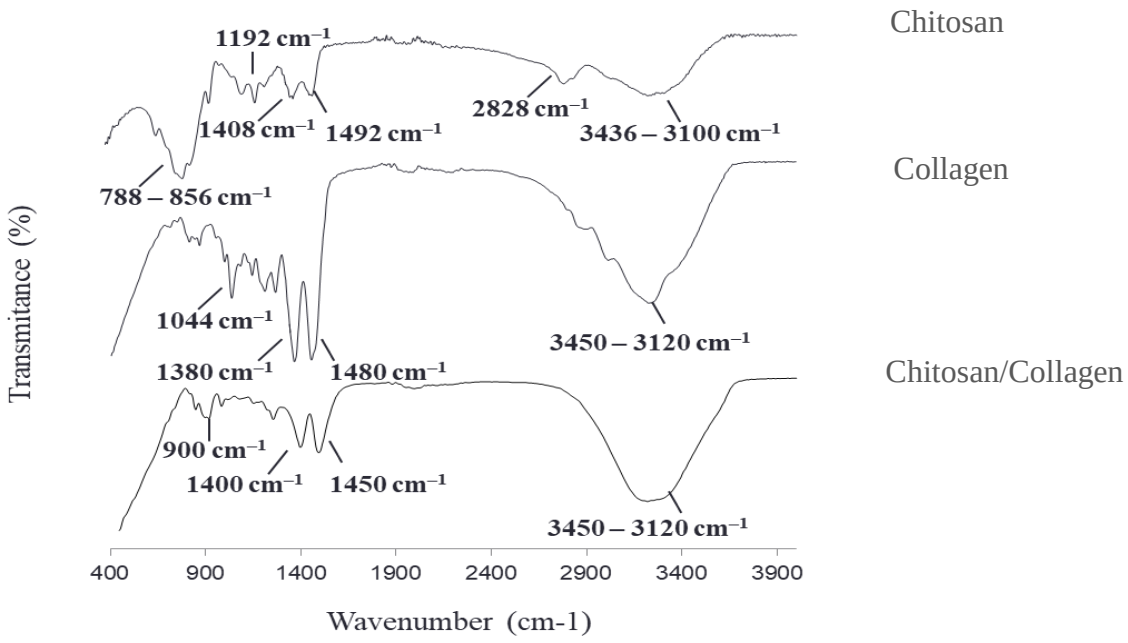
Characterization of polymers

Infrared spectroscopy

FT-IR was carried out with powder samples of chitosan, collagen and chitosan/collagen and the results are shown in figure 1. In chitosan infrared spectroscopy, a transition peak 3440 to 3100 cm^{-1} is associated with the OH and NH vibrations of the C-OH, from physically adsorbed of water and NH_2 groups, respectively. The peak 2828 cm^{-1} correspond to the symmetrical and asymmetric CH vibrations arising from the -CH_2 groups.

The peak 1492 cm^{-1} is relative to vibration mode of amide groups (amide I, stretching of C = O) and 1408 cm^{-1} corresponds to NH_2 bending vibration in amino group (amide band II). A vibration of OH, CH in the ring and a deformation at –CN of amino groups is showed by the peak 1192 cm^{-1}, and the transition peak at 856 to 788 cm^{-1} resulting from the polysaccharide structure. Since degree of deacetylation was not 100 % some amount of chitin is also presented along with chitosan. Similar results were demonstrated in characterization from shrimp chitosan (CAHÚ et al., 2012; MAREI et al., 2017; KUMARI et al., 2017; NOURI et al., 2016).

Figure 1. Fourier Transforms Infrared Spectra (FT-IR) of Chitosan, Collagen and the mixture of chitosan and collagen.



In collagen infrared spectroscopy a transition peak at 3450 to 3120 cm^{-1} is representative for O-H and N-H stretches from physically adsorbed of water molecules and amino groups and shows the existence of hydrogen bonds, probably with a carbonyl group of the peptide chain. The 1480 cm^{-1} peak occurs due to vibrations in the N-H bond plane and the C-N stretch in the amide band II. 1380 cm^{-1} corresponds to the vibrations of the proline and hydroxyproline pyrrolidine rings and 1235,94 cm^{-1} correspond to vibrations in the plane of amide III due to C-N stretching and N-H strain. The ratio of absorption intensity between 1240 cm^{-1} (amide III) and 1044 cm^{-1} (amide II) band was approximately equal to 1.0, which confirms the triple helical structure of collagen. The collagen sample from Nile tilapia skin

demonstrated the same vibrational profile of collagen from other fish species in the literature (HUANG et al., 2016; VEERURAJ; ARUMUGAM; BALASUBRAMANIAN, 2013; PATI; ADHIKARI; DHARA, 2010).

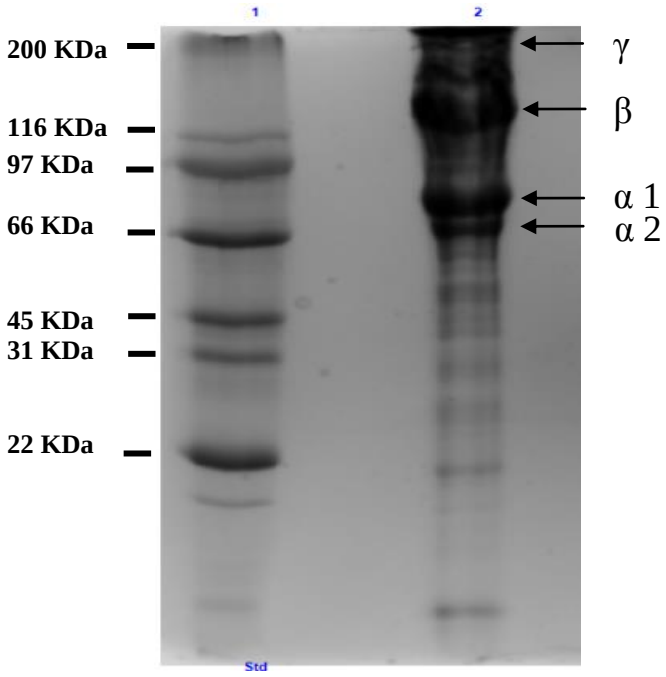
The FT-IR of chitosan/collagen mixture showed characteristic peaks from chitosan and collagen, with a transition peak at 3450 to 3120 cm^{-1} representative for O-H and N-H stretches from physically adsorbed of water molecules and amino groups and shows the existence of hydrogen bonds, probably with a carbonyl group of the peptide chain. Two peaks in 1450 and 1400 cm^{-1} that represents vibrations in the N-H bond plane and the C-N stretch in the amide band II and corresponds to the vibrations of the proline and hydroxyproline pyrrolidine rings, respectively. Also, a peak at 900 cm^{-1} resulting from the polysaccharide structure. These results could indicate that the interaction happened between acid soluble collagen and chitosan when the film was formed. The interactions between collagen and chitosan may occur by hydrogen bond formation. In the present study, the FT-IR spectrum of Chitosan/collagen solution showed no detectable changes which indicate that the collagen and chitosan interactions are polyelectrolytic with oppositely charged ionic polymers, particularly the cationic group of chitosan (NH^{3+}) and negative group in anionic collagen (COO^{-}) as also reported by Chen et al. (2015).

SDS-PAGE

Electrophoretic pattern of fish skin collagen from Nile tilapia is shown in figure 2. The sample analyzed is composed of α-chains, β (dimmer) and γ (trimer). This result suggests that collagen molecules are heterotrimers, which comprise at least two different types of α-chains, (α 1 and α 2). This pattern is the typical characteristic feature of type I collagen detected in Nile tilapia skin and also found in skin of most organisms. Generally, type-I collagen contains of cysteine and methionine, which play an important role in disulfide bond formation (RAMASAMY & SHANMUGAM, 2015).

The α-chains showed a molecular mass of approximately 70 kDa and 68 kDa for α 1 and α 2, respectively, and β and γ chains present 120 and 200 KDa respectively. The same kind of pattern and similar molecular weight were observed in collagen from other species of fish, like bigeye snapper (KITTIPHATTANABAWON et al., 2005), ocellate pufferfish (NAGAI, SUZUKI, 2002), Baltic cod (SKIERKA & SADOWSKA, 2007), eel-fish (VEERURAJ, ARUMUGAM, BALASUBRAMANIAN, 2013).

Figure 2. Electrophoresis gel SDS-PAGE method of collagen protein from Nile tilapia (*Oreochromis niloticus*) skin. Lane 1 contains Standard protein marker (1) whit different molecular weights from 22 KDa to 200 KDa, approximately, and lane 2 contains the collagen extracted in this study.



Moisture content

Water content is a parameter which provides information about the water molecules present in the film structure. The water content has an influence on the material properties as brittle fracture and the resistance on the external force (MICHALSKA-SIONKOWSKA et al., 2018). According to Altmann (2018), films produced from hygroscopic materials, such as chitosan, will always have high humidity values. The moisture content test was performed to determine the amount of water remaining after drying chi/col films in oven. The test determined a moisture content of 32.55% ± 0.76. The average film moisture test result was higher than the 11.97% ± 0.51 moisture content of films made from bovine skin collagen and commercial chitosan (ANDONEGI et al., 2020). This result may be justified by the hygroscopic character of chitosan.

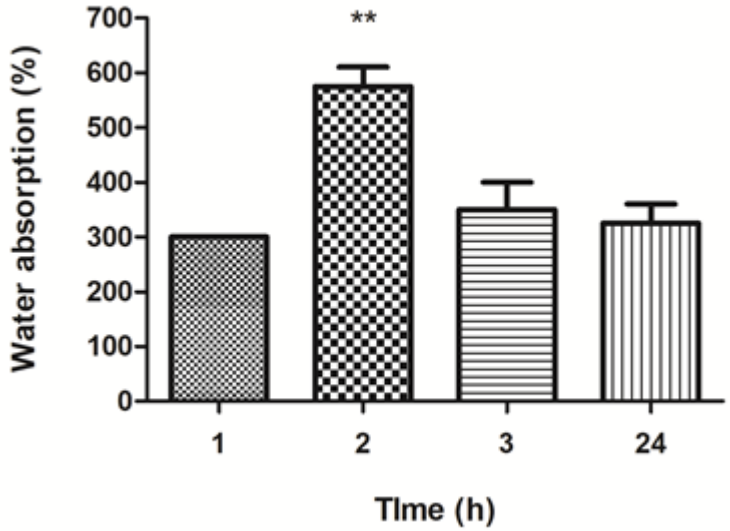
The final content of water in a polymeric film, especially those from natural and biodegradable sources, is of paramount importance in determining its application, since several processing techniques are related mainly to the water absorbed in the polymer and the hydrogen bonds present, as well as their water content interactions (SESSINI et al., 2018).

The effective management of the moisture content of a wound and of the surrounding skin is one of the most crucial requirements of any wound dressing types, mostly because a dry environment causes dehydration and consequent cell death, which would impair the healing process (SOUZA et al., 2018). In the case of exuding wounds, this implies the removal of excess wound fluid, but, in dry or lightly exuding wounds, the dressing may be required to conserve moisture to maintain the exposed tissue in the optimum state of hydration to facilitate epithelialisation or promote autolytic debridement (THOMAS, 2019; INDRANI, 2017).

Water absorption Test

High water/fluid holding capacity of film is very important for cell adhesion, proliferation, and growths. Also, moist healing prevents the formation of scar as the dressing absorbs wound exudate secreted from the ulcer. Chi/col films swelling was measured in three different intervals of time of incubation (figure 3). The analysis showed a highest film swelling in the second hour of incubation in PBS buffer, with 575 % ± 35.35. After three hours of incubation, the swelling rate decrease and became stable, probably as a result of film degradation since collagen has low stability in PBS buffer (KACZMAREK et al., 2018).

Figure 3. Water absorption of PBS buffer by chi/col films in different times of incubation.



Significance between values for water absorption from the film was considered as $^{**}p < 0.02$.

The capacity of these films to absorb water may be explained by the chitosan hydrophilic property due to its abundant hydroxyl groups. For wound closure, this could greatly accelerate healing by providing high oxygen permeability, exudate drainage and flexible wound closure (ABDEL-MOHSEN et al., 2020). Kaczmarek et al. (2018) reported a similar result by using chitosan/collagen/GAG films with a higher swelling percentage at 2 h incubation and a decrease of the rate in 24 and 48 h. Alagha, Nourallah and Sahar Hariri (2019), had demonstrated a swelling percentage smaller than the result for the present study with chitosan/collagen sponges (90 %) for one hour of PBS incubation.

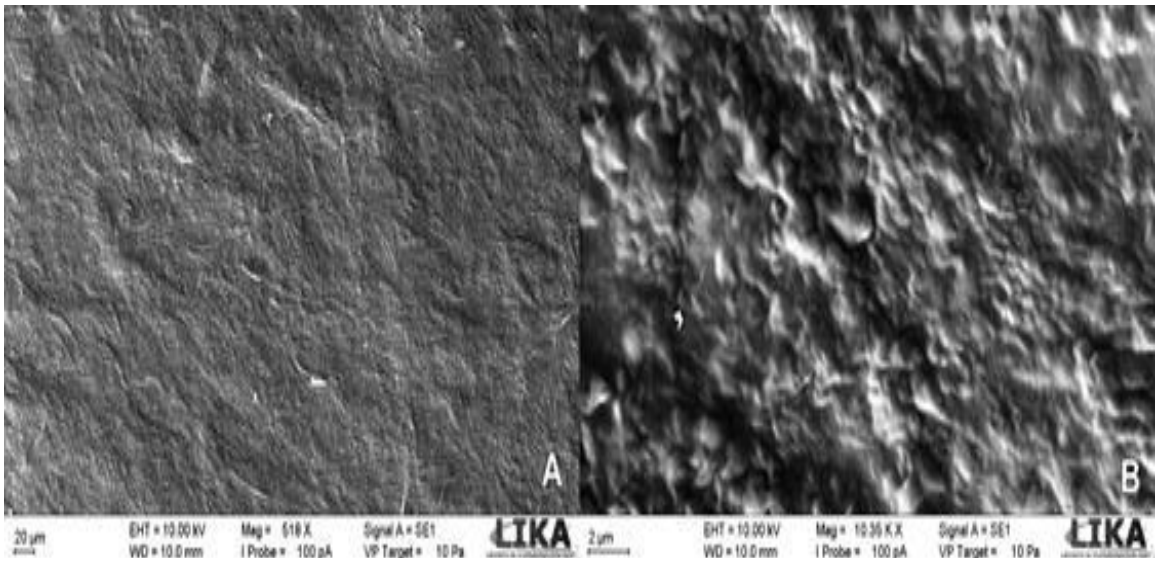
The prepared films should possess good water absorption capacity and it should also retain its shape intact for wound healing (SANDHIYA; NANDHINI; PANDIMADEVI, 2016). Swelling also increases the pore size and total porosity, maximizing the internal surface area of the wound dressing structure. Samples showing higher degree of swelling will have a larger surface area/volume ratio thus allowing the samples to have the maximum probability of cell infusion on the wound dressing as well as maximum cell growth by better attachment to its surface (PUPPI et al., 2010).

Scanning Electron Microscopy (SEM)

To form a clear understanding of the structure of cross-links formed during the interaction of chitosan/collagen, the microstructure of the films was evaluated by scanning electron microscopy (SEM) as shown in figure 4. SEM micrographs indicated the apparent compatibility between the solutions, and it can be inferred that a good miscibility occurred from the discontinuities observed in the film from the 500 and 10.000 X magnification.

The surface of chi/col film was rough with small ondulations, likewise was observed for Liu et al. (2017), with films also made by chitosan and collagen. Scanning electron microscopy had great importance because it enables the characterization of polymer surface morphology and observing the arrangement in the polymeric matrix. In addition, it also allows defining its shape, organization and particle size. The discontinuities perceived on the surface of the chi/col film of this study, in the increase of 10.000x magnification, indicate the possibility of cell attachment on the polymeric thin films and assisting in the relocation of cells at the time of tissue reconstruction (MICHALSKA-SIONKOWSKA et al., 2018; LOCILENTO, 2012).

Figure 4. Scanning Electron Microscopy (SEM) of the surface morphology of chi/col film with increase of 500X (A) and 10.000X (B) at magnification.



The characteristics of films surface depend on the film content. Michalska-Sionkowska et al., (2018), prove that pure chitosan film compared to collagen film has a smoother surface due to its microstructure, and collagen has fibril bundles in structure and also the surface is rougher.

Collagen incorporation may have influenced in mean pore size reduction in the composites and had formed cavities with interconnections but of different shapes and sizes. On contrary, the addition of chitosan resulted in compact and uniform pore structure which is best suited for tissue scaffold preparation (RADHIKA et al., 2019). The surface of chi/col film, associated with its capacity to maintain and absorb fluids, is an important indication of the film ability to incorporate new cells and auxiliate tissue repair.

Zeta potential

The results of zeta potential are shown in Table 1. Filmogenic solutions were analyzed separately and all of them are positively charged. For chistosan sample, the positive characteristic could be justified due to the ionization of amino groups of glycol chitosan (CHEN et al., 2015). Zeta potential is an important indicator of the stability of coloidal systems. It has also been used to characterize the surface charge of collagen (WANG et al., 2011). For pure collagen solution, the electrostatic interactions exert important impacts on fibrillogenesis. When collagen colloidal system is approaching its isoelectric point (pI), the

electrostatic repulsion would be minimized and the aggregation of collagen molecules would be favored (LI et al., 2009).

Table 1. Zeta potential of chitosan, collagen and chitosan/collagen solutions.

Samples	Zeta Potential (mV)
Chitosan	**+ 32.1 ± 0.92**
Collagen	**+ 58.2 ± 3.1**
Chitosan/Collagen	**+ 63.8 ± 1.12**

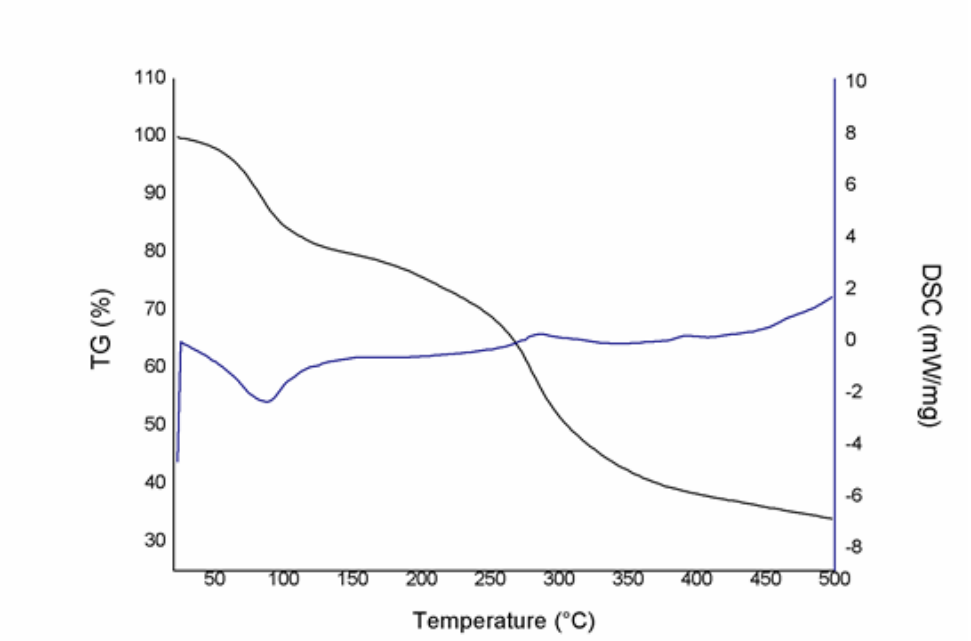
It is evident that the presence of cationic chitosan in the system would impose additional intermolecular electrostatic interactions, and also Wang et al. (2011) reported that the occurrence of collagen–chitosan complex via electrostatic interactions lessened the positive contribution to zeta potential increase by a higher chitosan addition which is probably due to an intensified competition between the formation of collagen–chitosan complex and collagen fibrillogenesis.

TG-DSC tests

The chi/col film was able to support a high change of temperature and and maintained about 34 % of its mass after incubation at 500 °C. These results are shown in figure 5. Thermal analyzes allow measuring changes in the physical or chemical properties of a material as a function of temperature. High-temperature exposure can alter the chemical structure and the physical properties of the materials, generating thermal degradation (SILVA et al., 2019). The thermal properties are strongly connected with the residual water content in the material which depends on the sample treatment (MUCHA & PAWLAK, 2005). The presence of peak below 100 °C on the DSC curve is caused by the water evaporation from the film structure (MICHALSKA-SIONKOWSKA, 2018).

Alagha et al. (2019) reported a similar result for DSC of chitosan/collagen film and related that DSC analysis confirmed that the structure of the collagen triple helix was preserved without deformation when mixing with chitosan because the collagen and collagen-chitosan scaffolds possessed a similar thermal peak and did not have any endothermal peaks in the 51- 47 °C region which is a changing area in gelatin (SHANMUGASUNDARAM et al., 2001). Ramasamy et al. (2015) also showed a single peak around 100 °C for chitosan/collagen films.

Figure 5. Curve of Thermogavimetry (TG) and Differential Scanning Calorimetry (DSC) from chi/col films.



Thermogravimetric analysis (TG) characterizes the thermal destruction of the sample. TG curve recorded for chi/col film is also presented in figure 5 and showed a mass loss of the film is iniciated around 100 °C, also related from the loss of structural bound water, and another considerable mass loss around 340 °C which is related with chitosan and collagen degradation. In comparison to the pure polymers, there is a slight change in the degradation temperature, indicating a possible interaction between them (FERNANDES et al., 2011). All sample was degraded to 500 °C and at the end of the degradation stage, chi/col film present 34 % of mass residue.

Porosity and surface area analysis

The porosity of samples was analyzed by nitrogen adsorption-desorption methodology. Results of Specific Surface Area (BET) and Specific Pore Volume Porosity (BJH) analyses of chi/col film were summarized in Table 2. Chi/col films presented a surface area of 3.6148 m^2/g and a specific pore volume of 0.000192 cm^3/g, higher parameters from those found in Chitosan-calcium phosphate composites for bone-graft substitutes (VAN DE GRAAF et al., 2015).

One way to characterize polymeric materials is through the geometry of the particles and their outer surface, down to the shape of the pores and the fraction of their accessible inner surface. The specific surface area range can vary widely due to the shape, particle size

and also its porosity. It can be used as a measure of the surface activity of various materials and is understood as the surface external to gas or molecular liquid (SANTANA et al., 2012).

Tabela 2. Values of Specific Surface Area (BET) and Specific Pore Volume Porosity (BJH) tested in chi/col film and its comparison with other results for the same parameters found in the literature.

Sample	**Specific surface area (m^2/g)**	**Specific pore volume (cm^3/g)**	**References**
Chitosan/collagen film	3.6148	0.000192	This study
Chitosan-calcium phosphate composites	1.0404	0.000118	van de Graaf et al., 2015
Extruded Poly(ethylene–co–vinyl alcohol) Composite Films	2.01	0.006	Nistico et al., 2016

The porosity of a particulate material is characterized by the amount of empty spaces existing in its total volume, being the ratio of the pore volume and the granular volume of the material. Porosity has a great effect on the mechanical properties of the material, such as: resistance to stress, hardness and deformation; physical-chemical properties, influence on dissolution characteristics and water holding capacity (SANTANA et al., 2012). Of the physical characteristics of a wound dressing, particle size and specific surface area are inextricably related and are thought to be critical to the determination of wound dressing surface coverage and thus its efficiency. These parameters also play an essential role for cell attachment and migration in wound healing (SANDHIYA; NANDHINI; PANDIMADEVI, 2016).

Enhancement in porosity could improve the water absorption of films and could easily absorb culture medium to facility the cell migration, adhesive and proliferation into and on their surface (ABDEL-MOHSEN et al., 2020). Well-designed wound dressing with a suitable porous structure will also support guided vascular infiltration, making it an ideal dermal substitute for wound regeneration (ROSTAMI et al., 2018).

Mechanical properties test

The results of elogation at break, tensile strenght and other mechanical properties of chitosan/collagen film are summarized in Table 3. Chi/col film showed a tensile strength of 28.2 ± 1.5 MPa and an elongation at break of 97.3 % ± 0.17. Also, the films could stretch at a maximum of 32.72 ± 2.3 mm.

Mechanical properties have proved to be crucial in biomaterials applications, being tensile strength and elongation at break important indices for skin substitutes (LIU et al., 2017). Prodpran et al. (2007) reported that chitosan's functional groups (hydroxyl and amino groups) can interact with the peptide chains of proteins, causing increase in films resistance. Hosseinin et al. (2013) also reported improvements in mechanical characteristics with the addition of chitosan to films prepared with fish gelatin.

The increment of film flexibility by adding chitosan can also be justified by its weakening participation or by reducing the number of hydrogen bonds, thus acting as a plasticizer (BATISTA, 2019). Films prepared from collagen were known to also have high mechanical strength (SANDHIYA; NANDHINI; PANDIMADEVI, 2016).

Table 3. Values of mechanical properties tested in chi/col film and its comparison with other results for the same parameters found in the literature.

Wound dressings materials	Tensile strenght (MPa)	Elongation at break (%)	References
Chitosan/collagen film	28	97.37	This study
Carboxymethyl cellulose films	38.9	43.8	Tavares et al., 2019
Agarose'silver particles films	22.0	37.3	Onofre-Cordeiro et al., 2018
Chitosan/collagen/GAG films	48	3.0	Kaczmarek et al., 2018
Grapheme oxide-modified collagen-chitosan films	25	2.5	Liu et al., 2017

Normally, the resistance to external forces is mainly due to chemical bonds, van der Waals forces and hydrogen bonds within the material (LIU et al., 2017). Tensile strength and mechanical stability are important parameters for a wound dressing material to ensure easy handling (SANDHIYA; NANDHINI; PANDIMADEVI, 2016; RADHIKA et al., 2019). From the values obtained in this test, it can be concluded that the combination of chitosan and

collagen for fabricating the biocomposites posses the required mechanical strength for wound dressings.

4 ARTIGO 2 - BIOCOMPATIBILITY AND IMMUNOSTIMULATORY PROPERTY PROMOTED BY COLLAGEN AND CHITOSAN BIOPOLYMERS IN PBMC

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Abstract

Collagen and chitin/chitosan are naturally occurring biopolymers and are considered co-products from fishery processing industry with potential to application in biomedical research. The aim of this work was to investigate the cytotoxicity and immunomodulatory activity of filmogenic solutions and films of chitosan and collagen on human peripheral blood mononuclear cells (PBMCs). Filmogenic solutions (Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3) and Chi/Col film were tested by measuring the changes in cell viability, cytosolic calcium concentration ([Ca2+] cyt), mitochondrial membrane potential (ΔΨm), reactive oxygen species (ROS) levels and cytokine production using PBMCs. The results were measured by flow cytometry. The differentiation and activation of T CD8+ and CD4+ subsets of lymphocytes treated with filmogenic solutions and film was also evaluated by immunophenotyping assays. In the cytotoxicity assay the tested samples induced a low rate of necrosis of PBMCs. For cytokines production, all the samples induced the release of IL-6 and IL-10; Chit 1, 2 and 3, Coll 1, 2 and 3 and Chi/Col film induced IFN-γ; Coll 1, 2 and 3 induced TNF-α; Chit 1, 2 and 3 and Chit/Coll 1, 2 and 3 induced IL-17 release. All cell treatments (except Chi/Col film) led to a high cytosolic ROS level, and Coll 1, Chit/Coll 1, 2 and 3 in all concentrations and Chi/Col film induced an increase in the level of mitochondrial ROS. Biomaterials, except Chi/Col film, did not affect [Ca^{2+}] cyt and ΔΨm levels. These results suggest that chitosan and collagen are biomaterials with immunostimulatory effects on PBMC with potential to be used on biomedical approaches.

Keywords: biomaterials; immune stimulation; proinflammatory

Introduction

The residues of fish processing are usually discarded in large amount by fishery industry since they are considered without commercial value (BEZERRA et al., 2005). However, several biomolecules, such as chitosan and collagen, can be reused from these residues since they have plenty of features favorable to many commercial applications.

Chitosan is a polysaccharide obtained from chitin deacetylation which can be found in a lot of sources, as in crustacean's carapace, insects and fungi (AHMED & IKRAM, 2016).

The use of heads and carapaces of crustaceans, obtained from crustacean processing, is an economic way to extract chitosan, once they are usually discarded (CAHÚ et al., 2017). Chitosan is a biodegradable, biocompatible, hydrophilic, low toxicity and mechanically stable biomolecule. Interestingly, chitosan exhibits considerable immunostimulatory activity, by inducing innate immune cells to release a wide range of pro- and anti-inflammatory cytokines, chemokines, growth factors and bioactive lipids (JENNINGS & BUMGARDNER, 2016).

Collagen is the main component of connective tissue. It consists of three polypeptide chains twisted into the shape of a three-wire rope and forms a triple helix. Currently, there are at least 29 known types of collagen (KACZMAREK & SIONKOWSKA, 2017). Fish waste, including skins, scales and bones, is increasingly considered a promising source of collagen and can be an economical alternative to mammalian sources (ENNAAS et al., 2016). Studies showed that collagen is a good protein for tissue remodeling, presenting low antigenicity and direct cell adhesion property, which contribute to its use in tissue reconstruction (SINTHUSAMRAN et al., 2014).

Some studies have shown that film or gel of chitosan and collagen can be formed by electrostatic interactions or hydrogen bonding, and can be used as bioactive substances (KACZMAREK & SIONKOWSKA, 2017). Hereby, this study aimed to evaluate chitosan and collagen solutions and chitosan/collagen film for its cytotoxic effect against human lymphocytes as well as its immunomodulatory properties on human peripheral blood mononuclear cells (PBMCs).

Material and Methods

Extraction of chitin and purification of chitosan from Pacific white shrimp (*Litopennaeus vannamei*)

The heads of the Pacific white shrimp were provided by Noronha Pescado Ltda, and stored in a freezer for the following steps. Then, enzymatic hydrolysis was performed at 40 °C for 3 hours with subsequent inactivation of the enzymes at 100 °C for 10 minutes. The peels were washed with distilled water, oven dried and stored at room temperature. Subsequently, they were submitted to a purification process, according to the methodology of Cahú et al. (2012), with slight modifications. After purification, chitosan was ressuspended in 1 % acetic acid (w/v) to be in a final concentration of 10 mg/mL. Chitosan sample was characterized by Fourier transform infrared spectroscopy (in KBr pellets, range 4000 – 400

cm−1) in a FT-IR Bomem MB100 spectrophotometer (data not shown). The degree of deacetylation (DD) was calculated by infrared spectra, according to Brugnerotto et al. (2001) and determined to be 79.6 % ± 0.5%.

Obtaining collagen from Nile Tilapia skin (*Oreochromis niloticus*)

Nile tilapia fish skins were provided by Noronha Pescado Ltda. The fish skin was removed and kept at 4 ºC until collagen extraction. Initially, the skin was cut into small pieces and stirred in 0.1 M NaOH solution for 1 hour (three changes) at 4 °C, for removing soluble proteins. After the last base change, 10 % butyl alcohol (to clean fat) was added and stirred for 12 h at 4 °C. Then 1 L of 0.5 M acetic acid was added for 12 h (two changes) at 4 °C. A change in collagen pH (around 8.0) was induced until its precipitation and then centrifuged. Thereafter, dialysis and lyophilization were performed. Lyophilized collagen was ressuspended in 1 % acid acetic (w/v) to preparer a solution with final concentration of 10 mg/mL and then was characterized by Fourier transform infrared spectroscopy (in KBr pellets, range 4000 – 400 cm−1) in a FT-IR Bomem MB100 spectrophotometer (data not shown).

Filmogenic solution and film obtainment

Collagen and chitosan powder were solubilized separately by dissolving it in 1 % (v/v) aqueous acetic acid with final concentration of 10 mg/mL and were used as filmogenic solution. For the analysis these solutions were diluted until have final concentration of 0.2, 0.1 and 0.05 mg/mL and were named Chit 1 (0.2 mg/mL), Chit 2 (0.1 mg/mL) and Chit 3 (0.05 mg/mL) for chitosan and Coll 1 (0.2 mg/mL), Coll 2 (0.1 mg/mL) and Coll 3 (0.05 mg/mL) for collagen. In addition, filmogenic solution were mixed and diluted at the same concentration of 0.2, 0.1 and 0.05 mg/mL and were named Chit/Coll 1 (0.2 mg/mL), Chit/Coll 2 (0.1 mg/mL) and Chit/Coll 3 (0.05 mg/mL). The mixed solution was also used to prepare chitosan/collagen films with final concentration of 1 % (10 mg/mL). For this, the mixed filmogenic solutions were cast in molds (lids of microplates of 96 wells) and placed in dry oven at 37 °C until being dried to form chitosan/collagen films in final concentration, named Chi/Col film. After, they were neutralized with 0.3 M NaOH in ethanoic solution (1:2 water:ethanol), then washed with distilled water and dried. To complete discs formation, the films were placed in the fridge overnight and were kept at 4 °C until being used. The disc area was 34.79 mm^2.

Isolation of peripheral blood mononuclear cells (PBMCs)

Blood was collected from six voluntary donors in heparin tubes (Vacuette) after obtaining a "term of free and informed consent" from all donors. The Ethics Committee from the Universidade Federal de Pernambuco approved (number 1.870.360/2016) the experimental protocols. PBMCs were obtained through a gradient concentration technique with addition of Ficoll-Paque^{TM} Plus (GE Healthcare Life Sciences, Sweden) and centrifugation (400 ×g, 30 min, 20 °C). The ring of PBMCs was collected, washed twice with sterile phosphate-buffered saline (PBS) and counted using a Neubauer chamber. Cell viability was assessed by the trypan blue dye exclusion method (Sigma-Aldrich, USA). Cells were used for the assays only if the viability was > 98 %. PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, USA) supplemented with 10% (w/v) fetal bovine serum (Sigma-Aldrich, USA) in 24-well plates (TPP Techno Plastic Products, Switzerland) at a density of 10^6 cells/well.

Evaluation of cytotoxicity of filmogenic solutions and film against PBMCs

PBMCs (10^6 cells/well) were cultured in RPMI medium for 24 h in the absence and presence of Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3 and Chi/Col film. Cells were centrifuged at 450 ×g for 10 min at 4°C. The supernatant was discarded and the pellet ressuspended in 1 mL of PBS (1x). Cells were re-centrifuged at previous conditions. The pellet obtained was resuspended in 300 μL apoptosis buffer (Apoptosis Detection Kit I from BD Biosciences, Franklin Lakes, NJ, USA) and transferred to a labeled cytometer tube with annexin V conjugated with fluorescein isothiocyanate (AnnV-FITC) and propidium iodide (Apoptosis Detection Kit I from BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry was performed in a FACSCalibur platform (BD Biosciences, San Jose, USA) and the results were analyzed using CellQuest Pro software (BD Biosciences). AnnV–/PI+ cells were considered as necrotic. Double negative cell was considered as viable.

Measurement of cytokines levels

PBMCs (10^6 cells/well) were cultured in the absence and presence of Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film in RPMI medium for 24 h. Supernatants of cultures were collected for the quantification of cytokines using the cytometric bead array (CBA) Human Th1/Th2/Th17 Cytokine Kit (Becton Dickinson Biosciences, USA) for simultaneous detection of interleukins (IL-2, IL-4, IL-6, IL-10, IL-17A), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ). Assays were

performed according the manufacturer's instructions and data acquired on FACSCalibur platform. Six individual cytokine standard curves (0–5000 pg/mL) were run in each assay.

Lymphocytes immunophenotyping assay

PBMCs were incubated (10^6 cells/well) for 24 h in RPMI medium in presence and absence of Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film. Following incubation, cells were removed from the plate using ice-cold 1 % PBS-Wash (140 mM NaCl, 2.7 mM KCl,10 mM disodium phosphate [Na_2HPO_4], and 1.8 mM monopotassium phosphate [KH_2PO_4]; BD Biosciences) and transferred to 15-mL polypropylene tubes containing 6 mL of PBS-Wash for centrifugation (400 ×g for 10 min). The supernatant was discarded and cell pellet washed with 2 mL PBS-Wash, followed by centrifugation at 400 ×g for 5 min. The supernatant was discarded and the cells were treated with surface monoclonal antibodies conjugated with peridinin chlorophyll (PerCP) and FITC (anti-CD4-PerCP and anti-CD8-FITC, respectively; BD Biosciences) for 30 min. Following incubation, cells were washed twice with 1 mL PBS-Wash, followed by centrifugation at 400 ×g for 5 min. Cells were fixed for 15 min with 150 mL Cytofix solution (BD Biosciences), washed with 2 mL PBS-Wash, and centrifuged at 400 ×g for 5 min. After removal of the supernatant, 300 mL PBS-Wash was added to each tube and the solution loaded onto the FACSCalibur platform.

Evaluation of reactive oxygen species (ROS) levels and cytosolic Ca^{2+} concentrations

Cytosolic and mitochondrial levels of Reactive Oxygen Species (ROS) were measured by flow cytometry, using Dihydroethidium (DHE) (Sigma Aldrich-USA) and MitoSox Red (Thermo Fisher Scientific-USA) probes, respectively, as described before by Melo et al., 2010. PBMCs (10^6 cells/well) were incubated in plates (24 wells) with RPMI 1640 medium and Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film in a humidified CO_2 incubator (5 % CO_2) for 24 h. Cells only with medium were used as positive control. After time incubation, PBMCs were washed with 1 x PBS (2000 rpm/5 min). Pellet of cells was inserted in new plates (24 wells) and cells were incubated in a humidified CO_2 incubator with 5 μM of DHE (40 min) or with 5 μM of MitoSox Red (10 min). After time incubation, cells were centrifuged with 1x PBS (2000 rpm/5 min) and transferred to cytometer tubes. Fluorescence intensity was analyzed using a FACSCalibur flow cytometer (BD Biosciences-USA) with excitation at 488 nm and emission at 620 nm. A minimum of 10,000 events were collected. Results are presented as single-parameter histograms or scatter grams of cellular events versus fluorescence intensity. Cytosolic Ca^{2+} concentrations ($[Ca^{2+}]$cyt) were measured

using Fluo-3AM (5 μM) probe (Thermo Fisher Scientific-USA). PBMCs (10^6 cells/well) were incubated in plates (24 wells) with RPMI 1640 medium and of Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film in a humidified CO_2 incubator (5 % CO_2) for 24 h. Cells only with medium were used as positive control. After time incubation, PBMCs were washed with 1 x PBS (2000 rpm/5 min). Pellet of cells was inserted in new plates (24 wells) and cells were incubated in a humidified CO_2 incubator with 5 μM of Fluo-3 AM containing 1 μM of pluronic acid F-127 (Sigma Aldrich-USA) and 30 μg/mL of Bovine Serum Albumin (BSA) (Sigma Aldrich-USA) in a humidified CO_2 incubator (5 % CO_2) at 37 °C for 40 min. After incubation time, cells were centrifuged with 1x PBS (2000 rpm/5 min) and transferred to cytometer tubes. Maximum fluorescence was measured using tubes with cells incubated with ionomycin (1 μM) for 2 min and minimal fluorescence was measured using tubes with cells incubated with EDTA (8 mM) also in 2 min of incubation. Fluorescence intensity was analyzed using a FACSCalibur flow cytometer (BD Biosciences-USA) with excitation at 395 nm and emission at 525 nm. A minimum of 10,000 events were collected. $[Ca^{2+}]_{cyt}$ was calculated through equation:

$$[Ca2+]_{cyt} = Kd(F_{sample} - F_{min})/(F_{max} - F_{sample})$$

where F is Fluorescence and Kd value is 390 nM (MELO et al., 2010; TSIEN, 1988; DEGASPERI et al., 2006). Results are presented as single-parameter histograms or scatter grams of cellular events versus fluorescence intensity.

Determination of the mitochondrial transmembrane potential (ΔΨm)

Changes in mitochondrial transmembrane potential (ΔΨm) were measured using MitoStatus probe (BD Biosciences-USA). PBMCs (10^6 cells/well) were incubated in plates (24 wells) with RPMI 1640 medium and Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film in a humidified CO_2 incubator (5% CO_2) for 24 h. Cells only with medium were used as positive control. After time incubation, splenocytes were washed with 1x PBS (2000 rpm/ 5 min). Pellet of cells was inserted in new plates (24 wells) and cells were incubated with 100 nM MitoStatus in a humidified CO_2 incubator (5% CO_2) at 37 °C for 30 min. After incubation time, cells were centrifuged with 1x PBS (2000 rpm/5 min) and transferred to cytometer tubes. Fluorescence intensity was analyzed using a FACSCalibur flow cytometer (BD Biosciences-USA) with excitation at 488 nm and emission at 620 nm. A

minimum of 10,000 events were collected. Results are presented as single-parameter histograms or scatter grams of cellular events versus fluorescence intensity.

Statistical analysis

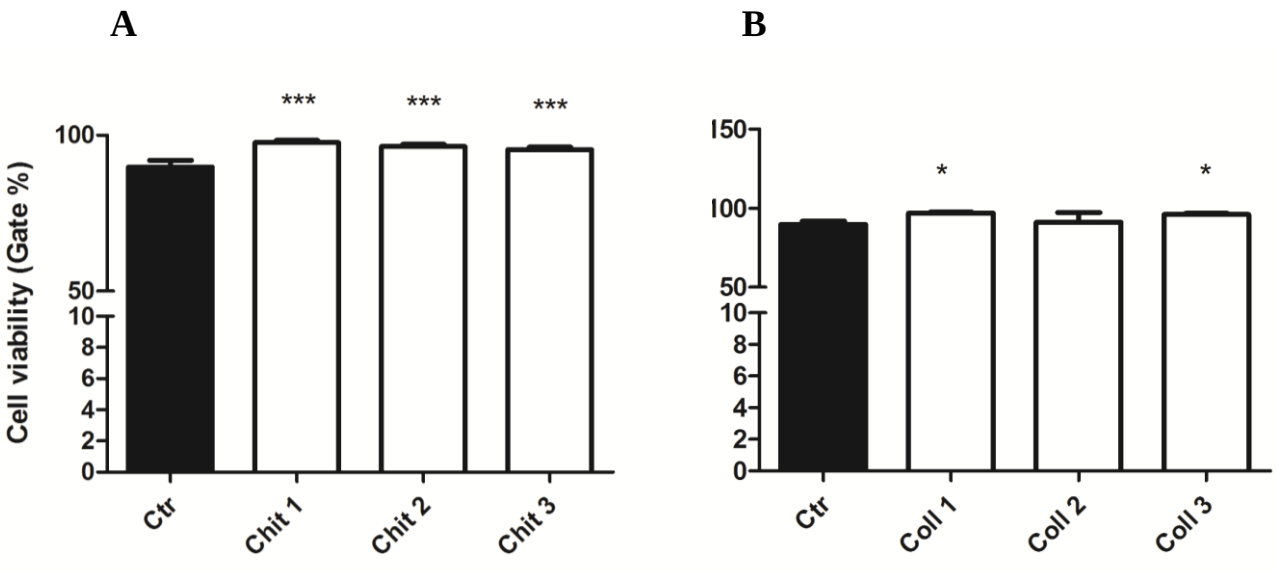
The statistical difference between two groups was analyzed by Student's T-test and among more groups by ANOVA. Significance level considered was 5 %. GraphPad Prim 5.01 software was used in statistical tests.

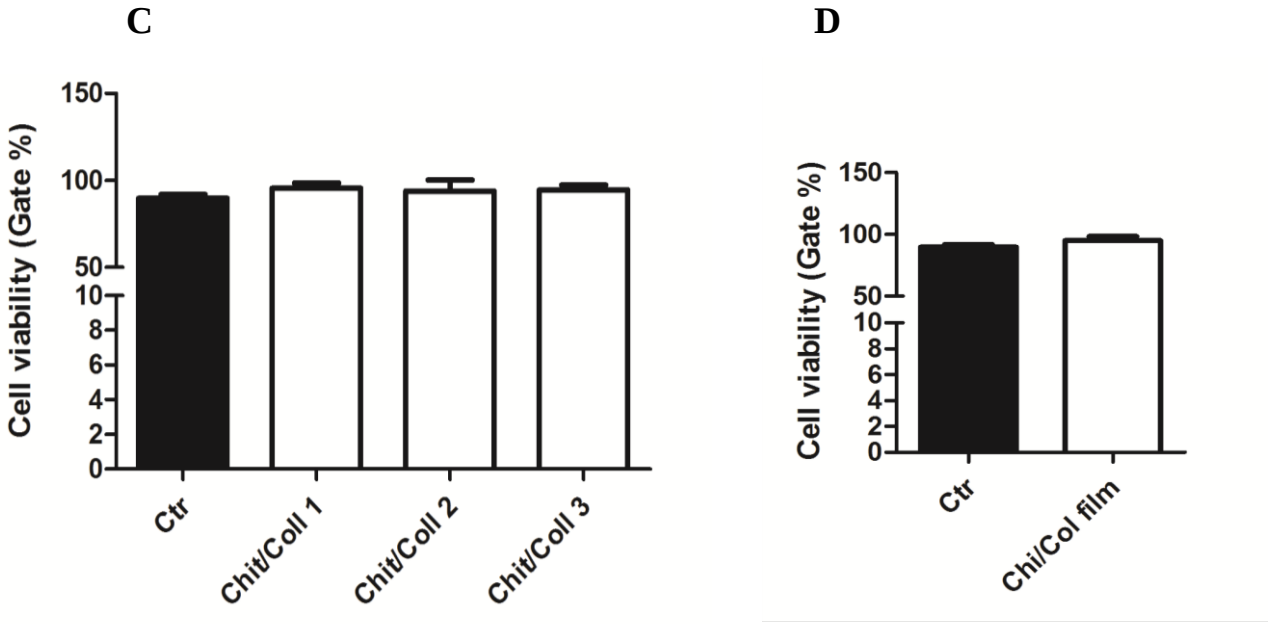
Results and Discussion

Evaluation of cytotoxicity of filmogenic solutions and film against PBMCs

PBMCs necrosis rate (%) was evaluated in the presence of Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film and the results are shown in figure 1. The principal cells involved in the repair of a dermal wound include peripheral blood mononuclear immune cells (PBMCs), fibroblasts and keratinocytes. There may be benefits for having elevated PBMCs during the initial stages of wound healing, in order to mitigate infection (LEVI-POLYACHENKO et al., 2016). Neither filmogenic solutions nor the film were able to promote significant death in lymphocytes when compared to control, which proved that the samples were nontoxic for the PBMCs.

Figure 1. Cell death investigation promoted by solutions and film: A - Chit 1, 2 and 3, B - Coll 1, 2 and 3, C - Chit/Coll 1, 2 and 3, and D - Chi/Col film using and propidium iodide staining. The bars represent three independent experiments. Significance differences values between all groups were considered as ***p < 0,0001 and *p < 0,01.





Chitosan and chitosan-based materials are reported to have a relation between its high degree of deacetylation and an effect on viability of fibroblast cells (Grobler et al., 2008). It seems that as higher is the deacetylation degree, better is for cell viability. Therefore, it is possible to relate the high deacetylation degree of chitosan used in this experiment with the low rate of cell necrosis observed in cytotoxicity test. Brito et al. (2013), using collagen and chitosan nanostructured membranes, also showed a non-toxic characteristic for human fibroblasts cells.

Measurement of cytokines levels

Cytokines play an important role in inflammatory and immune response, and they regulate both innate and adaptive immunity (CHANGSONG et al., 2019). The production of cytokines was determined to evaluated the functionality of the cells treated with Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film. All samples were able to stimulate the production of some cytokines by PBMCs (figure 2). Nevertheless, not all cytokines were produced with significant difference in relation to the control. All samples had the ability to induce the production of TNF-α, which can promote wound healing by indirectly stimulating inflammation and increasing macrophage produced growth factors (BARRIENTOS et al., 2008).

The results presented a not significant increase of IL-6 and IL-10 with statistical difference from the control (except for Chit 1 and Chi/Col film that did not exhibit difference

in relation to control). IL-6 has been shown to be important in initiating the healing response. Its expression is increased after wounding and has a mitogenic and proliferative effect on keratinocytes and is chemo attractive to neutrophils (BARRIENTOS et al., 2008). IL-10 family cytokines exert essential functions to maintain tissue homeostasis during infection and inflammation through restriction of excessive inflammatory responses, upregulation of innate immunity, and promotion of tissue repairing mechanisms (OUYANG & O'GARRA, 2019).

An increase in production of TNF-α and IFN-γ was also reported for Chit 1, 2 and 3, Coll 1, 2 and 3 and Chi/Col film samples and just Coll 1, 2 and 3 showed a production of IFN-γ with no statistical difference from the control. In addition, a higher production of IL-17 compared to control was found in the presence of chit 1, 2 and 3, and Chit/Coll 1, 2 and 3.

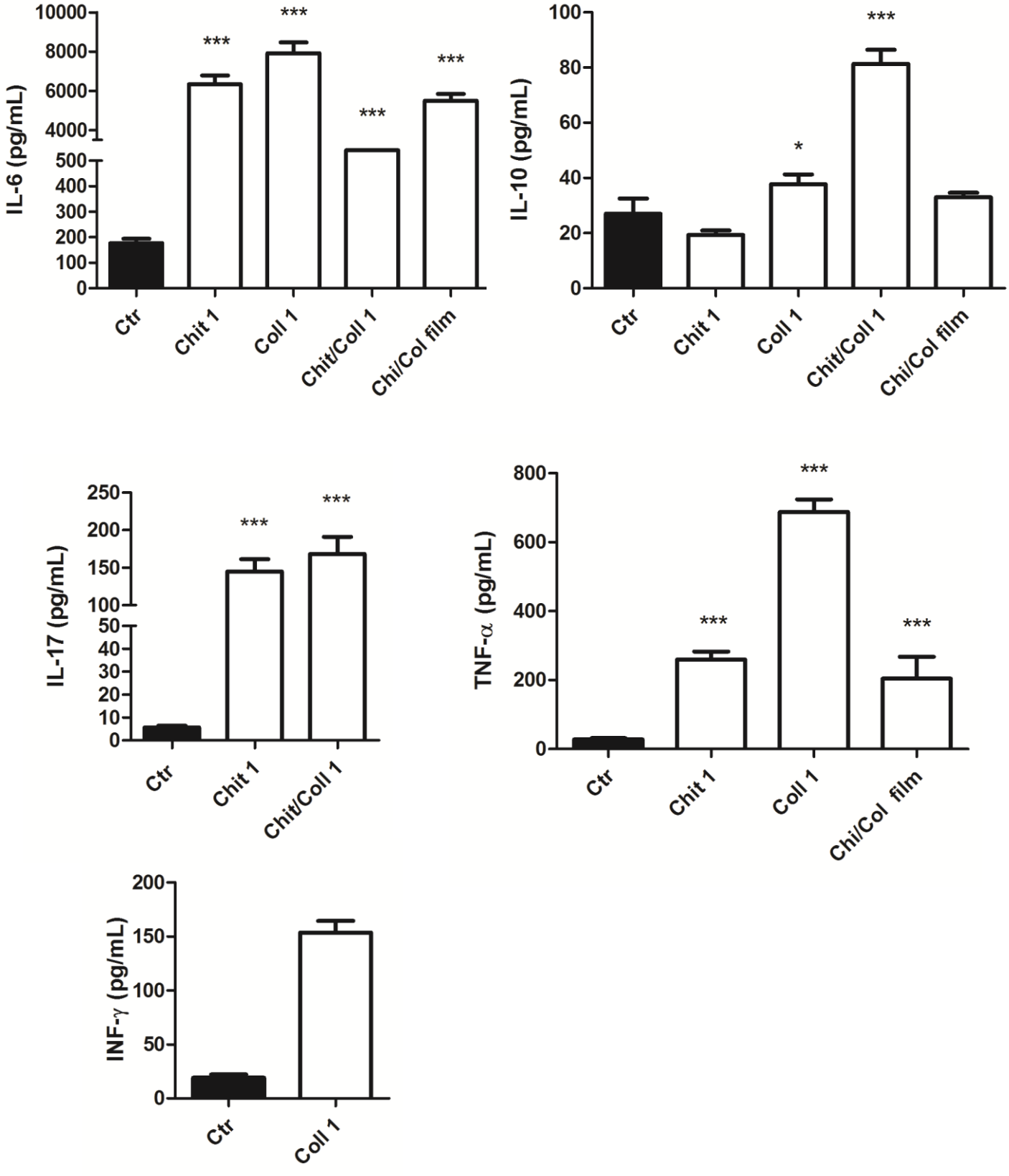
Liu and Sun, 2019, showed that in presence os hydrolyzed fish collagen induced the production of IL-6 in the osteogenically differentiated cells and a slight, but non-significant, increase in the level of IL-10. Jellyfish collagen also stimulated production of cytokines such as tumor necrosis factor (TNF-α) and interferon γ (IFN-γ) by human peripheral blood lymphocytes (SUGAHARA et al., 2006), enhanced the antigen specific immune response *in vivo* (MORISHIGE et al., 2011) and highly stimulated production of TNF-α and IL-6 by J774.1 cells in mouse primary peritoneal macrophages (PUTRA et al., 2012).

It is likely that the stimulating effect of chitosan on the number of B splenocytes is due to the activation of T lymphocytes and macrophages through the cytokine cascade (IL-1, CSF and IFN-γ) that is enhanced by this agent. These factors activate the functions of polymorphonuclear cells (PMNs) and macrophages. Interferons have a significant effect in B-cell growth and differentiation (OBMIŃSKA-MRUKOWICZ; SZCZYPKA; GAWĘDA, 2006).

The immunostimulatory effect of chitosan derivatives has been attributed to the presence of the N-acetyl-D-glucosamine groups, which act by stimulating immune cells, such as neutrophils, macrophages and fibroblasts (PORPORATTO et al., 2003). Chitosan also increases the production of interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), nitric oxide (NO) and interleukin-6 by macrophages (UENO et al., 2001a).

Cytokines are secreted from an inflammatory response at sites of infection and injury, and favors the healing cascade (OLIVEIRA et al., 2011). Cytotoxic tests made with chitosan-collagen films have shown that IL-6 cytokines are released from fibroblasts after exposure to collagen, and these are growth mediators and differentiation of numerous cell types (SUN et al., 2009).

Figure 2. Evaluation of the effect of the treatment on the production of cytokines on PBMCs cultures in 24 h of assay in comparison with control cells. The bars represent three independent experiments. Significance differences values between all groups were considered as ***$p < 0{,}0001$ and *$p < 0{,}01$.

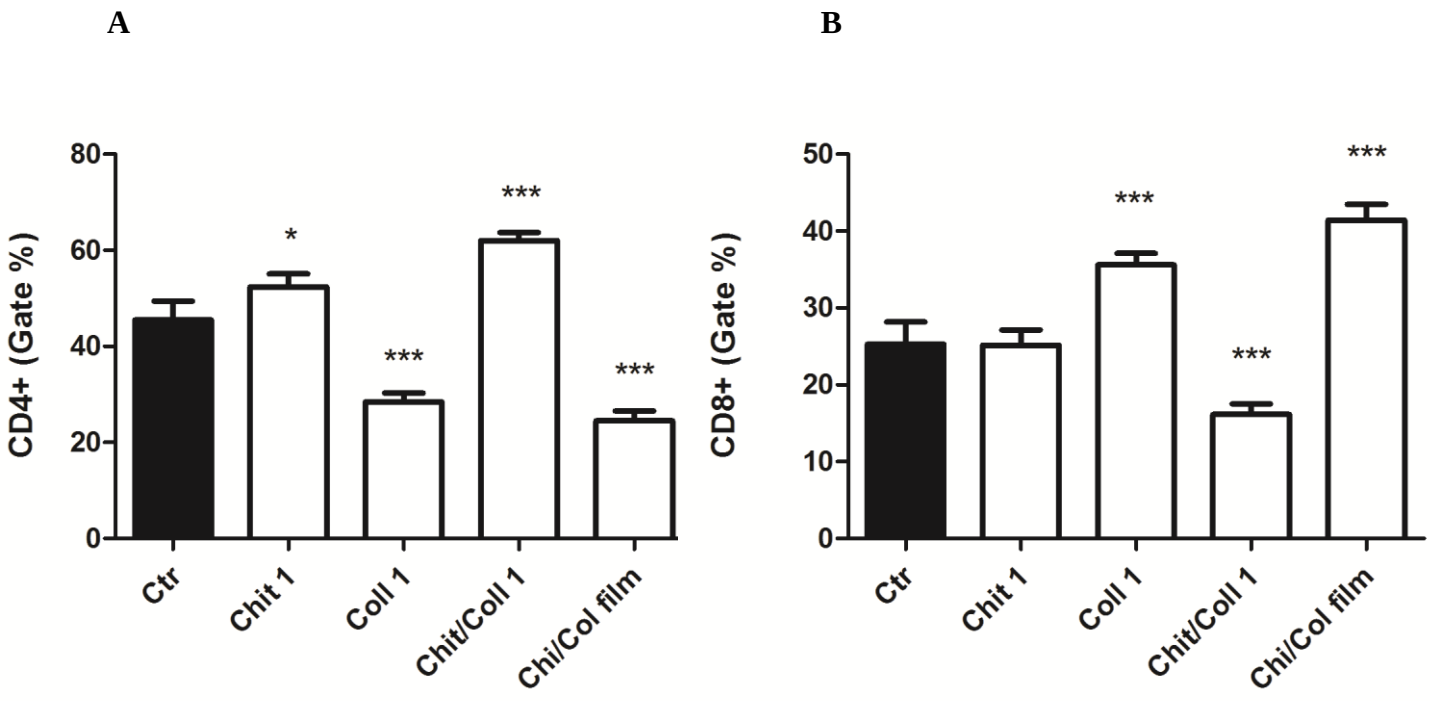


Lymphocytes immunophenotyping assay

T cells are divided into two types: $CD4^+$ T cells and $CD8^+$ T cells. $CD8^+$ T cells are cytotoxic T cells, which express the CD8 surface glycoprotein. $CD4^+$ T cells, also termed T helper cells, express the CD4 surface glycoprotein and help other T cells in directing B- and T-cell responses (LIU et al., 2011). Lymphocyte immunophenotyping assay showed that Chit 1, 2 and 3, and Chit/Coll 1, 2 and 3 stimulate the proliferation of $CD4^+$ and Coll 1, 2 and 3, and Chi/Col film stimulate the proliferation of $CD8^+$ as compared to control (Figure 3).

$CD4^+$ Th cells are required for effective immunity against an array of pathogens from protozoa, viruses, bacteria through to helminths, and natural killer (NK) cells. Is the main Th1 cytokine responsible for macrophage activation and differentiation as well as for the induction of the expression of pro-inflammatory proteins, while $CD8^+$ are critical for effective defense against intracellular pathogens and cancer (MORAN et al., 2018; BRITO et al., 2017). The results found for the samples in this study prove that chitosan and collagen could improve proliferation of $CD4^+$ and $CD8^+$ and aggregate its immunomodulatory effects by being blended in a solution or a film.

Figure 3. Samples stimulation of the proliferation of T $CD4^+$ (A) and $CD8^+$ (B) subsets as compared with the control. The bars represent three independent experiments. Significance differences values between all groups were considered as ***$p < 0{,}0001$ and *$p < 0{,}01$



Changes in ROS, in cytosolic calcium levels and in mitochondrial membrane potential

These analysis are mostly used to investigate changes in mitochondrial membrane potential, ROS release and cytosolic calcium $[Ca^{2+}]^{cyt}$ levels, to evaluate if a compound is able to promote cellular activation, without damaging it (CRUZ FILHO et al., 2019).

The results obtained in this study showed that cells were activate in presence of Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film without being harmful (results confirmed with cytotoxicity assay). All samples were able to produce cytosolic and mitochondrial ROS (except for Chi/Col film in cytosolic ROS production and Chit 2 for mitochondrial ROS production) (figures 4A and 4B).

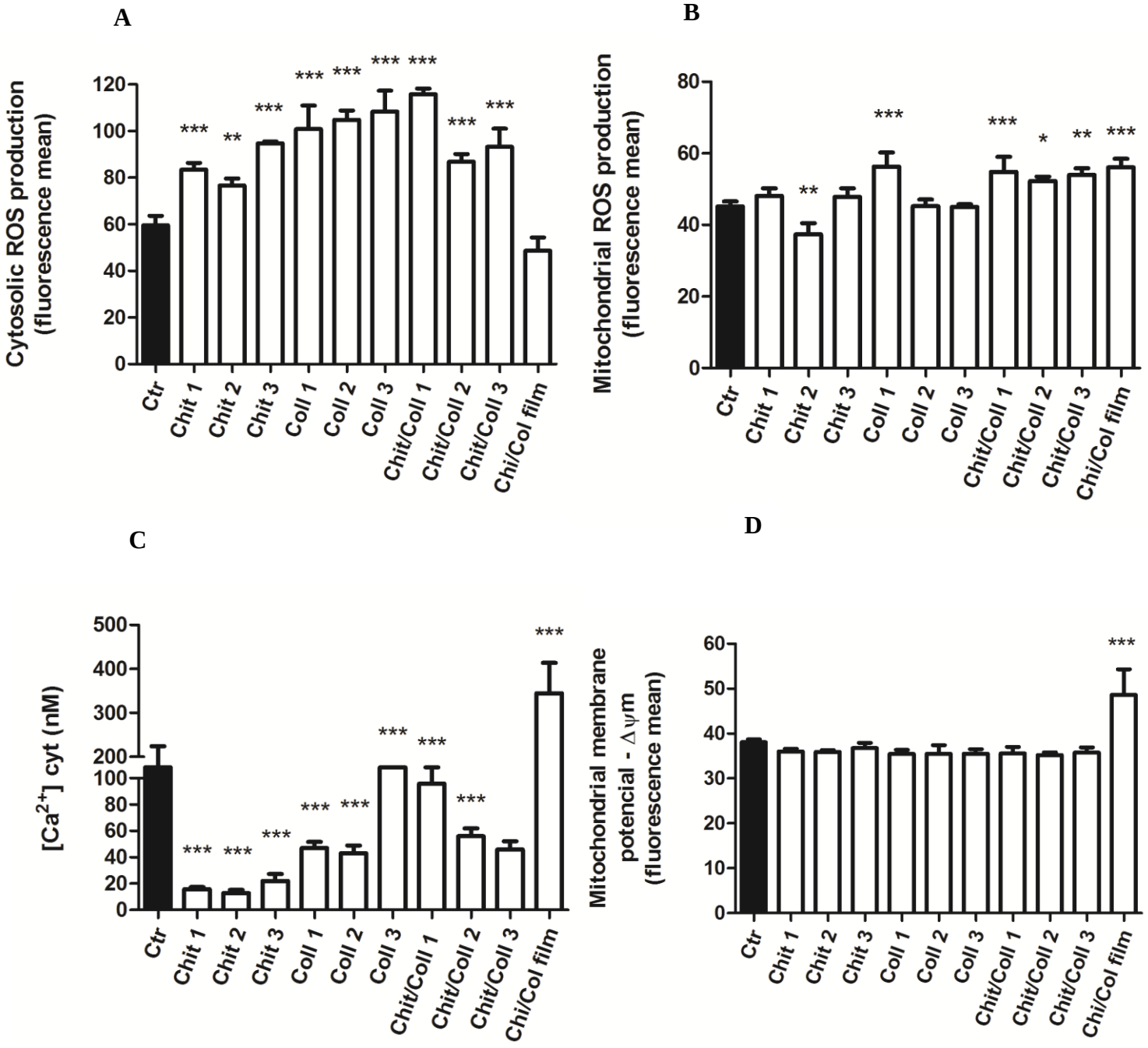
ROS play a vital role in the development of many inflammatory diseases and tumors (DAVALLI et al., 2016; SÁ JR. et al., 2017). It is reported that ROS trigger pro-inflammatory processes (LI et al., 2016). Zhang et al. (2019) hypothesized that the up-regulation of the ROS levels might be associated with the increased secretion of pro-inflammatory cytokines. These researchers also related that the collagen-stimulated generation of ROS regulates signal transduction in platelets. The collagen-induced platelet aggregation has been shown to be closely associated with H_2O_2 production (Zhang et al., 2019).

Moran et al. (2018) showed that ROS production has also been implicated in type I IFN production in response to chitosan and it is possible that the generation of ROS in presence of chitosan could be a shared attribute of both signaling pathways and the point at which both pathways diverge.

A high value of cytosolic calcium release, in comparison with control, was only observed in cells treated with Chi/Col film (Figure 4C). The level of $[Ca^{2+}]$cyt can signal for cell activation, differentiation, proliferation or apoptosis depending on the concentration (MELO et al., 2010). Since there was no significant increase in necrosis rate of cells cultured in presence of the film, we can conclude that the increase in the level of $[Ca^{2+}]$cyt is an indication that the film was providing cell activation and/or proliferation.

A significant increase of mitochondrial membrane potential in comparison to control was also only observed in presence of Chi/Col film (Fig. 4D), confirming its potential to activate the cells. All these combined results could be affirmative for the potential of Chit 1, 2 and 3, Coll 1, 2 and 3, and Chi/Col film to promote oxidative stress without causes PBMCs damage.

Figure 4. Oxidative stress measurement promoted Chit 1, 2 and 3, Coll 1, 2 and 3, Chit/Coll 1, 2 and 3, and Chi/Col film, in PBMCs cultures in 24 h of assay. Induction of cytosolic (A) and mitochondrial (B) ROS levels. Induction of cytosolic calcium release (C) and significant values of ΔΨm (D). The bars represent three independent experiments. Significance differences values between all groups were considered as ***$p < 0{,}0001$, $p < 0{,}001$ and *$p < 0{,}01$.



5 ARTIGO 3 - EVALUATION OF CHITOSAN/COLLAGEN/TRYPSIN FILMS AND THEIR POTENTIAL FOR WOUND HEALING

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Abstract

The potential to accelerate wound healing process in Wistar male rats was tested using Chitosan/Collagen and Chitosan/Collagen/Trypsin films produced from fishery industry subproducts. Hemmaglutination test was carried out with chitosan and collagen filmogenic solution in different concentrations 10, 5, 2.5, 1.25, 0.625 mg/mL and hemagglutination activity was observed, comparing with control behavior. Antimicrobial activity of filmogenic solutions was also tested and chitosan (3 mg/mL) and Chitosan/Collagen (2, 4 and 6 mg/mL) showed a notable inhibitory activity in *Staphylococcus aureus* and *Staphylococcus epidermidis* growth compared to a positive control with Penicillin antibiotic. Protein release was tested to observe if collagen and trypsin were being released from the films in different time intervals and the result showed an increase in supernatant protein content in 3h and 24h of PBS buffer incubation. A specific trypsin activity was measured in the same interval and showed that trypsin maintained enzymatic activity in all tested hours with a small decrease rate. In wound healing procedure, chitosan/collagen (chi/col film) and chitosan/collagen/trypsin (chi/col/try film) films showed a higher wound contraction (73.04 ± 7.8 and 77.43 ± 2.37 %, respectively) in comparison with control treated only with saline buffer (54.5 %), after 6 days. Also, treated-film groups showed a faster remodeling process of wound tissue. These findings demonstrated that an effective wound dressing could be produced from fishery industry subproducts.

Keywords: Biopolymers, Fishery industry, wound dressing.

Introduction

The skin is a dynamic protective tissue that acts as a tough barrier to prevent invasions of various pathogens and hazardous substances, and also serves as a mechanical absorber (PRODPRAN; BENJAKUL; ARTHARN, 2007). However, the skin is subjected to various injuries by physical or chemical means. A damaged or wounded area of the skin initiates a series of complicated and well-designed events to reestablish its integrity and to restore normal function (HOSSEINI et al., 2013).

Wound healing is a complex and dynamic biological process that involves cells, mediators, growth factors, and cytokines (KIM et al., 2012). Typically, the healing of a wound injury proceeds through three distinctive overlapping major stages, as follows: inflammation, formation of new tissue in the processes of extracellular matrix (EMC) deposition and epithelialization, and tissue remodeling (GURTNER et al., 2008; YATES et al., 2007).

In order to have a fast and effective tissue restoration via the healing process, an appropriate microclimate with optimal moisture and oxygen levels and physical barriers to pathogenic microorganisms are critical (ISHIHARA et al., 2002). Recently, tissue-engineered substitutes have become an option to regenerate skin tissue, particularly for skin defects caused by burns, trauma, or non-healing chronic wounds. (CHANDIKA; KO; JUNG, 2015)

Hence, the use of biologically active natural and synthetic materials has also evolved in wound healing and skin tissue regeneration applications. As per current scenario, waste utilization strategy includes recovery of value added products from fishery industry waste as it is potential source of proteins, minerals, bioactive peptides, fish oils, enzymes, amino acids and others biomaterials, which present so many characteristics that could be applied in skin wound healing and skin tissue regeneration (BHUIMBAR; BHAGWAT; DANDGE, 2019).

The use of relatively low cost, low pollution biomaterials with specific properties has great potential for the development of a new generation of prosthetic medical suppliers (RAMASAMY & SHANMUGAM, 2015). Collagen is a protein that naturally occurs in human body and is able to interact with cells and regulates cell anchorage, migration, proliferation and survival (MICHALSKA-SIONKOWSKA; WALCZAK; SIONKOWSKA, 2017). Chitosan is a linear polysaccharide obtained during deacetylation of chitin. Chitosan used as component of wound dressing can prevent wound dehydration, can accelerate tissue regeneration as well as can inhibit infection by stimulating the macrophages to release cytokines (ORYAN & SAHVIEH, 2017).

Also, trypsin (E.C. 3.4.21.4) is an enzyme that maintains its catalytic efficiency in different situations, such as changes in temperature and pH and other factors. Its application in healing dressings has also been investigated and this enzyme has been shown to be effective as a biomaterial since it helped the healing of skin lesions in mice (MONTEIRO et al., 2007).

The aim of the study was to investigate clinical and histological aspects of cutaneous wounds performed experimentally in healthy male Wistar rats evaluating the effects of films composed by chitosan/collagen or chitosan/collagen/trypsin as potential biopolymers to injury repair as wound dressing.

Material and Methods

Extraction and purification of chitosan from Pacific white shrimp (*Litopennaeus vannamei*)

The heads of the Pacific white shrimp were provided by Noronha Pescado Ltda, and stored in a freezer for the following steps. Then, enzymatic hydrolysis was performed at 40 °C for 3 hours with subsequent inactivation of the enzymes at 100 °C for 10 minutes. The peels were washed with distilled water, oven dried and stored at room temperature. Subsequently, they were submitted to a purification process, according to the methodology of Cahú et al. (2012), with slight modifications. After purification, chitosan was resuspended in 1 % acetic acid (w/v) to be in a final concentration of 10 mg/mL. Chitosan sample was characterized by Fourier transform infrared spectroscopy (in KBr pellets, range 4000 – 400 cm^{-1}) in a FT-IR Bomem MB100 spectrophotometer. The degree of deacetylation (DD) was calculated by infrared spectra, according to Brugnerotto et al. (2001) and determined to be 79.6 % ± 0.5%.

Obtaining collagen from Nile Tilapia skin (*Oreochromis niloticus*)

The specimens of Nile tilapia fish (*Oreochromis niloticus*) were provided by Noronha Pescado Ltda. The fish skin was removed and kept at 4 ºC until collagen extraction. Initially, the skin was cut into small pieces and stirred in 0.1 M NaOH solution for 1 hour (three changes) at 4 °C, for removing soluble proteins from fish skin. After the last base change, 10 % butyl alcohol (to clean fat) was added and stirred for 12 h at 4 °C. Then 1 L of 0.5 M acetic acid was added for 12 h (two changes) at 4 °C. A change in collagen pH (around 8.0) was induced until its precipitation and then all samples was centrifuged. Thereafter, dialysis and lyophilization were performed. Lyophilized collagen was resuspended in 1 % acid acetic

(w/v) to preparer a solution with final concentration of 10 mg/m Land then was characterized by Fourier transform infrared spectroscopy (in KBr pellets, range 4000 – 400 cm^{-1}) in a FT-IR Bomem MB100 spectrophotometer and by SDS-PAGE electrophoresis were performed according to Laemmli method (1970).

Trypsin extraction and purification

Specimens of Nile tilapia (*Oreochromis niloticus*) were kindly provided by Noronha Pescado Ltda and were sacrificed in an ice bath and the intestine was collected. Immediately after desiccation, the tissues were homogenized in 100 mM Tris-HCl pH 8.0, using a tissue homogenizer (IKA RW 20D S32, China). The resulting homogenate was centrifuged (Sorvall RC-6 Superspeed Centrifuge – North Carolina, USA) at 10,000 xg for 15 min at 4 °C to remove cell debris and nuclei. The supernatant (crude extract) was used for the further purification steps (BEZERRA et al., 2005).

The enzyme was purified following four-step procedure described by Marcuschi, 2010 with slight modification. The crude extract was first incubated for 30 min at 45 °C (heat treatment) and centrifuged at 10,000 xg for 15 min at 4 °C. The supernatant was collected and fractionated with ammonium sulphate for 2 h at 4 °C for the final salt saturation of 0–30% and 30–60%. After each fractionation, the extract was centrifuged at 10,000 xg for 15 min at 4 °C, the pellets were collected, resuspended with 4 mL of 100 mM Tris-HCl pH 8.0 and dialyzed against this same buffer for 24 h at 4 °C. After dialyzation step, the sample was placed on an affinity column (2 cm^3, 1 mL) of benzamidine-agarose, Sigma-Aldrich, USA). The fractions with trypsin activity were pooled and dialyzed against 10 mM Tris-HCl pH 8.0, lyophilized and resuspended on 100 mM Tris-HCl pH 8.0 for further assays.

Film obtainment

Collagen (1 % w/v) and chitosan (1 % w/v) solution was prepared separately by dissolving it in 1% v/v aqueous acetic acid. Both solutions, with final concentration of 10 mg/mL, were stirred overnight using magnetic stirrer, in room temperature for chitosan and 4 °C for collagen. After solubilization, chitosan and collagen solution were mixed and cast in molds (lids of microplates of 24 and 96 wells) and placed in dry oven at 37 °C until being dryed. The chitosan/collagen discs were then neutralized with 0.3 M NaOH in ethanolic solution (1:2 water:ethanol) then washed with distilled water and dried. To complete discs formation, the films were place in the fridge overnight and were kept at 4 °C until being used. The discs areas were 190.79 mm^2, for tests *in vivo* and 34.79 mm^2, for tests *in vitro*. Same

parameters and procedures were carried out to produce films including trypsin (1 mg/mL) from Nile tilapia intestine. Films were named as chi/col film, containing chitosan and collagen, and chi/col/try film, containing chitosan, collagen and trypsin.

Characterization of the films

Haemagglutination Test

Peripheral blood samples were collected from healthy donors in plastic tubes containing 3.2% sodium citrate (1:10) and homogenized manually for 1 minute prior to avoid coagulation. For preparation of red blood cells suspension (RBC) whole citrated blood was centrifuged 300 g for 5 min at 18°C for separation of RBC and supernatant plasma. RBC were washed several times with sterile PBS and stored at 4°C as a 10% (v/v) suspension as a stock solution until use within one week. Hemaglutination assay was carried out in round-bottomed 96-well plates with a serial 2-fold sample dilution by adding 50 µL PBS in each well. Samples were placed in the first column (50 µL), homogenized and transferred to the adjacent well on the same row until the end of the row, discarding the last volume. Then, 50 µL of RBC 2.5% (v/v) prepared from stock solution was added to each well and the plate was mixed gently, and left in room temperature for 60 min to develop. Negative results were observed as dark spots in the center of the well and positive results formed a uniform reddish color across the well.

Antimicrobial activity

Antimicrobial activity was tested using *Staphylococcus aureus* (UFPEDA 02) and *Staphylococcus epidermidis* (UFPEDA 08) strains, provided by Departamento de antibióticos, Universidade Federal de Pernambuco, Brazil in Difco Nutrient Agar and stored at 4°C. To each well was added (n=6) 200 µL of bacteria cultures (in Muller Hilton Nutrient Broth) and chitosan (0.2 % w/v), collagen (0.2 % w/v), chitosan/collagen (0.125 % v/v) and chitosan/collagen (0.1 % v/v) solutions. Inhibitory assays on bacterial growth were performed in sterile 96 well microplates and the measurement of absorbance at 600 nm was made using a microplate spectrophotometer (xMark™, Bio-Rad) every hour during 10 hours and in 24 and 48 h at 37 °C. A negative control was carried out using bacterial suspensions in Muller Hilton Nutrient Broth, and a positive control was carried out using penicillin.

Protein release and specific trypsin activity in the films

Protein release from the chi/col/try films was tested in triplicate in different time intervals. Films were placed in microtubes with 1 mL of 0.1 M Tris-HCl buffer pH 8.0 for 24h. The total protein content of the samples was estimated (Smith et al., 1985) using a Pierce® BCA kit for protein analyses (Thermo Scientific, Waltham, MA, USA) in different time intervals, 1h, 3h and 24h. Trypsin, release from the films were tested to show if it still present tryptic activity following the procedure described by Bezerra (2001), with the chromogenic substrate N-α-benzoyl-L-arginine p-nitroanilide (BApNA), using a microplate reader (BioRad Model X-MarkTM, Brazil). One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing one μmol of BApNA per minute under the established conditions, using the molar extinction coefficient of 9,100 mM^{-1} cm^{-1}.

Wound healing property of films

Rat Model of Wound Healing

All animal treatments were carried out according to ethical principles for experimental work (Brazilian Society for Science of Laboratory Animals, SBCAL, São Paulo, Brazil, 2013) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The experimental procedure was approved by the Ethic Committee of the Federal University of Pernambuco, Brazil (Process No. 0035/2019). Three treatments were performed, named group control (with 0.9 % saline buffer without film), group 1 (chi/col film) and group 2 (chi/col/try film).

In each experiment, 3 adults male Wistar rats (350 g, 90 days old) were anesthetized; the dorsal area was trichotomized and cleaned with povidone-iodine and then 70% ethanol (v/v). Skin was excised from the back below the neck using a steel punch (1 cm), creating a full-thickness wound. The film was applied over the wounds and immediately adhered to the wound cavity. Animals were maintained in individual cages with full access to water and food. After 3, 6, 9 and 14 days, lesions were measured (length and width) and removed with at least 2 mm of border skin using surgical scissors. The percentage of wound contraction for circular areas was calculated according to the formula:

Wound contraction (%) = (wound area day 0 – wound area day 6/wound area day 0)*100

Histopathological section

Immediately after removal, skin samples were placed onto squares of filter paper and fixed in buffered (PBS pH 7.2) 10% formalin solution. After a maximum of 48 h, histological

slices were prepared (dehydrated in a graded ethanol series, cleared in xylene) and then embedded in paraffin wax. Sections (5-μm thick) were stained with hematoxylin and eosin (H&E) and mounted onto glass slides.

Results and Discussion

Haemagglutination Test

Chitosan, collagen and chitosan/collagen samples were tested for hemagglutination test. Chitosan and chitosan/collagen samples were capable of agglutination at concentration of 10, 5, 2.5, 1.25, 0.625 mg/mL. At low concentrations, the solution did not cause hemagglutination (0.3125 to 0.0097 mg/mL). Collagen did not cause hemagglutination at any concentration.

The hemagglutinating activity of chitosan occurs through the interaction of its positive charges with the negative charges of the erythrocyte membrane (RAO & SHARMA, 1997). The result of the association of the two polymers would be the most suitable for this processing, since collagen in the blend would join with the fibrin present at the wound site, forming a protective "network" where the red cells clump together, creating a clot (AMABIS, 2004). The development of a clot also provides a temporary matrix for fibroblasts, endothelial cells and keratinocytes to reach the wound (MANDELBAUM et al., 2003).

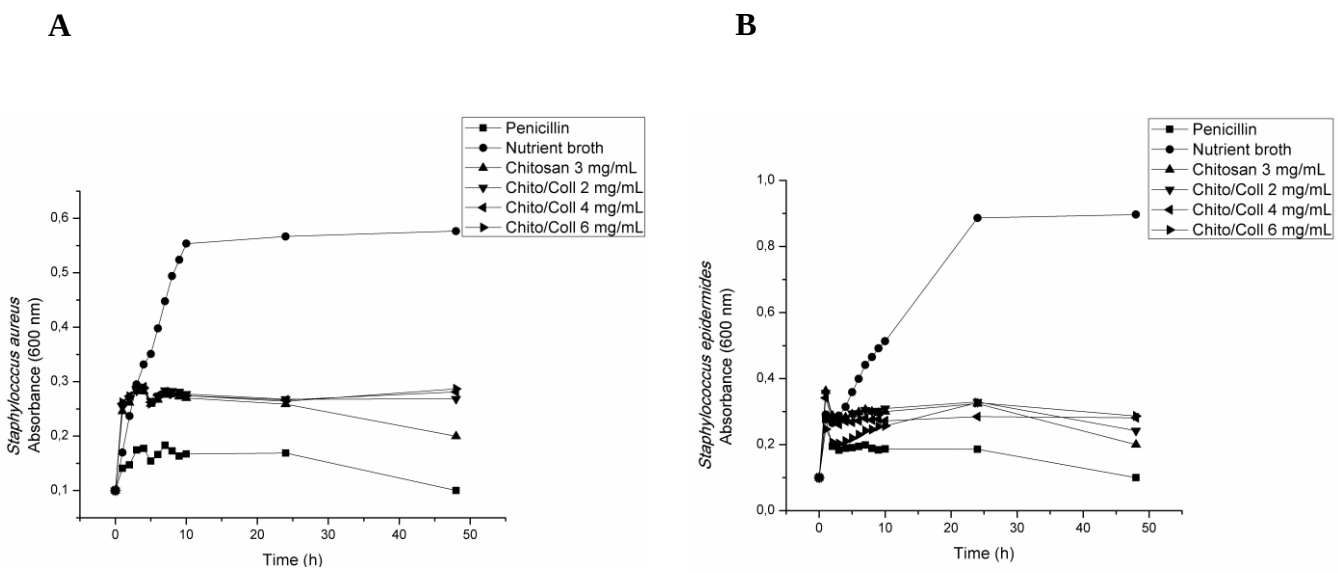
Antimicrobial activity

The antimicrobial activity from filmogenic solutions were evaluated using *Staphylococcus aureus* and *Staphylococcus epidermidis* strains (figure 1A and 1B, respectively). Chitosan (3 mg/mL), and chitosan/collagen (2, 4, and 6 mg/mL) solutions inhibited the growth of these bacteria.

Wounds are characterized by skin damage with the loss of the normal skin functions, in particular, its self-defense capabilities. In addition to being unable to serve as a barrier for bacterial invasion, the injured part of the body generally exudes a large amount of fluid, which together with the warm body temperature and rich nutritional components serves as an ideal place for bacterial growth, leading eventually to wound infection and cross infection in hospital wards. Of the various types of bacteria that can cause wound infection, *S. aureus* is a gram-positive bacterium that exists as a skin commensal, with the ability to cause a wide range of infections. The most effective way to control the spreading of bacteria from wound sites is to incorporate antimicrobial agents in wound dressings or use antimicrobial biomaterials to produce the dressings (QIN, 2019).

The colonization of the wound by bacteria can interrupt or delay the healing process and even progress to life-threatening complications. The wound dressing can avoid the bacteria penetration and wound colonization through its structural properties (pore size, wettability and surface charge) (JOO & OTTO, 2012; MIGUEL et al., 2017).

Figure 1. Inhibitory activity of *Staphylococcus aureus* growth (A) and *Staphylococcus epidermidis* growth (B) from chitosan (3 mg/mL) and chitosan/collagen (2, 4 and 6 mg/mL) solutions using Penicillin (antibiotic) as a positive control and Muller Hilton Nutrient Broth as a negative control.



Chitosan possess antimicrobial activity against a number of Gram-negative and Gram-positive bacteria (QI, 2004). It is generally accepted that the main antimicrobial effect of chitosan is related to the ability of its positively charged amine groups to bound onto the negatively charge surface of bacterial wall or plasma membrane. This causes a modification of the cell permeability, leading to an osmotic damage with the efflux of ions and proteins from the cytoplasm to the extracellular space (LIU et al., 2004). Therefore, all intrinsic factors of chitosan influencing its positive charge density are of critical importance. In fact, higher the positive charge density of chitosan, stronger the electrostatic interactions with the bacterial cell surface.

Also, some studies in the literature generally agree that chitosan with higher deacetylation degree shows more pronounced antimicrobial activity (KONG et al., 2008; TAKAHASHI et al., 2008; MELLEGÅRD et al., 2011) due to the higher number of free amino groups on the polymer backbone. It was found that Gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumonia* and *S. typhi*) have a higher susceptibility to chitosan compared to

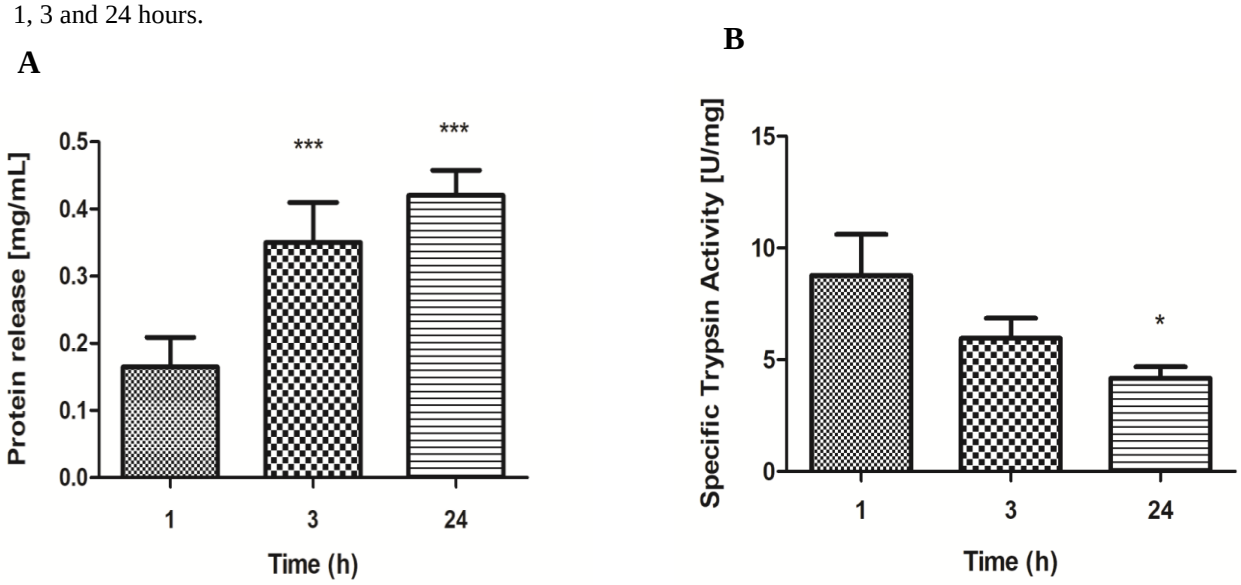
Gram-positive bacteria (*S. aureus, B. cereus, E. faecalis* and *M. luteus*) (YOUNES et al., 2014).

Protein release and specific trypsin activity in the films

To evaluate protein release, chi/col/try films were incubated in PBS buffer for 24 h. The measurement of protein release in different time intervals of 1, 3 and 24 h showed an increase in protein content in supernatant buffer analyzed with the dosage protein kit. It was observed an increase from 0.2 mg/mL to 0.4 mg/mL (100 %) in the initial 3 h of incubation (figure 2A). A small increase was observed in the final of 24 h (0.5 mg/mL). For trypsin specific activity in the same time interval, it was observed a decrease in the activity over time (figure 2B). Chi/col/try film allowed the release of enzyme to the external medium and the initial increase is probably due to the cumulative release of those molecules allocated in film surface.

Monteiro et al., (2007) showed an increase of 121.7 % of swine trypsin release from film of polysaccharide and cellulose in the first 24 h, as we have observed in the present study. Markvicheva et al. (2000) with bovine trypsin immobilized by entrapment in PVCL–Ca alginate and PVCL–chitosan sulphate polymers both magnetized showed a partial enzyme release from the beads in the supernatant and the loss of relative activity was 80 % and 70 %, respectively.

Figure 2. Protein release (A) and Specific trypsin activity (B) from chi/col/try films in different time intervals of 1, 3 and 24 hours.

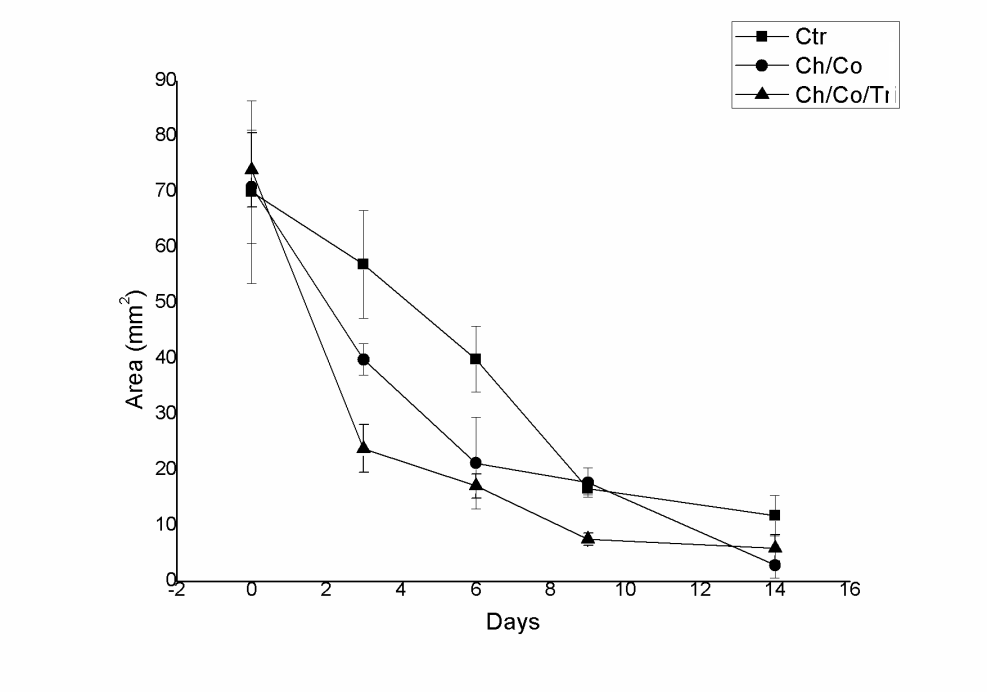


Significance differences values between all groups were considered as ***p < 0,0001 and *p < 0,01.

Wound healing property of films

Chi/col and chi/col/try films were in the healing cutaneous surgically caused in rats and the results were followed in the days 3th, 6th, 9th and 14th after surgery. Figure 3 shows the area of wound contraction in all days studied.

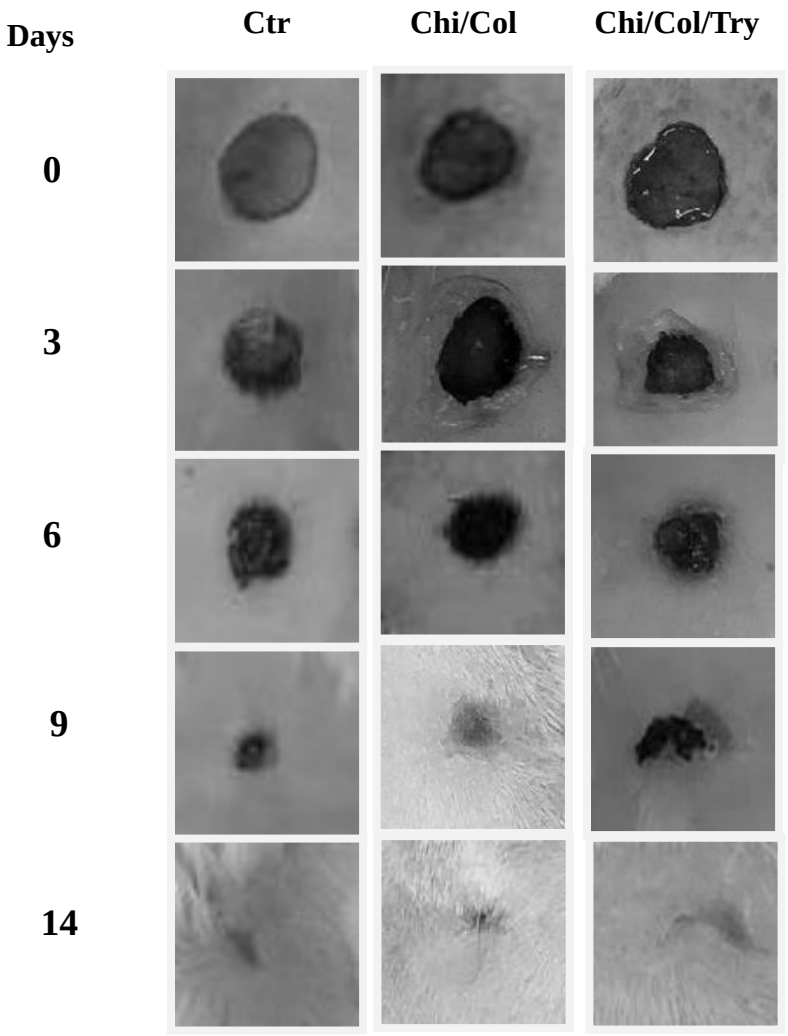
Figure 3. Graphic of the quantification analysis of wound closure areas (mm^2) at 0, 3, 6, 9 and 14 days post-injury in each treated groups chito/coll film, chito/coll/tryp film and control (0.9 % saline buffer).



Wound reduction is a necessary feature of healing process, and the films tested showed an increase in wound reduction when compared to 0.9 % saline buffer control. The contraction percentage of wounds for control group was 54.5 ± 3.7 %, chi/col film was 73.04 ± 7.8 % and higher contraction was observed from chi/col/try film with 77.43 ± 2.37 %. Wound contraction of both film treatment were very similar and significantly higher than the control ($p < 0.003$), which suggest we can assume that the films tested are effective to be a wound dressing of healings.

Moreover, figure 4 showed the images of wounds made by incisions in rats back, and it is possible to observe that the groups with the films showed a higher capability to decrease the wound area in less time and with less scar formation, especially for chi/col/try films.

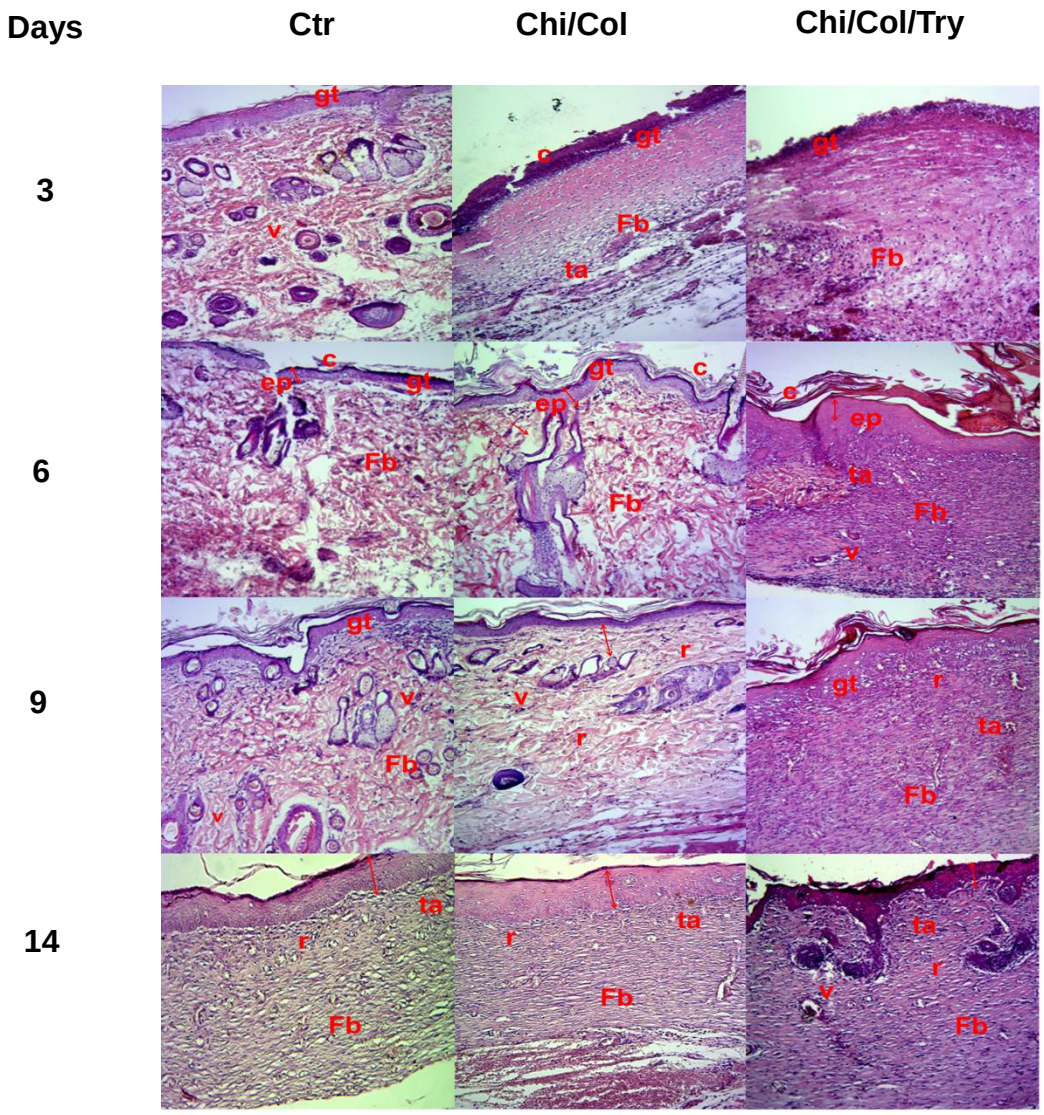
Figure 4. Wound photographs of *in vivo* wound healing effect of control (0.9 % saline buffer), chi/col film and chi/col/try film at 0, 3, 6, 9 and 14 days post-injury.



Before wound contraction analysis animals were euthanized on the 3th, 6th, 9th and 14th days and fragments of tissue biopsy were collected to histological (H&E staining) process. In figure 5 the results were showed in a 100 x magnification in optical microscopy. Histological sections of epithelial samples revealed tissue reepithelization in the fourteen days for control group and chi/col and chi/col/try films groups.

In the 3th day (first day of analysis) it was possible to observe that film groups present a more significant and higher layer of granulation tissue compared with control, also an abundance of fibroblasts cells and crust were observed in treated groups, especially chi/col film group. During stages of wound healings, the granulation tissue newly formed consists of invasion of endothelial cells, macrophages, and fibroblasts to restore tissue integrity (CORIOLANO et al., 2014).

Figure 5. Hematoxylin-eosin stained histological micrographs (magnification of 100 x) from control, chi/col and chi/col/try films groups treated during 14 days. Granulation tissue (gt), Fibroblasts (Fb), Crust (c), Vascularization (v), Transition area (ta), Epithelization (ep), Remodeling tissue (r), Double arrows (new tissue).



The formation of new tissue initiated in day 6 for all groups, including control, but is more evident for treated groups. Chi/col film present a more organized cell and tissue deposition, also the formation of hair follicles, which has been seen as a sign of healthy repair and complete tissue healing of the dermis (TAGHIZADEH & DAVARI, 2006). Chi/col/try showed large amounts of fibroblasts and a great quantity of crust.

In the 9th the remodelation phase starts for film treated groups and the muscle remodelation is more evident for chito/coll group. In the last day (14th) it is possible to

observe that treated groups had a more organized tissue remodelation and a thicker layer of cells forming the new tissue for the group of chi/col films.

These findings suggest that there is some relation to the macroscopic observation saw in figure 4 when the treated groups with films performed a better and faster closure and recuperation of wounds in comparison to the control group, untreated, which leads us to the conclusion that film-treated groups contributed to a faster wound healing process, with more organized re-epitelization and less scar formation.

An ideal wound dressing should be antimicrobial and biocompatible, it should remove excess of exudates and allow gaseous exchange, have suitable mechanical properties, be non-toxic and easily removed without trauma and, most of all, and it should create a proper moisture balance or moist environment in order to enhance healing (COLOBATIU et al., 2019). In this study, it was confirmed that both films used in the treatment present all the features previously cited.

These results could be explained by the fact that in skin wound healing, growth factors act as stimulators of fibroblast proliferation and synthesis of extra cellular matrix (ECM) proteins and chitosan has been reported to indirectly enhance cell proliferation in vivo (AZUMA, 2015). Also, in the inflammatory phase, chitosan accelerates the migration of cells to the wound area, ensuring that macrophages destroy microorganisms, remove dead cells, and stimulate other immune-mediating cells. These stimulated cells help tissue organization and angiogenesis (AZUMA, 2015). Chitosan also interacts with glucosaminoglycans through its cationic charge, and activates growth factors (TORT et al., 2019).

Among the bioactive and biocompatible materials for dressings, collagen is probably the most promising biomaterial, due to the minimal inflammatory response, low cytotoxicity and its property to promote cell growth. In addition to providing strength and integrity to the tissue matrix, collagen also plays a role in hemostasis (LOCKHART et al., 2001).

The collagen triple helix interacts with a large number of molecules that trigger biological events. The interactions of collagen with cell surface receptors regulate many cellular processes, including adhesion, proliferation and migration (LEITINGER & HOHENESTER, 2007).

In recent years, the use of proteases, such as trypsin, has been investigated when applied to skin lesions, with the aim of assessing their potential for healing. These studies demonstrate the efficacy of trypsin in the recovery of injured tissue, since it is able to assist in blood clotting, in the lysis of the fibrin clot and in the transport of membrane proteins (RAO et al., 1998). Studies show the participation of trypsin, together with thrombin, in the

regulation of target cells from the cleavage and activation of proteases coupled to protein G (PARs), which contribute to the defense of the body in response to inflammation and invasion of pathogens (VERGNOLLE et al., 2001).

The presence of trypsin in chi/col films presented bigger tissue fragility with characteristic of a process less developed in the 3th day. However, in the following days, chi/col/try films showed good results, helping to restore tissue formation. Similar results were found in Markvicheva et al. (2000) and Monteiro et al (2006) were a trypsin in hydrogel and film, respectively, present a remarkable accelerated in wound healing in skin excision with same pattern of regeneration.

6 CONCLUSÕES

Os biopolímeros quitosana, colágeno e tripsina foram isolados de subprodutos do processamento do pescado, com um bom grau de rendimento e alto grau de acetilação para a quitosana. Filmes poliméricos à base de quitosana e colágeno foram formados e suas características físicas foram avaliadas. Os filmes de quitosana/colágeno apresentaram bom teor de umidade e alto grau de absorção de líquidos, o que possibilita a drenagem de exsudatos em lesões.

A avaliação da superfície do filme por MEV, BET e BJH demonstrou boa miscibilidade das soluções, além de área superficial e porosidade que podem contribuir para a realocação das células durante a cicatrização tecidual. A análise térmica inferiu que houve boa miscibilidade entre os filmes, pela presença de apenas um pico em aproximadamente 100 ºC e mostrou que o filme manteve até cerca de 34 % de sua massa após incubação a 500 ºC. Os testes mecânicos mostraram que os filmes de quitosana/colágeno têm boa resistência à tração e elasticidade, o que é importante para acompanhar a contração da pele durante o processo de cicatrização.

As soluções filmogênicas e filme de quitosana/colágeno apresentaram efeitos imunomoduladores em células mononucleares de sangue periférico (PBMCs), com boa viabilidade celular e indução na produção de citocinas.

A atividade hemaglutinante positiva das soluções filmogênicas sugere que estas proporcionariam a supressão do sangramento da ferida de maneira mais rápida. As soluções filmogênicas apresentaram atividade antimicrobiana inibindo o crescimento de *S. aureus* e *S. epidermidis* o que contribui para a manutenção de um microamiente livre de infecções para que a cicatrização ocorra mais adequadamente. Filmes formados por quitosana/colágeno e quitosana/colágeno/tripsina foram eficientes no processo de cicatrização em lesões cutâneas tópicas em ratos, com taxa de fechamento das lesões significantemente maiores que o controle.

A análise histológica dos cortes de pele coletados após 4 tempos de tratamento mostrou que houve maior remodelamento e tecido de granulação no grupo tratado com filmes de quitosana/colágeno, mas houve uma formação de crosta mais significativa para o grupo tratato com quitosana/colágeno/tripsina. A avaliação destes filmes como curativo tópico, demonstrou que os subprodutos do processamento do pescado podem ser utilizados como fonte para obtenção de biomateriais com potencial para aplicação na área médica e em engenharia de tecidos.

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ANEXOS A - PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DO CENTRO DE BIOCIÊNCIAS DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (CEUA-UFPE)

Processo n° 0035/2019

Universidade Federal de Pernambuco
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Recife, 02 de julho de 2019

Ofício nº 35/19

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Ranilson de Souza Bezerra**
Departamento de Bioquimica
Universidade Federal de Pernambuco
Processo **nº 0035/2019**

Certificamos que a proposta intitulada **"Imobilização de tripsina do peixe tilápia do Nilo (Oreochromis niloticus) em filmes de quitosana/colágeno e avaliação de seu potencial na cicatrização de lesões cutâneas."** registrado com o **nº 0035/2019** sob a responsabilidade de **Prof. Rannilson de Souza Bezerra** o que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 02/07/2019

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	junho de 2019 a janeiro 2020
Espécie/linhagem/raça	Rato heterogenico.
Nº de animais	96
Peso/Idade	350g/ 90 dias
Sexo	Machos (96)
Origem: Biotério de Criação	Biotério do Departamento de Nutrição
Destino: Biotério de Experimentação	Laboratorio de Enzimologia do Depatamento de Bioquimica.

Atenciosamente,

Prof. Sebastião R. F. Silva
Vice-Presidente CEUA/UFPE
SIAPE 2345691

ANEXO B - MATERIAL ADICIONAL

Figura 1. Aglutinação de hemácias na presence de colágeno, quitosana, quitosana/colágeno em diferentes concentrações (10, 5, 2.5, 1.25, 0.625, 0.31, 0.16, 0.08, 0.04, 0.02 2 0.01 mg/mL) comparando ao controle.