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NÍVEL MESTRADO

AVALIAÇÃO DA ATIVIDADE CICATRIZANTE DA MEMBRANA DE XILOGLUCANA COM OU SEM CRAMOLL 1,4 EM MODELOS DE CAMUNDONGOS DIABÉTICOS

FERNANDA MIGUEL DE ANDRADE

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Dissertação apresentada ao Programa de Pós-graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como pré-requisito para obtenção do título de Mestre em Ciências Biológicas.

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Recife, 29 de Fevereiro de 2016.

A comissão examinadora, composta pelos professores e doutores abaixo, sob a presidência do primeiro, considera a mestranda FERNANDA MIGUEL DE ANDRADE como:

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“Você sabe que alguém te ama não pelo que ele fala, mas pelo que ele faz. O amor não sobrevive de teorias.” (Padre Fábio de Melo).

RESUMO

ANDRADE, Fernanda Miguel. **Avaliação da atividade cicatrizante da membrana de Xiloglucana com ou sem Cramoll 1,4 em modelos de camundongos diabéticos.** 2016. Dissertação (Mestrado em Ciências Biológicas) – Universidade Federal de Pernambuco – UFPE, Recife, 2016.

Lectinas podem ser compreendidas como sendo proteínas (ou glicoproteínas) de origem não imunológica que se ligam reversivelmente a carboidratos, são encontradas em forma livre ou ligadas a superfícies celulares por meio de sítios de ligação em sua estrutura tridimensional. Estudos demonstraram que a lectina de *Cratylia mollis*, Cramoll 1,4, em solução salina promoveu em um menor tempo o efetivo fechamento e reparo de feridas em ratos imunocomprometidos quando comparado com grupo controle (solução salina). Uma estratégia para aumentar a potência de drogas é o seu enclausuramento em membranas polissacarídicas, as quais têm se mostrado eficientes na veiculação e liberação controlada de drogas. Frequentemente, feridas diabéticas envolvem extensas necroses e processos infecciosos que podem levar a amputação de membros, afetando de forma devastadora a qualidade de vida dos indivíduos afetados. O presente estudo teve como objetivo avaliar o potencial cicatrizante da membrana de Xiloglucana extraída de *Hymenaea courbaril* contendo ou não Cramoll 1,4 em modelos de camundongos diabéticos induzidos. Foram utilizados 60 camundongos Swiss albino machos, divididos ao acaso em 4 grupos experimentais cada um contendo 15 animais: **Grupo Controle** – Animais diabéticos tratados diariamente com 100 µL solução salina; **Grupo Cramoll** – Animais diabéticos tratados diariamente com 100 µL Cramoll 1,4; **Grupo Xilo** – Animais diabéticos tratados com membrana de xiloglucana e **Grupo Xilo+Cramoll** – Animais diabéticos tratados com membrana de xiloglucana contendo 100 µL Cramoll 1,4. Os resultados mostraram que nos grupos Cramoll, Xilo e Xilo+Cramoll houve uma aceleração na formação de crostas, na reepitelização e desprendimento, uma maior retração das feridas, e que os eventos histológicos e hematológicos típicos do processo de cicatrização ocorreram precocemente nesses grupos quando comparado com o grupo Controle. De acordo com os resultados, o tratamento com membranas de Xiloglucana com ou sem Cramoll 1,4 em feridas de camundongos diabéticos é eficaz, tornando esses compostos interessantes para a indústria farmacêutica.

Palavras-chaves: Adesivos biológicos, Cramoll 1,4, Diabetes, Xiloglucana.

ABSTRACT

ANDRADE, Fernanda Miguel. **Evaluation of the healing activity of the membrane of Xiloglucana with or without Cramoll 1,4 in models of diabetic mice.** 2016. Dissertation (MA in Biology) – Federal University of Pernambuco – UFPE, Recife, 2016.

Lectins can be understood as proteins (or glycoproteins) of non-immune origin which bind reversibly to carbohydrates, could be find in free form or bound to cell surfaces by means of binding sites on its three dimensional structure. Studies have demonstrated that the lectin *Cratylia mollis*, Cramoll 1,4, in saline soluction promoted in a shorter time the effective closure and wound repair in immunocompromised mice compared with control group (saline). A strategy to increase the potency of drugs is their enclosure in polysaccharide membranes, which have been shown to be effective in serving and controlled release of drugs. Often, diabetic wounds involve extensive necrosis and infectious processes that can lead to amputation of limbs, affecting a devastating effect on the quality of life of affected individuals. This study aimed at the evaluation of the healing potential of membrane based on the Xyloglucan extracted from *Hymenaea courbaril* seeds containing or not Cramoll 1,4 in models of induced diabetic mice. Sixty Swiss male mices were used divided randomly into 4 experimental groups each containing 15 animals: Control group - diabetic animals treated daily with 100 µL saline solution; Cramoll group - diabetic animals treated daily with 100 µL Cramoll 1,4; Xylo Group – diabetic animals treated with xyloglucan membrane; and Xylo + Cramoll Group - diabetic animals treated with xyloglucan membrane containing 100 µL Cramoll 1,4. The results showed that Cramoll, Xylo and Xylo + Cramoll groups there was an acceleration in the formation of crusts, in reepithelialization and detachment, a greater contraction of wounds, and the hematological and histological events of the healing process occurred early in these groups as compared to the control group. According to the results, treatment with Xiloglucana membranes with or without 1,4 Cramoll wounds in diabetic mice is effective, making these compounds interesting for the pharmaceutical industry.

Keywords: Wound dressings, Cramoll 1,4, Diabetes, Xyloglucan.

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CDR – Domínio de reconhecimento de carboidrato;

COBEA – Colégio Brasileiro de Experimentação Animal;

EGF – Fator de crescimento epidérmico;

FGF – Fator de crescimento de fibroblastos;

GFs – Fatores de crescimentos;

IL-1 – Interleucina-1;

LIKA – Laboratório de Imunopatologia Keizo Asami;

NO – Óxido nítrico;

PDGF – Fator de crescimento derivado de plaquetas;

ROS – Espécies reativas de oxigênio;

TGF-α – Fator de crescimento transformante- α;

TGF-β – Fator de crescimento transformante- β;

TNF-α – Fator de necrose tumoral;

VEGF – Fator de crescimento de células endoteliais;

XG – Xiloglucana.

SUMÁRIO

1. INTRODUÇÃO

Lectinas podem ser compreendidas como sendo proteínas (ou glicoproteínas) de origem não imunológica que se ligam reversivelmente a carboidratos (GHOSH et al., 1999; SUN et al., 2007), encontradas em forma livre ou ligadas a superfícies celulares por meio de sítios de ligação em sua estrutura tridimensional. A capacidade das lectinas para ligar carboidratos presentes em superfícies celulares possibilita as mesmas a desempenharem um importante papel em eventos celulares como aglutinação, reconhecimento celular, simbiose, estimulação da proliferação, opsonização, metástase e apoptose (WITITSUWANNAKUL; WITITSUWANNAKUL; SAKULBORIRUG, 1998).

Estas proteínas estão amplamente distribuídas entre plantas, animais e micro-organismos (KAWAGISHI et al, 2001). As plantas constituem ricas fontes de lectinas e sua distribuição ocorre principalmente nas raízes, folhas, flores, frutos, sementes, tubérculos, bulbos, rizomas e entrecascas. As lectinas são particularmente abundantes em sementes de leguminosas (SOL; CAVADA; CALVETE, 2007), chegando a constituir até 10 % da proteína total (SHARON; LIS, 1990).

Cratylia mollis (feijão camaratu) é uma planta forrageira da Região Semiárida do estado de Pernambuco e de suas sementes têm sido purificadas e caracterizadas formas múltiplas de lectinas: Cramoll 1, Cramoll 2, Cramoll 3 e Cramoll 4. Uma preparação contendo as formas 1 e 4 associadas denominada Cramoll 1,4 ou Cramoll apresenta especificidade glicose/manose (CORREIA; COELHO, 1995), a mesma observada para Cramoll 1, Cramoll 2 e Cramoll 4, enquanto Cramoll 3 é uma glicoproteína, específica para galactose (PAIVA; COELHO, 1992).

Vários estudos têm demonstrado o perfil imunomodulatório de Cramoll (MELO et al., 2010a; MELO et al., 2010b), como caracterização de tecidos cancerígenos humanos (BELTRÃO et al., 1998; LIMA et al., 2010), e atividade mitogênica em linfócitos humanos (MACIEL et al., 2004). Estudos revelaram que Cramoll 1,4 promoveu 41% de inibição tumoral (sarcoma 180) quando aplicada na forma livre; quando encapsulada esta atividade aumentou para cerca de 80%, além de eliminar efeitos hepatotóxicos provocados pela lectina livre (ANDRADE et al., 2004). Estudos demonstraram que Cramoll 1,4 em solução salina promoveu em um menor tempo o efetivo fechamento e reparo de feridas em ratos imunocomprometidos quando comparado com grupo controle (solução salina) (MELO et al., 2011a).

O termo lesões cutâneas pode ser definido como qualquer rompimento que leve a uma descontinuidade cutânea (STRODTBECK, 2001). O surgimento de uma ferida em um organismo desencadeia uma cascata de reações celulares e bioquímicas com o objetivo de reparar o tecido lesionado, porém em lesões diabéticas, este reparo é lento. Clinicamente, divide-se o processo cicatricial em três fases: fase Exsudativa ou Inflamatória; fase Proliferativa ou Fibroplasia e fase de Maturação ou Remodelação (ROBBINS, 2000; JORGE; DANTAS, 2003). Compreender o processo cicatricial é de grande importância para a avaliação evolutiva da ferida e fundamental para um diagnóstico correto a respeito da fase em que esse processo se encontra e consequentes medidas terapêuticas que podem ser prescritas para o tratamento ideal da lesão.

Segundo a Federação Internacional de diabetes (2016), haviam 14,2 milhões de casos de diabetes no Brasil em 2015. Entre as complicações que mais frequentemente afetam os indivíduos diabéticos estão as cardiopatias, retinopatias, neuropatias e as feridas nos membros inferiores, que representam a maior carga dos pacientes no sistema de saúde (SINGH; ARMSTRONG; LISY, 2005). Tais feridas são usualmente crônicas, a maioria delas é encontrada nos pés e o tratamento é difícil, muitas vezes, essas feridas não são curadas primariamente (ARONOW, 2004). O simples controle dos níveis sanguíneos de glicose, embora importantes, não são necessariamente seguidos pela cicatrização dessas úlceras (JEFFCOATE; PRICE; HARDING, 2004). Frequentemente, feridas envolvem extensas necroses e processos infecciosos que podem levar a amputação de membros (MARKOWITZ et al., 2006).

Feridas crônicas têm um efeito devastador sobre a saúde e o estilo de vida de mais de 8 milhões de americanos por ano. Cerca de 900 mil pessoas por ano nos EUA sofrem de úlceras diabéticas, 915 mil sofrem de úlceras de estase venosa e 6,5 milhões de pessoas sofrem de úlceras de pressão. Feridas crônicas podem levar anos para cicatrizar e geralmente há uma alta taxa de recorrência da ferida (RODGERS et al, 2011).

Pacientes com úlceras crônicas necessitam de um longo tempo de tratamento, isso resulta em um alto custo com cuidados médicos. Um dos principais desafios é a busca por novas substâncias terapêuticas, visando a neoformação tecidual (FONDER et al., 2007; SEHN et al., 2009). Diante disso, a pesquisa por compostos naturais não-tóxicos com propriedades estimuladoras da reparação tecidual se faz necessária, visando principalmente encontrar recursos para confecção de produtos cicatrizantes feitos a

partir de matéria-prima de baixo custo (MANDELBAUM; DI SANTIS; MANDELBAUM, 2003; SEHN et al., 2009).

O uso de polissacarídeos como curativos biológicos é vantajoso, uma vez que essas substâncias apresentam livre administração, simplicidade de operação, e diminui os custos dos produtos (OKAMOTO et al., 1995). Entre as várias aplicações dos polissacarídeos, destaca-se a aplicação como veículo de liberação de fármacos (CARNEIRO-DA-CUNHA et al., 2009). Membranas de polissacarídeos têm sido amplamente utilizadas no campo alimentício (CERQUEIRA et al, 2012) e farmacêutico como curativo tópico (MONTEIRO et al., 2007).

Estudos relatados por Arruda et al., (2015) afirmam que a xiloglucana extraída de *Hymenaea courbaril* é um polissacarídeo promissor para indústrias alimentícia e farmacêutica. Diante do exposto, o presente trabalho visou avaliar a atividade cicatrizante de uma membrana polissacarídica contendo Cramoll 1,4 em lesões cutâneas experimentais em camundongos diabéticos induzidos.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar o potencial cicatrizante da membrana de Xiloglucana extraída de *Hymenaea courbaril* com ou sem Cramoll 1,4 em modelos de camundongos diabéticos.

2.2 Objetivos Específicos

2.2.1 Purificar Cramoll 1,4 por protocolo previamente estabelecido;

2.2.2 Extrair e purificar os polissacarídeos provenientes das sementes de *Hymenaea courbaril* (Xiloglucana);

2.2.3 Imobilizar por enclausuramento Cramoll 1,4 em membranas da Xiloglucana obtida de sementes *H. courbaril*;

2.2.4 Induzir diabetes em camundongos *Swiss albino*;

2.2.5 Induzir lesões cutâneas experimentais em camundongos *Swiss albino* diabéticos induzidos;

2.2.6 Realizar o tratamento tópico das lesões cutâneas experimentais utilizando a Cramoll 1,4 livre, a membrana contendo Cramoll 1,4 e controles durante 12 dias;

2.2.7 Acompanhar a evolução do processo de reparo tecidual do ponto de vista clínico (avaliação clínica das feridas e mensuração de sua área) durante 12 dias;

2.2.8 Acompanhar a cicatrização do ponto de vista histopatológico, através de biópsias no 2º, 7º e 12º dias de pós-operatório, onde serão analisados o esquema de tratamento e o comportamento hematológico.

3. REFERENCIAL TEÓRICO

3.1 Morfofisiologia da pele

O tecido epitelial desempenha várias funções no organismo de acordo com o órgão no qual se localiza. Ele pode atuar na proteção do corpo, na absorção e secreção de substâncias do meio e na percepção de sensações. É classificado em epitélio glandular, especializado na produção e eliminação de secreções, e em epitélio de revestimento, que tem como principal função a proteção das estruturas internas do organismo (KUMAR et. al., 2006).

A pele é composta por tecido epitelial de revestimento estratificado pavimentoso queratinizado, sendo considerada o maior órgão do corpo, cobrindo toda superfície externa (SANTOS et. al., 2004). É definida como o manto de revestimento do organismo, indispensável à vida, pois isola os componentes orgânicos do meio exterior. Em sua constituição se encontra uma estrutura complexa composta de diferentes tecidos, que executam suas funções harmonicamente (SAMPAIO; RIVITTI, 2007).

Além de ser um órgão sensorial especializado (JUNQUEIRA; CARNEIRO, 2008), a pele desempenha múltiplas funções, como termo regulação, síntese de vitamina D, reservatório de água, minerais e gorduras (BRAGULLA et. al. 2004), prevenção contra desidratação, proteção contra raios ultravioleta (UV), absorção, cicatrização entre outras (CLARK; GHOSH; TONNESEN, 2007; JUNQUEIRA; CARNEIRO, 2008; THEORET, 2009; GÁL et. al., 2011). É considerada a primeira linha de defesa do organismo, pois atua como uma barreira mecânica flexível, que promove proteção imunológica, contra infecções de microrganismos e toxinas do meio externo (SANTOS et. al., 2004; GÁL et. al. 2011).

Histologicamente, a pele é composta por duas camadas distintas, a epiderme e a derme (BRAGULLA et al., 2004; JUNQUEIRA; CARNEIRO, 2008), que estão firmemente unidas entre si (SANTOS et. al., 2004). A camada subcutânea, situada abaixo da derme e conhecida como hipoderme, tem a mesma origem embrionária da derme, porém não faz parte dela (DE ALMEIDA, 2009) (**Fig. 1**).

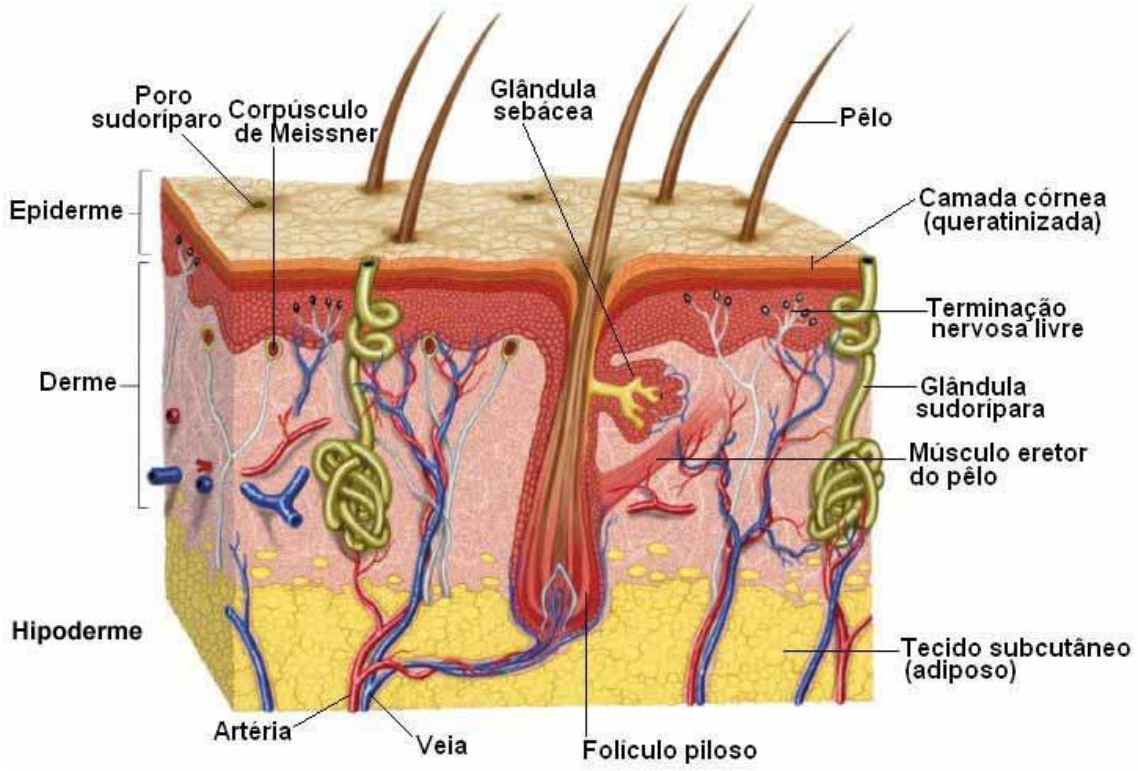

Figura 1. Principais estruturas e camadas da pele. Fonte: Adaptado de http://www.vidacentro.com.br/wp-content/uploads/2011/06/camadas_da_pele.jpg

3.1.1 Epiderme

A epiderme, camada mais externa da pele, é avascular e constitui-se de tecido epitelial estratificado pavimentoso queratinizado (SAMPAIO; RIVITTI, 2007; BORGES, 2010). Sua nutrição é realizada por meio de vasos sanguíneos da derme (BORGES, 2010). Ela serve como barreira que protege o organismo contra invasões por microrganismos, desidratação e injúrias mecânicas (DE ALMEIDA, 2009).

É responsável pela origem dos seguintes apêndices: glândulas sudoríparas, folículos pilosos, unhas e glândulas sebáceas (BLANPAIN; FUCHS, 2009). Tais apêndices fazem com que a epiderme desempenhe bem suas funções (DE ALMEIDA, 2009).

As células que constituem a epiderme são os queratinócitos, melanócitos, células de Langerhans e células neuroendócrinas de Merkel (ALMEIDA, 2011). Os queratinócitos produzem a proteína queratina e apresentam função de barreira protetora. Os melanócitos são células o tecido conjuntivo, responsáveis pela produção de melanina, pigmento da pele que age como protetor natural contra os efeitos nocivos de raios UV. As células de Langerhans atuam como mediadoras da resposta inflamatória, e

as células neuroendócrinas de Merkel são mecanoreceptores (EHRENREICH; RUSZCZAC, 2006; ALMEIDA, 2011).

A epiderme é subdividida em cinco camadas ou estratos, que refletem a progressão das células germinativas, encontradas na base, para células corneificadas diferenciadas, presentes na superfície (DE ALMEIDA, 2009) (**Fig. 2**). A partir do estrato mais interno para o estrato mais superficial, encontram-se:

1. Estrato basal ou germinativo;
2. Estrato espinhoso;
3. Estrato granuloso;
4. Estrato lúcido;
5. Estrato Córneo.

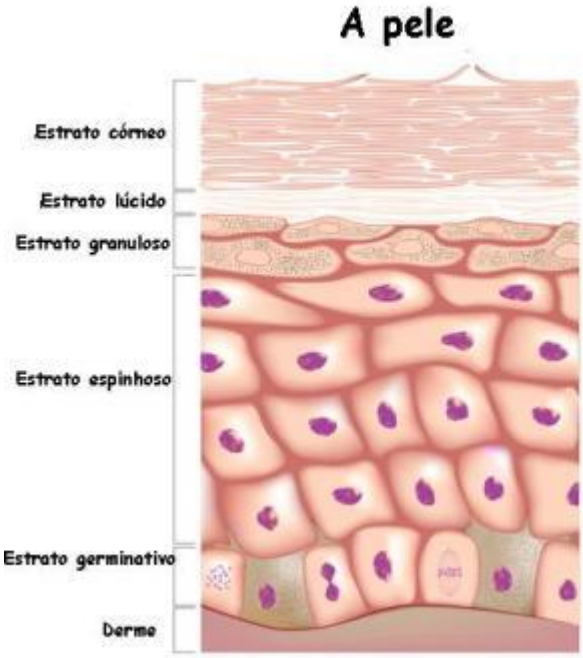

Figura 2. Camadas ou estratos da epiderme. Fonte: Adaptado de http://brasilescola.uol.com.br/upload/conteudo/images/camadas-pele-224ac06c3d10.jpg

Na diferenciação da epiderme, os queratinócitos saem da camada basal e movem-se em direção a camada córnea. Esse processo é conhecido como diferenciação terminal, onde os queratinócitos perdem a capacidade de se dividir mitoticamente, sofrem modificações morfológicas e bioquímicas, e transformam-se em estruturas multilamelares de corneócitos envolvidos por matriz extracelular lipídica, formando a barreira de proteção contra agentes externos (ELIAS, 2007; KIM; YUN; CHO, 2011).

3.1.2 Derme

A derme é formada por tecido conjuntivo constituído de fibras colágenas, fibras elásticas e glicosaminoglicanas, produzidos por fibroblastos dermais, e que conferem estrutura, apoio, consistência física e elasticidade a pele. É bem vascularizada e inervada, contém folículos pilosos e glândulas sebáceas e sudoríparas, que se estendem até a epiderme. Ela também é responsável pela nutrição da epiderme através da difusão de pequenas moléculas (ARNOLD; ODOM; JAMES, 2002; JUNQUEIRA; CARNEIRO, 2008; THEORET, 2009).

A derme é dividida em duas camadas:

1. Camada papilar: é constituída por tecido conjuntivo frouxo, com fibroblastos e delgadas fibras colágenas e elásticas, que estão perpendiculares à junção dermoepidérmica. Apresentam saliências chamadas de papilas dérmicas e é bastante vascularizada (EHRENREICH; RUSZCZAK, 2006; DE ALMEIDA, 2009).
2. Camada reticular: nela são encontradas as fibras conjuntivas não remodeladas e fibras elásticas em plexos. Feixes de fibras colágenas e elásticas estão localizadas na substância fundamental amorfa a qual é constituída por glicosaminoglicanas (DE ALMEIDA, 2009).

3.2 Lesões cutâneas

O termo lesões cutâneas pode ser definido como qualquer rompimento que leve a uma descontinuidade cutânea, sendo provocada frequentemente por traumas (mecânico, físico ou químico), isquemia, pressão no local e devido a procedimentos cirúrgicos (STRODTBECK, 2001).

As lesões constituem um dos principais problemas de saúde pública, pois além de afetar a qualidade de vida do paciente, algumas são de difícil cicatrização devido a vários fatores intrínsecos e extrínsecos e por isso necessitam de um período prolongado de tratamento (FALANGA, 2005). Quando na fase crônica, a dificuldade de cicatrização aumenta, agravando a doença e debilitando ainda mais a qualidade de vida do indivíduo afetado (GARY-SIBBALD; WOO, 2008).

A cicatrização de feridas pode decorrer de três formas: de primeira intenção, quando não há perda de tecido e as extremidades da pele ficam justapostas uma à outra (lesões cirúrgicas); de segunda intenção, quando há perda de tecido e as extremidades

da pele ficam distantes uma da outra, sendo necessária a formação de tecido de granulação, a contração e a reepitelização da lesão; e de terceira intenção, quando há fatores como infecção da lesão, má nutrição, baixa perfusão de oxigênio nos tecidos, doença endócrina como diabetes e obesidade, terapia em curso com glicocorticóides, radioterapia e quimioterapia, que retardam a cicatrização de uma lesão inicialmente submetida a um fechamento por primeira intenção (BORGES et al., 2001).

3.3 Cicatrização

O processo de cicatrização tem a finalidade de curar feridas (ISAAC et al., 2010), é dinâmico e envolve fenômenos bioquímicos e fisiológicos que se comportam de forma harmoniosa para promover a restauração tissular (MANDELBAUM et al., 2003). Nesse processo ocorre uma complexa interação entre diferentes tipos celulares, citocinas, e moléculas da matriz extracelular, que atuam no local, em conjunto com numerosos fatores sistêmicos como as plaquetas, a cascata de coagulação e componentes celulares e humorais do sistema imune (SOO et al. 2002).

Na cicatrização um tecido lesado é substituído por tecido conjuntivo vascularizado, quer a lesão tenha sido traumática ou necrótica (PANOBIANCO et al., 2010). Esse processo consiste na sequência das seguintes etapas: inflamação, quimiotaxia, proliferação celular, diferenciação e remodelação. Essas etapas ocorrem de maneira interdependentes, se sobrepõe, e se agrupam nas fases: inflamatória, fibroplasia ou proliferativa e maturação ou remodelação (MARTINS et al., 2006; ISAAC et al., 2010) (**Fig. 3**). A fase inflamatória dura em média de 2 a 3 dias, a fibroplasia ou proliferativa, geralmente se estende até o 10º dia, e por fim a de maturação, que dependendo da lesão, pode durar até mais de um ano (BALBINO; PEREIRA; CURI, 2005).

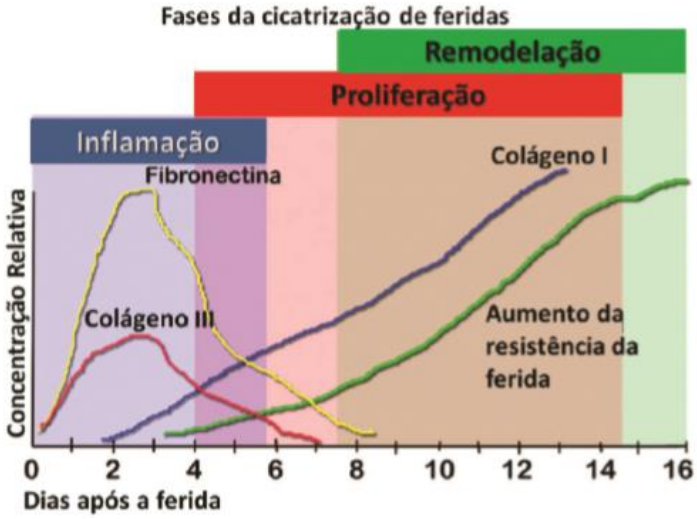

Figura 3: Fases da cicatrização e a deposição dos componentes da matriz cicatricial ao longo do tempo. Fonte: Adaptado de BROUGHTON, G.; JANIS J. E.; ATTINGER, C. E., 2006.

3.3.1 Fase inflamatória

Após a ocorrência do ferimento, inicia-se o extravasamento sanguíneo que preenche a área lesada com plasma e elementos celulares, principalmente plaquetas. A fase inflamatória inicia-se imediatamente após o trauma, provocando a vasoconstrição para conter a hemorragia local. Simultaneamente, ocorre a deposição e ativação plaquetária com infiltração de fibrina e eritrócitos, os quais formam um primeiro tampão na lesão que se torna mais consistente conforme a fibrina vai se polimerizando pelas vias da coagulação. Esse tampão além de restabelecer a hemostasia e formar uma barreira contra a invasão de microrganismos, organiza a matriz provisória necessária para a migração celular. Essa matriz servirá também, como reservatório de citocinas e fatores de crescimento que serão liberados durante as fases seguintes do processo cicatricial (WERNER; GROSE, 2003; EMING; KRIEG; DAVIDSON, 2007).

A ativação plaquetária libera mediadores quimiotáticos que vão promover a migração de células inflamatórias para o local lesionado (BALBINO; PEREIRA; CURI, 2005). Induzidas pela trombina sofrem a degranulação plaquetária e liberam vários fatores de crescimento, como o derivado de plaquetas (PDGF), o de crescimento transformante-β (TGF-β), o de crescimento epidérmico (EGF), o de crescimento transformante-a (TGF-α) e o fator de crescimento de células endoteliais (VEGF), além de glicoproteínas adesivas como a fibronectina e trombospondina, que são importantes

constituintes da matriz extracelular provisória (ARNOLD; WEST, 1991; STREIT et al., 2000).

Nesta fase, predominam neutrófilos e macrófagos (BALBINO; PEREIRA; CURI, 2005), que migram em direção ao leito da ferida em resposta aos agentes quimiotáticos após a saída das plaquetas (CLARK, 1996). Essas células vão digerir e fagocitar bactérias e restos celulares (**Fig. 4**). Além disso, os macrófagos secretam fatores quimiotáticos que atraem outras células inflamatórias ao local da ferida e produzem prostaglandinas, que funcionam como potentes vasodilatadores, afetando a permeabilidade dos microvasos (ARNOLD; WEST, 1991; SINGER; CLARK, 1999; EMING et al., 2007), e produzem diversos fatores de crescimento, tais como o PDGF, o TGF-β, fator de crescimento epidérmico (EGF), o fator de crescimento de fibroblastos (FGF) e o VEGF, que se destacam como as principais citocinas necessárias para estimular a formação do tecido de granulação (SINGER; CLARK, 1999), que por sua vez, atraem para o local lesionado fibroblastos e queratinócitos (principais células de regeneração da pele) e os induzem a proliferação (BALBINO; PEREIRA; CURI, 2005) iniciando assim a fase proliferativa.

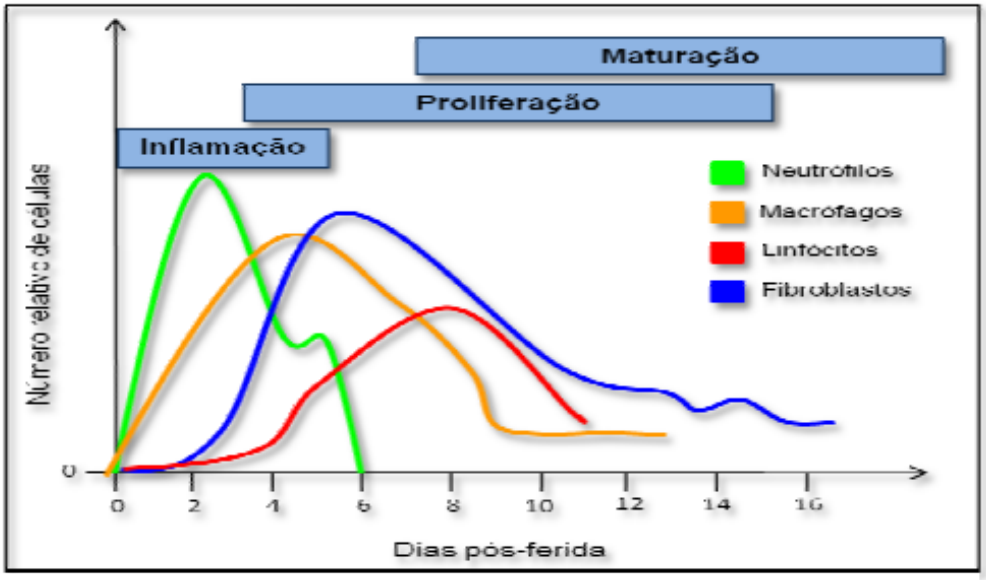

Figura 4: Representação gráfica da resposta fisiológica da pele a feridas, mostrando o número relativo de células sanguíneas e fibroblastos nas fases do processo de cicatrização. Fonte: Biologia da ferida e cicatrização, 2008.

3.3.2 Fase proliferativa

A fase proliferativa é caracterizada pela formação de tecido de granulação contendo células inflamatórias, fibroblastos e vasos sanguíneos neoformados em uma

matriz de colágeno frouxa (MILORO, 2008). É responsável pelo fechamento da lesão propriamente dito. Compreende: reepitelização, que se inicia horas após a lesão, com a movimentação das células epiteliais oriundas tanto da margem como de apêndices epidérmicos localizados no centro da lesão; fibroplasia e angiogênese compõem o tecido de granulação, que é responsável pela ocupação do tecido lesionado cerca de quatro dias após a lesão (SINGER; CLARK, 1999).

A fase de proliferação epitelial, no caso da pele, inicia-se por estimulação mitogênica e quimiotática dos queratinócitos pelo TGF-α e EGF (DVORAK et al., 1999; DVORAK, 2002). Os queratinócitos migram das bordas da ferida e multiplicam-se, reestruturando as camadas da epiderme e promovendo a reepitelização tecidual. Ocorre a angiogênese, ou seja, a abundante formação de vasos novos e finos a partir de vasos íntegros adjacentes, estes com a função de fornecer oxigênio e nutrientes para cicatrização. Simultaneamente ocorre a deposição e proliferação de fibroblastos sobre a rede de fibrina formando um tecido frouxo, denominado tecido de granulação. Os fibroblastos secretam elastina, fibronectina, glicosaminoglicana, proteases e colágeno, principalmente colágeno tipo III, os quais são responsáveis pela regeneração tecidual (MANDELBAUM et al., 2003 A; ISAAC et al., 2010).

3.3.3 Fase de maturação ou remodelação

Na fase de remodelação ocorre uma tentativa de recuperação da estrutura tecidual normal (GABBIANI, et al., 1972). Envolve um minucioso e organizado equilíbrio entre degradação e formação de matriz (MILORO, 2008; ACKERMANN, 2009), é marcada por maturação dos elementos e alterações na matriz extracelular, ocorrendo o depósito de proteoglicanas e colágeno (GABBIANI, et al., 1972). Influenciados por citocinas e fatores de crescimento, a matriz de colágeno é continuamente degradada, resintetizada, reorganizada e reestabilizada (MILORO, 2008; ACKERMANN, 2009).

Nessa fase, surgem as primeiras fibras de colágeno tipo I, o qual é mais resistente e maduro em substituição ao colágeno tipo III depositado durante a fase proliferativa (CLARK; HENSON, 1988; ACKERMANN, 2009). O colágeno é uma proteína fibrosa insolúvel de função estrutural, que aumenta com uma taxa relativamente constante a partir do 3º dia de lesão (JUNQUEIRA; CARNEIRO, 2008). Essa proteína é classificada em diferentes grupos, conforme sua sequência de

aminoácidos. Os colágenos presentes no tecido conjuntivo são os tipos I e III. Em um tecido normal cerca de 90% é colágeno tipo I e 10% colágeno tipo III, mas quando o tecido é lesado, o tipo III predomina na fase proliferativa, porém na fase de remodelagem é substituído ao longo do processo de reparo tecidual pelas fibras do tipo I (SAMPAIO, 2000; JUNQUEIRA; CARNEIRO, 2008).

Na fase mais tardia, os fibroblastos do tecido de granulação transformam-se em miofibroblastos comportando-se como um tecido contrátil responsivo aos agonistas que estimulam o músculo liso. Ocorre, concomitantemente, reorganização da matriz extracelular, que se transforma de provisória em definitiva, cuja intensidade fenotípica, observada nas cicatrizes, reflete a intensidade dos fenômenos que ocorreram, bem como o grau de equilíbrio ou desequilíbrio entre eles (GABBIANI et al., 1972).

Assim que a demanda metabólica da lesão em processo de cicatrização diminui, a alta rede de capilares, fibroblastos e células inflamatórias desaparece do local da ferida mediante processos de emigração, apoptose ou outros mecanismos desconhecidos de morte celular (ARNOLD; WEST, 1991; MILORO, 2008; ACKERMANN, 2009). Esse fato leva à formação de cicatriz com reduzido número de células. Por outro lado, se persistir a celularidade no local, ocorrerá a formação de cicatrizes hipertróficas ou queloides. As principais citocinas envolvidas nessa fase são: fator de necrose tumoral (TNF-α), interleucina (IL-1), PDGF e TGF-β produzidas pelos fibroblastos, além das produzidas pelas células epiteliais como EGF e TGF-b (KARUKONDA et al., 2000).

O processo de maturação da ferida é lento, podendo levar meses para reorganizar as fibras colágenas e atingir as quantidades adequadas de colágeno tipo I e III que irão aumentar a força da cicatriz, diminuir sua espessura e fornecer elasticidade. Assim, a cicatrização normal pode atingir características semelhantes ao tecido íntegro e sua resistência pode chegar a 80% da força de tensão fisiológica original (MANDELBAUM; SANTIS; MANDELBAUM, 2003).

3.4 Lesões diabéticas e o processo de cicatrização

O diabetes mellitus é uma doença crônica sistêmica decorrente de uma deficiência parcial ou absoluta de insulina, que compromete o metabolismo de carboidratos, gorduras e proteínas. Esse comprometimento metabólico é provocado pela hiperglicemia resultante da ausência de secreção de insulina ou da resistência a sua ação nos tecidos (PÖPPL; GAONZÁLEZ, 2005; PINTO et al., 2009).

O surgimento de uma ferida em um organismo desencadeia uma cascata de reações celulares e bioquímicas com o objetivo de reparar o tecido lesionado, porém em lesões diabéticas, este reparo é lento. Vários mecanismos são apontados como fatores importantes na diminuição do processo de cicatrização em indivíduos diabéticos, entre eles estão: a produção excessiva de espécies reativas de oxigênio (ROS), diminuição do óxido nítrico (NO), diminuição da reposta aos fatores de crescimento (GFs) e das proteínas da via de sinalização da insulina (BREM; TOMIC-CANIC, 2007; KOLLURU; BIR; KEVIL, 2012).

Entre as complicações que mais frequentemente afetam os indivíduos diabéticos, as feridas nos membros inferiores são a maior carga dos pacientes no sistema de saúde (SINGH; ARMSTRONG; LISY, 2005). Tais feridas são usualmente crônicas, a maioria delas é encontrada nos pés e o tratamento é difícil, muitas vezes, essas feridas não são curadas primariamente (ARONOW, 2004). O simples controle dos níveis sanguíneos de glicose, embora importantes, não são necessariamente seguidos pela cicatrização dessas lesões (JEFFCOATE; PRICE; HARDING, 2004). Frequentemente, feridas crônicas envolvem extensas necroses e processos infecciosos que podem levar a amputação de partes do corpo, até mesmo de membros (MARKOWITZ et al., 2006).

3.5 Lectinas

Lectinas são definidas como proteínas ou glicoproteínas de origem não imunológica que se ligam reversivelmente e especificamente a carboidratos (GOOSH et al., 1999; CAVADA et al., 2001; SUN et al., 2007). Os sítios de ligação a carboidratos, presentes na molécula de lectina interagem com o carboidrato específico através do mecanismo do tipo chave-fechadura, envolvendo pontes de hidrogênio, ligação metálica, interações de Van der Waals e interações hidrofóbicas (KENNEDY et al., 1995). O termo lectina (originado no latim *lectus*, que significa escolhido, selecionado) foi primeiro empregado por Boyd; Shapleigh em 1954, para designar um grupo de proteínas que apresentava a característica comum de seletividade na interação com carboidratos (PEUMANS; VAN DAMME, 1998; PEUMANS et al., 2001).

O primeiro relato sobre lectinas ocorreu em 1888, quando Stillmark, ao estudar a toxicidade de extratos de *Ricinus communis L*. (mamona), observou sua capacidade para aglutinar eritrócitos, devido à presença de uma proteína extraída, a ricina, descoberta que marcou o início das pesquisas envolvendo lectinas (KENNEDY et al., 1995). Pouco

tempo depois, outra hemaglutinina, chamada abrina, foi encontrada em sementes de *Abrus precatorius L*. (jequiriti). Entretanto, o estudo sobre estas proteínas só começou a ganhar ímpeto em 1960, abrindo uma vasta área de aplicação para as lectinas (GABOR et al., 2004).

Devido a habilidade de se ligar especificamente a carboidratos, as lectinas apresentam alto grau de especificidade em suas reações com grupos sanguíneos do sistema ABO e MN, estabelecendo interação com os carboidratos através de ligações de hidrogênio e interação hidrofóbicas em uma porção da proteína denominada de Domínio de Reconhecimento a Carboidrato (CDR) (SHARON; LIS, 1990; KENNEDY et al., 1995; NISHIMURA et al., 2006). Propriedade esta que resulta na capacidade de aglutinar seletivamente eritrócitos **(Fig. 5)**, o que destingue as lectinas de outras macroléculas ligantes de açucares como as glicosidades e glicotransferases. (GOLDSTEIN et al., 1980; ELIFIO et al., 2000).

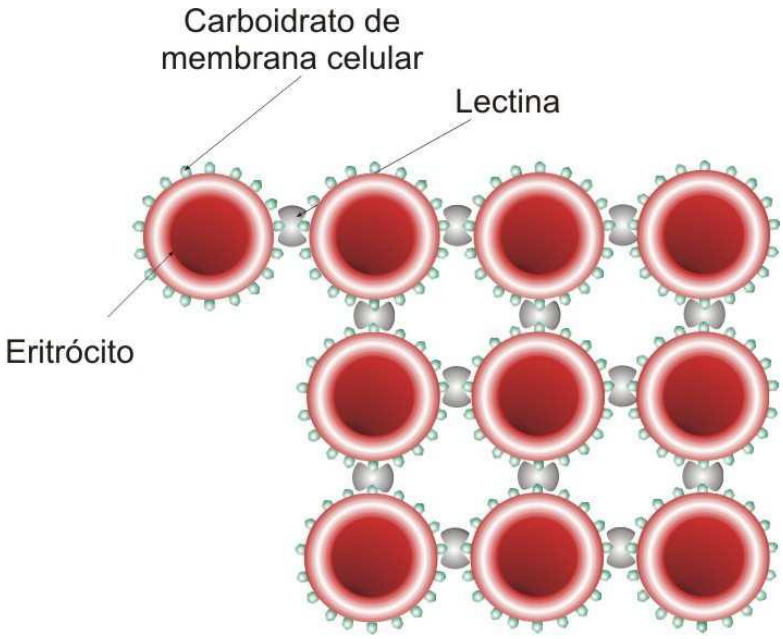

Figura 5: Rede de hemaglutinação mediada por lectinas. Fonte: **Pimentel, J. C., 2006.**

As lectinas são encontradas em forma livre ou ligadas a superfícies celulares por meio de sítios de ligação em sua estrutura tridimensional (WITITSUWANNAKUL; WITITSUWANNAKUL; SAKULBORIRUG, 1998). Isso ocorre porque a maioria das proteínas da superfície celular e muitos lipídios nas membranas celulares são glicosilados e estes glicanos são sítios de ligação para lectinas (BIES; LEHR; WOODLEY, 2004). Estruturalmente possuem ampla variedade, sendo comum entre

elas a presença de, ao menos, um sítio de ligação a carboidrato (Domínio de Reconhecimento de Carboidrato- CDR) (WEIS; DRICKAMER, 1996).

A combinação de uma pequena quantidade de açúcar pode produzir uma vasta gama de estruturas químicas diferentes, com isso diferentes tipos de células expressam diferentes matrizes de glicano e em particular, células doentes, tais como células transformadas ou cancerosas, frequentemente expressam diferentes glicanos em comparação com células normais. Por isso, as lectinas podem ser utilizadas como moléculas transportadoras para alvejar drogas especificamente a diferentes células e tecidos (BIES; LEHR; WOODLEY, 2004). A capacidade das lectinas para ligar carboidratos presentes em superfícies celulares possibilita as mesmas a desempenharem um importante papel em eventos celulares como aglutinação tanto de células animais como vegetais, especialmente eritrócitos de diferentes espécies animais (CORREIA; COELHO, 1995; CAVADA et al., 2001), reconhecimento celular, simbiose, estimulação da proliferação, opsonização, metástase, apoptose (WITITSUWANNAKUL; WITITSUWANNAKUL; SAKULBORIRUG, 1998), e precipitar polissacarídeos, glicoproteínas ou glicolipídeos (CAVADA et al., 2001).

As lectinas possuem ampla distribuição, sendo encontradas tanto em organismos eucariontes como em procariontes. As plantas constituem ricas fontes de lectinas e sua distribuição ocorre principalmente nas raízes, folhas, flores, frutos, sementes, tubérculos, bulbos, rizomas e entrecascas. As lectinas são particularmente abundantes em sementes de leguminosas (SOL; CAVADA; CALVETE, 2007), chegando a constituir até 10 % da proteína total (SHARON; LIS, 1990).

Lectinas de plantas podem assumir diferentes papeis biológicos. De maneira abrangente as lectinas podem ser organizadas em dois grupos segundo seu ligante: podem assumir papéis exógenos ao se associarem a ligantes externos, por exemplo, atividade antifúngica contra fitopatógenos, ou podem assumir papéis endógenos se interagirem com ligantes do próprio organismo, por exemplo, auxiliar a deposição de proteínas de reserva nos corpos protéicos (SHARON; LIS, 2004).

3.5.1 Lectina de *Cratylia mollis* (Cramoll)

A *Cratylia mollis* **(Fig. 6),** conhecida popularmente como feijão camaratuba ou camaratu, é uma planta forrageira nativa do Semi-Árido do Nordeste do Brasil, no Estado de Pernambuco. Pertencente à família leguminosa Papilionoideae, tribo

Phaseoleae, subtribo Diocleinae que compreende 13 gêneros, dos quais se destacam os gêneros: *Canavalia, Cratylia, Calopogonium, Dioclea, Galactia* e *Herpyza* (POLHILL *et al.*, 1981).

As sementes de *C. mollis* têm sido consideradas fontes importantes de lectinas (Cramoll), fornecendo múltiplas formas moleculares com especificidades diferentes para carboidratos (Cramoll 1, Cramoll 2, Cramoll 3, Cramoll 4, além de uma preparação que contém as isoformas Cramoll 1 e Cramoll 4, a Cramoll 1,4 (PAIVA; COELHO, 1992; CORREIA; COELHO, 1995). A Cramoll 1,4, apresenta especificidade glicose/manose (CORREIA; COELHO, 1995), a mesma observada para Cramoll 1, Cramoll 2 e Cramoll 4, enquanto Cramoll 3 é uma glicoproteína, específica para galactose (PAIVA; COELHO, 1992).

Figura 6: *Cratylia mollis*. Arbusto (à esquerda) e sementes (à direita). Fonte: **Pereira, D.S.T.**

As lectinas das sementes de *C. mollis* vêm sendo estudadas em diversas áreas biotecnológicas. Vários estudos têm demonstrado o perfil imunomodulatório de Cramoll (MELO et al., 2010a; MELO et al., 2010b), como caracterização de tecidos cancerígenos humanos (BELTRÃO et al., 1998; LIMA et al., 2010), atividade mitogênica em linfócitos humanos (MACIEL et al., 2004). Estudos demonstraram que Cramoll 1,4 promoveu 41% de inibição tumoral (sarcoma 180) quando aplicada na forma livre; encapsulada esta atividade aumentou para cerca de 80 %, além de eliminar efeitos hepatotóxicos provocados pela lectina livre (ANDRADE et al., 2004). Há experimentos envolvendo isolamento de inibidores de tripsina (PAIVA et al., 2003), de glicoproteínas do plasma (LIMA et al., 1997) e da soja (SILVA et al., 2011); na indução

de resposta mitogênica e produção de citocinas (DE MELO et al., 2011); como biosensores na identificação de diferentes sorotipos da dengue (OLIVEIRA et al., 2011); como indutoras de morte de epimastigotas de *Trypanossoma cruzi* por necrose (FERNANDES et al., 2010), como potencial anti-hemintíco contra *Schistosoma mansoni* (MELO et al., 2011). Estudos demonstraram que Cramoll 1,4 em solução salina promoveu em um menor tempo o efetivo fechamento e reparo de feridas em ratos imunocomprometidos quando comparado com grupo controle (solução salina) (MELO et al., 2011). Estudos in vitro mostraram os efeitos citoprotetores de pCramoll e rCramoll contra danos oxidativos induzidos por peróxido de hidrogênio (H2O2) em células Vero (DA SILVA et al., 2015).

3.6 Polissacarídeos como curativos biológicos

Há uma grande variedade de curativos sintéticos e biológicos que têm sido desenvolvidos e caracterizados com o objetivo de proporcionar propriedades ideais para o tratamento de lesões. Os curativos biológicos ou biomateriais são empregados na tentativa de estabelecer um suporte biológico tridimensional saudável, mimetizando uma matriz cutânea original que possibilite a migração, proliferação e uma organização ideal das células no microambiente fisiológico cutâneo alterado (GOMES et al., 2003; SILVA, 2008), e caracterizam-se por terem origem de produtos renováveis e biodegradáveis (PAUL; SHARMA, 2004; WIEGAND; HIPLER, 2010).

Para a utilização de um novo curativo na cicatrização de lesões é preciso cumprir várias exigências, dentre elas pode-se citar a capacidade do novo curativo de atuar como barreira contra patógenos do meio externo, não ser tóxico, manter o ambiente úmido, promover cicatrização, e ao mesmo tempo manter uma boa troca de gases. Também devem ter a capacidade de absorver o excesso de exsudato, não se aderir ao leito da lesão, ser de fácil manipulação e de baixo custo (WIEGAND; HIPLER, 2010).

Existe uma grande variedade de curativos em formas de géis, espumas e filmes de diferentes biomateriais. Alguns obtiveram êxito, mas ainda não representam um curativo ideal. Por isso, se intensificaram as pesquisas que visam aprimorar a produção de um curativo biológico ideal para o tratamento de lesões e queimaduras (WIEGAND; HIPLER, 2010).

Curativos biológicos (biomateriais) produzidos a base de polímeros como a celulose, colágeno, alginato e quitosana, têm a capacidade de envolvimento natural no

tecido lesado, assim como no tecido em formação, para promover a cicatrização. Eles também apresentam propriedades antimicrobianas intrínsecas e distintas possiblidades de ligações para mediadores inflamatórios (citocinas, proteases e radicais livres), cujas concentrações são elevadas em úlceras crônicas de difícil cicatrização (WIEGAND; HIPLER, 2010).

O uso de polissacarídeos como curativos biológicos é vantajoso, uma vez que essas substâncias apresentam livre administração, simplicidade de operação, e diminui os custos dos produtos (SHIGEMASA; MINAMI, 1995). Os polissacarídeos são polímeros difundidos na natureza e especialmente disponíveis a partir de plantas e microrganismos, representando uma fonte inesgotável de biopolímeros geralmente não tóxicos, biodegradáveis, biocompatíveis (MKEDDER et al., 2013). Devido a facilidade de se obter polissacarídeos a partir de fontes naturais, estes se tornam mais acessíveis do que os polímeros sintéticos (COVIELLO et al., 2007).

Polissacarídeos de varias fontes possuem uma ampla gama de aplicações, especialmente, nas áreas alimentícias, biomédicas, farmacêuticas e cosméticas. Na área biológica, eles são aplicados em engenharia de tecidos, como matriz para imobilização de moléculas e construção de biossensores, assim como veículo de liberação de fármacos (CARNEIRO-DA-CUNHA et al., 2009). Membranas de polissacarídeos têm sido amplamente utilizadas no campo alimentício (CERQUEIRA et al., 2012) e farmacêutico como curativo tópico (MONTEIRO et al., 2007).

3.7 Xiloglucanas

Xiloglucanas (XG) são heteropolissacarídeos normalmente encontrados nas paredes celulares primárias de plantas e nas sementes, com funções estruturais e de armazenamento, respectivamente. Apresentam em sua estrutura química uma cadeia central de (1→4)- β-D-glicose, ligada por pontes de hidrogênio na posição O-6 por ramos α-D-xilose e parcialmente ligada em O-2 por unidades de β-D-galactose (HAYASHI, 1989; BUCKERIDGE et al., 2010).

As XG podem ser utilizadas em alimentos e indústrias farmacêuticas para processos biotecnológicos, devido às suas características, como a capacidade de retenção de água e de sua resistência contra o calor, sal e pH. Na indústria de alimentos, XG são amplamente utilizados como espessantes, estabilizantes, substitutos de gordura, ou modificadores de amido em muitos produtos, por exemplo, sorvete, molho,

maionese, macarrão, guisado, etc. para melhorar as propriedades reológicas e térmicas de produtos. Além disso, várias atividades biológicas, como efeitos hipolipemiantes e antidiabéticos, foram relatadas para esse polissacarídeo (LUCYSZYN et al., 2011).

Estudos relatados por Arruda et al., (2015) afirmam que a XG extraída de *Hymenaea courbaril* é um polissacarídeo promissor para alimentos e indústria farmacêutica. A *H. courbaril* var. *courbaril* pertence à família Fabaceae e subfamília Caesalpiniaceae, é popularmente conhecido por Jatobá, e apresenta distribuição ampla, desde o sul do México até o Centro-Oeste do Brasil, ocorrendo também na Floresta Amazônica, no Cerrado e na Caatinga, no Nordeste Brasileiro (LEE; LANGENHEIM 1975).

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5. ARTIGO CIENTÍFICO QUE SERÁ ENVIADO PARA A REVISTA

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EVALUATION OF THE HEALING ACTIVITY OF THE MEMBRANE OF XILOGLUCANA WITH OR WITHOUT CRAMOLL 1,4 IN MODELS OF DIABETIC MICE.

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Abstract

This study aimed at the evaluation of the healing potential of membrane based on the xyloglucan extracted from *Hymenaea courbaril* seeds containing the lectin from *Cratylia mollis* (Cramoll 1,4) in models of induced diabetic mice. Sixty *Swiss albino* male mices were used divided randomly into 4 experimental groups each containing 15 animals: Control group - diabetic animals treated daily with 100 µL saline solution; Cramoll group - diabetic animals treated daily with 100 µL Cramoll 1.4; Xylo Group – diabetic animals treated with xyloglucan membrane; and Xylo + Cramoll Group - diabetic animals treated with xyloglucan membrane containing 100 µL Cramoll 1,4. The groups were subdivided into three subgroups, each containing 5 animals, which correspond to the times of biopsies, performed at 2, 7 and 12 days after surgery. The results showed that Cramoll, Xylo and Xylo + Cramoll groups there was an acceleration in the formation of crusts, in reepithelialization and detachment, a greater contraction of wounds, and the hematological and histological events of the healing process occurred early in these groups as compared to the control group. According to the results, treatment with Xiloglucana membranes with or without Cramoll 1,4 wounds in diabetic mice is effective.

Keywords: Wound dressings, Cramoll 1,4, Diabetes, Xyloglucan.

1. Introduction

The emergence of a lesion in a body triggers a cascade of cellular and biochemical reactions in order to repair the injured tissue, but in diabetic lesions, this repair is slow. Several mechanisms are seen as important factors in reducing the healing process in diabetic subjects, among them are: the excessive production of reactive oxygen species (ROS), decreased nitric oxide (NO), decreased response to growth factors (GFs) and the insulin signaling pathway proteins (Brem and Tomic-Canic, 2007; Kolluru et al., 2012).

According to the International Diabetes Federation (2016), there were 14.2 million cases of diabetes in Brazil in 2015, generating an average of spending on health amounting to 1,527.4 dollars per diabetic person. Among the complications that often affect diabetic individuals are the wounds of the lower extremities (Singh et al., 2005). Such wounds are usually chronic and difficult to treat (Aronow, 2004) and may take years to heal and generally having a high rate of recurrence (Rodgers et al., 2011).

Wounds commonly involve extensive necrosis and infectious processes that can lead to amputation of body parts (Markowitz et al., 2006). Chronic wounds have a devastating effect on the health and lifestyle of more than 8 million americans each year. About 900 thousand people per year in the USA suffering from diabetic ulcers, 915 thousand suffer from venous stasis ulcers, and 6.5 million people suffer from pressure sores (Rodgers et al., 2011).

Therefore, the search for non-toxic natural compounds with stimulating properties of tissue repair is needed, aimed especially to find resources for making healing products made from raw material cost (Mandelbaum et al., 2003; Sehn et al., 2009).

Lectins are defined as proteins or glycoproteins of non-immune origin which bind carbohydrates specifically and reversibly (Ghosh et al., 1999; Dug et al, 2001; Sun et al., 2007). Lectins of *Cratylia mollis* seeds have been studied in several biotechnological areas, among these studies highlight the use of Cramoll 1,4 in wound healing, where it was seen that the saline solution of the lectin promoted a preview and effective closure of topical wounds in immunocompromised mice when compared with the control group (Melo et al., 2011). It was also seen that the topical application of hydrogel containing Cramoll 1,4 in the treatment of second degree burns accelerated

granulation, the process of re-epithelialization and wound contraction (Pereira et al., 2012).

The use of polysaccharides as a biological healing tool is advantageous, since these substances have unfettered, simplicity of operation and reduces costs of products (Okamoto et al., 1995). Among the various applications of polysaccharides, it is important to note their application as a vehicle for drug delivery (Carneiro-da-Cunha et al., 2009), where polysaccharide films have been widely used in the food industry (Cerqueira et al., 2012) and pharmaceutical and dressing topic (Monteiro et al., 2007).

Xyloglucans are polysaccharides extracted from the seeds of plants that have the ability to form matrix for the immobilization of bioactive molecules (Lucyszyn et al., 2011). Rosario et al. (2008) reported the extraction of xyloglucan from the seeds of *Copaifera langsdorffii*, *Hymenaea courbaril* and *Mucuna sloanei*, and their evaluation on the recruitment of macrophages into the peritoneum of rats. They observed that all xyloglucans promoted an increase in the number of peritoneal macrophages in dose dependent manner, characteristic associated to polysaccharides with immunomodulatory activity. Arruda et al. (2015) reported that the xyloglucan extracted from *H. courbaril* seeds is a promising polysaccharide for food and pharmaceutical industries.

In view of the effective potential of wound repair demonstrated by Cramoll 1,4 and the inherent characteristics of xyloglucan, immobilizing the lectin into polysaccharide membranes could accelerate the topical wound healing activity, especially as regards the assessment of the healing process in models with slow repair, such as diabetic lesions. This study aimed to evaluate the healing potential of xyloglucan membrane extracted from *H. courbaril* containing the lectin obtained from *C. mollis* seeds (Cramoll 1,4) in models of induced diabetic mice.

2. Material and methods

2.1 Cramoll 1,4 purification

C. mollis seeds were ground to obtain a flour, which was dissolved in 0.15 M NaCl, 10% (w / v) under magnetic stirring (500 rpm) for 18h at 4 ° C. This extract was fractionated by saturation with ammonium sulphate (0-40% and 40-60%), producing three fractions (F): F0-40 and F40-60 (precipitated fractions) and SF40-60 (supernatant fraction). F40-60 was dialyzed and purified by affinity chromatography on Sephadex G-75 column (Correia and Coelho, 1995).

2.2 Extraction of the xyloglucan from *H. courbaril* seeds

The seeds of Jatobá, *H. courbaril*, were washed and boiled in distilled water at 100°C for 30 minutes for enzyme inactivation and softening of the hull. Once stripped, the seeds were dried until constant weight (dry weight) and then subjected to exhaustive extraction with 0.1 M NaCl [15% (w/v)] at 25 ° C and ground to obtain a greenish crude extract. Then, the residues were removed by centrifugation for 20 min at 1500 rpm, and the supernatant was filtered through a voil tissue and a screen printing clothe (type 90), respectively, and precipitated with ethanol 46% (1:3 v/v) for 16h. The obtained precipitate was filtered using a screen printing clothe (type 110), washed consecutively with 100% ethanol and twice with acetone PA to remove possible protein contaminants, been filtered through a screen printing clothe (type 110) between each wash, and finally dried at 30°C, pulverized and maintained in amber glass vials (Arruda et al., 2015).

2.3 Preparation of xyloglucan membranes with Cramoll 1,4

The filmogenic solution of xyloglucan was prepared in 0.5% (w/v) of distilled water under stirring for 16h at 25 °C, and added to the plasticizer glycerol at 0.3% (v/v), under magnetic stirring for two hours at 25 °C. Cramoll 1,4 was incorporated at 0.5 mg.mL-1 to the filmogenic solution and the mixture was held at 25 ° C for 30 min and the pH was adjusted to 5.8 with 1.0 M NaOH. Subsequently, 5 ml of each solution was placed in Petri plates (3 cm) and the membranes were dried at 37 ºC for 24 h and used within a maximum interval of 48h.

2.4 Animals

There were used a total of 60 mice *Swiss albino* male, 2 months old, weighing between 28 and 32g, from the vivarium of the Laboratory of Immunopathology Keizo Asami (LIKA) of the Federal University of Pernambuco, kept at a constant temperature (22 ± 2 ° C), brightness controlled (12 hours of light and 12 hours of dark) and with food and water ad libitum. The experiments were performed obeying the Brazilian College of Animal Experimentation standards (COBEA). The study was approved by Animal committee of the Federal University of Pernambuco, Brazil (proc. 23076.018754/2013-61).

The animals were randomly divided into 4 experimental groups of 15 rats each: Control group - Diabetic animals treated daily with 100 µL saline solution; Cramoll

group - Diabetics animals treated daily with 100 µL Cramoll 1,4; Xylo group - Diabetic animals treated with xyloglucan membrane; and Xylo + Cramoll group - Diabetic animals treated with xyloglucan membrane containing 100 µL Cramoll 1,4. Each group was subdivided into three groups containing 5 animals, which correspond to times of biopsies that were performed on the 2nd, 7th and 12th day after surgery.

2.5 Diabetes induction

Diabetes was induced by a single intraperitoneal injection of alloxan in a dose of 120 mg/kg. For this, the animals were kept in a solid fast for 24 h. The solution of alloxan monohydrate (Sigma-AldrichInc, St. Louis, MO, USA) was prepared minutes before application by dilution of the drug in saline 0.15 M. Glucose levels were determined 7 days after induction by the Labtest kit for glucose. For this procedure, the animals were subjected to 12 h of fasting solid. Animals who obtained blood glucose above 250 mg/dL were considered diabetic (Malerbi and Franco, 1992).

2.6 Wounds induction

The wounds were induced 7 days after confirmation of diabetes. The animals were anesthetized intramuscularly with 2% xylazine (10 mg/kg) and ketamine hydrochloride 10% (115 mg/kg). After the anesthesia, each animal was submitted to trichotomy and antisepsis with povidone iodine 1% in the dorsal region. With the aid of a metal punch (No. 8mm), the skin was marked for the production of cutaneous wound. The animals were kept in individual cages and treated in accordance with the corresponding group.

2.7 Clinical evaluation of experimental lesion

Clinical characteristics of experimental wounds were observed daily, considering the following parameters: swelling, redness, discharge, scabies, granulation, epithelialization and tissue healing.

2.8 Microbiologic evaluation

The microbiological evaluation was performed with swab in the lesion area at the time of surgery and the corresponding biopsies days. This review was conducted to assess the degree of contamination of injuries.

2.9 Evaluation of the retraction of the wounds

To evaluate the retraction of the wounds the measurement was carried out of its borders, from the measurements of the area with a digital caliper, observing the largest and smallest diameter. From these elements, the area was calculated, using the recommended mathematical equation: $A = \pi . R . r$, where "A" is the area "R" and the largest radius "r" the smallest radius of the lesion. The calculation of the degree of contraction was expressed as a percentage using the equation of Ramsey (1995): $100 \times (Wo - Wi)/Wo = M \pm DP$, where Wo = the initial wound area; Wi = Wound area at day of biopsy (T_2, T_7 e T_{12}).

2.10 Biopsy of the lesion and histopathological evaluation

Skin biopsies were performed aiming at the removal of all skin fragment and the wound area, including one centimeter apart from each dorsal and ventral margin in the caudal direction. Immediately after removal of the skin, the samples were placed on filter paper and fixed in 4% formaldehyde for a period of 48 h replaced by a 70% ethanol solution for processing of histological slides. These were submitted to staining with HE, for histopathological analysis. Slides were photographed at the Leica DM-500 microscope coupled with Leica ICC 50 camera and imaging software at the same magnification.

2.11 Hematologic parameters evaluation

The blood of the animals in each group was collected via cardiac puncture (T2, T7 and T12). Hematological parameters (erythrocytes, leukocytes and platelets) were determined for the immediate collection of blood and analyzed by Sysmex XT 4000i equipment. Four experimental groups of animals were sacrificed by injection of 30 mg / kg-1 of thiopental sodium.

2.12 Statistical analysis

For statistical analysis, non-parametric tests were used. To detect differences between groups was used analysis of variance Two-way ANOVA using GraphPad Prism 5.0 program. All results are expressed as mean values ± standard deviation of the groups and analyzed considering $p < 0.05$ as statistically significant.

3. Results and Discussion

3.1 Clinical evaluation of the experimental wounds

The presence of edema and hyperemia was observed in the groups Control and Cramoll 1,4, however those symptoms had become more pronounced in the Control group. The formation of primary and secondary crusts was observed in all groups; it was formed earlier in the treated groups, thus promoting reepithelialization and detachment of the wounds (Fig. 1). It is well known that the presence of edema and hyperemia are key-characteristics of the inflammatory process. Inflammation is crucial in the wound healing cascade, to prevent infection, stimulate cell proliferation, revascularization and remodelling of extracellular matrix (Chen et al., 2014; Bielefeld et al., 2013). Our results corroborate those found by Melo et al. (2011), who observed edema and hyperemia in all experimental groups when evaluating the healing activity of Cramoll 1,4 in immunocompromised animal wounds.

3.2 Microbiological evaluation

The microbiological evaluation showed that the lesions of the experimental groups had absence of contamination during the 12 days of treatment. Our results corroborate the studies by Pereira et al. (2012), that reported no infection in experimental groups when evaluating the healing ability of the hydrogel of Cramoll 1,4 in second degree burns in rats. Melo et al. (2011), in turn, studied the healing process in imunocompromised mices and observed contamination of the lesions of Control groups during the 12 days of treatment, since the lesions treated with 1,4 Cramoll were not contaminated. The infection associated with excessive inflammation (Sibbald et al, 2011), is considered the most likely cause of delayed wound healing in chronic and acute wounds, despite the wide availability of antimicrobial agents for the prophylaxis and treatment (Farrar, 2011; Sen, 2011).

3.3 Evaluation of the retraction of the wounds

The area of the wound gradually decreased in all groups, however in the T2 period, the Xylo group (54.97 ± 1.97) had the highest rate of decline, while the Control group (41.15 ± 7.51) and the Xylo + Cramoll (44.96 ± 4.60) had a higher decrease compared to Cramoll 1,4 group (28.00 ± 2.86). According to the results reported by Rosario et al. (2008), xyloglucans extracted from *C. langsdorffii*, *C. courbaril* and *M.*

sloanei promoted an increase in the number of peritoneal macrophages, featuring xyloglucan with an immunomodulating activity. This immunomodulatory activity on macrophages may explain the higher retraction of the Xylo group during this period, since the activated macrophage is the main effector cell in the tissue repair process, degrading and removing components of the damaged tissue, such as collagen, elastin and proteoglycans (Singer and Clark, 1999). These cells act in the vascular endothelium, inducing the expression of adhesion molecules (Medzhitov, 2008), furthermore, they produce and export lipid mediators, peptide mediators such as cytokines and growth factors, and other proteins such as clotting factors, and enzymes related to the remodeling of the extracellular matrix, such as collagenases and metalloproteases (Mantovani et al., 2013; Pasparakis et al., 2014).

de Melo et al. (2010a) evaluated Cramoll 1,4 in murine lymphocyte cultures as an immunomodulatory agent. The results demonstrated that Cramoll 1,4 has anti-inflammatory response by suppressing nitric oxide (NO), and then the study concludes that Cramoll 1,4 could be considered a lectin with immunomodulatory activity. Our results demonstrates that Cramoll 1,4 may have modulated the inflammatory response in order to equilibrate this response; the wound retraction during this period was not significant, since it is known that NO is important in mediating the immune response, and when produced at high levels by macrophages, has cytotoxic activity against microorganisms, parasites and tumor cells, and also plays an important role in modulating the immune system (Moncada et al., 1991). In vitro studies revealed that when peritoneal exudate cells infected with *Staphylococcus aureus* were treated with different concentrations of pCramoll (native Cramoll) and rCramoll (recombinant Cramoll), NO was significantly produced (Silva et al., 2010).

Cramoll 1,4 group (77.84 ± 6.1) presented the highest retraction wound at T7, and the wounds of Xylo + Cramoll group (72.22 ± 3.16) retreated more than the wounds of the groups Control (61.65 ± 0.75) and Xylo (52.93 ± 4.99). At T7, the Control group showed a greater retraction compared with the Xylo group. The healing process in chronic wounds is complex and many factors such as diabetes, for example, contribute to delayed healing, they act extending inflammation in the wound causing an incomplete and disorganized cure (Bayat et al. 2003; Fonder et al ., 2008).

After 12 days of treatment (T12 period), the groups Cramoll 1,4 (92.36 ± 3.05) and Xylo (89.62 ± 0.98) had a retraction rate higher than the Control group (83.30 ±

0.83) (Fig. 2). The healing activity of Cramoll 1,4 has been well studied and the lectin has been also reported with an effective and earlier wound repair in immunocompromised mice when compared with Control group (treated with saline) (Melo et al., 2011). The acceleration of the healing process of wounds by topical application of a hydrogel containing Cramoll 1,4 was also reported in the treatment of second degree burns (Pereira et al., 2012).

Several polysaccharides have proven to be effective in the treatment of wounds, as an example we can mention alginate, which proved to be effective in burn wound healing process (Kim et al., 2008), and chitosan due to its immunomodulating property that enables almost exclusively macrophages (Senel and McClure, 2004).

3.4 Histopathological evaluation

Animals of all groups presented a vascular granulation tissue during the assessment period T2, but Cramoll 1,4 group this tissue proved to be vascular and floppy (Fig. 3B). In this time interval, the inflammatory infiltrate was acute on all the animals of the experimental groups, but proved to be intense in the Xylo group (Fig. 3C), and moderately intense in Xylo + Cramoll 1,4 group (Fig. 3D).

According to Bird et al. (2012) and Tam et al. (2014), hyperglycemia enhances the production of pro-inflammatory cytokines and reactive oxygen species (ROS) while decreases the production of NO, resulting in difficulty to promove the healing process in diabetic wounds. The excessive generation of oxidative stress active leukocytes and causes damage to endothelial cells (Granger, 2002). An in vivo study showed that a single administration of Cramoll 1,4 resulted in an increase in cytosolic and mitochondrial ROS in lymphocytes of rats, but no damage was observed on the morphology of apparent cells (de Melo et al., 2010b). Silva et al. (2015) contrasted this information when performed in vitro studies and evaluated the cytoprotective effects of pCramoll and rCramoll against oxidative damage induced by hydrogen peroxide (H_2O_2) in Vero cells. The results revealed that the pre-treatment of cells with lectins (pCramoll and rCramoll) for 30 minutes reduced significantly the production of mitochondrial ROS.

When an injury occurs, keratinocytes produce cytokines that contribute to the beginning of the inflammation (Pasparakis, et al., 2014); activated macrophages, in turn, produce pro-inflammatory cytokines that act amplifying the inflammatory process (Li et

al., 2007). Our results demonstrate that the Xylo group showed an intense inflammatory infiltrate due to the action of the xyloglucan in the recruitment of macrophages and activity of these cells in inflammation.

The experimental groups in T7 presented a fibrovascular granulation tissue, characteristic of the proliferative phase of the wound healing. The inflammatory infiltrate proved to be acute in the Control group (Fig. 4A) and discreet in the treated groups. Control, Xylo, and Xylo + Cramoll 1,4 groups showed early reepithelialization at the edges of the lesions, however the Cramoll 1,4 group presented a reepithelialization correlated with keratinization (Fig. 4A, 4B and 4D).

As already mentioned before, the healing process in chronic wounds is complex and there are factors that act extending inflammation in the wound, causing an incomplete and disorganized cure (Bayat et al. 2003; Fonder et al., 2008). Our results corroborate this information, since the Control group presented an acute and reminiscent inflammatory infiltrate, implying the need for more time for the wound healing.

The evaluation on T12 showed fibrous tissue for Control, Cramoll and Xylo groups, however the fibrous tissue was at the beginning of its formation in the Control group (Fig. 5A), and it was mature in Cramoll, Xylo and Xylo+Cramoll groups (Fig. 5B, 5C and 5D). In all groups the reepithelialization was complete (Fig. 5D).

In the remodeling or maturation phase, a recovery of the normal tissue structure is attempted, most of the vessels, fibroblasts and inflammatory cells disappear from the lesion site through the process of migration, apoptosis and other mechanisms of cell death, resulting in scar with fewer cells (Mendonça and Coutinho Netto, 2009). In this phase there is a detailed and organized balance between degradation and formation of the matrix (Miloro, 2008; Ackermann, 2009), and is marked by aging of elements and changes in the extracellular matrix, affecting the deposition of proteoglycan and collagen (Gabbiani et al., 1972).

3.5 Haematological evaluation

The haematological values showed no significant differences in hematocrit, eosinophils, basophils, and lymphocytes parameters for the entire experimental interval (Table 1).

In T2, the platelets were in higher quantities in the Xylo + Cramoll 1,4 group compared to the Control group (Table 1). In the healing process, platelets are activated

by substances secreted by extracellular matrix surrounding the endothelium, resulting in the start of cellular adhesion and aggregation for the platelet plug formation (Silva et al., 2006). The association of xyloglucan and Cramoll 1,4 may modulate the recruitment of these blood components. The study performed by Serra et al. (2012) used platelet rich plasma (PRP) for the treatment of wounds of 26 diabetic patients submitted to underwent amputation and demonstrated that, after a month of treatment, there was a cure rate of 96.15% compared to the Control group, which achieved a cure rate of 59.37%.

The Control group had a higher percentage of neutrophils compared to Cramoll group, and monocytes compared to Xylo and Xylo + Cramoll 1,4 groups (Table 1). In the inflammatory phase of the wound healing, once the platelets come out of the vascular bed, neutrophils and monocytes migrate to the central of the wound in response to chemotactic agents, but the absence of neutrophils in the blood did not affect the repair process in the absence of infection (Werner and Grose, 2003; Simpson and Ross, 2009).

Our results showed that the Cramoll 1,4 group had a greater number of total leukocytes compared with the Xylo group, confirming what was discussed in the previous section, that the Cramoll group had a floppy vascular tissue probably due to excessive generation of oxidative stress, which consequently activates leukocytes.

In T7, Cramoll group showed a greater amount of red blood cells when compared to the other groups, and a higher level of hemoglobin than that provided by Xylo and Xylo + Cramoll 1,4 groups. Regarding the platelets level, it was observed that Xylo and Xylo + Cramoll 1,4 groups presented higher level of than those of the groups Control and Cramoll 1,4. Total leukocytes was higher for Cramoll 1,4 group (Table 1). The proliferative phase is responsible for the closure of the injury, and also covers the reepithelialization, fibroplasia and angiogenesis. The granulation tissue presented fibroplasia and angiogenesis even for four days after injury. The fibroblasts produce the new extracellular matrix required for cell growth and new blood vessels carry oxygen and nutrients to the local cellular metabolism (Mendonca and Coutinho-Netto, 2009).

In T12, platelets presented higher quantities in the Control group compared with Cramoll and Xylo + Cramoll 1,4 groups. (Table 1). As previously metioned, at the remodeling phase, the majority of vessels, fibroblasts and cells of the inflammatory phase disappears from the site of the injury (Mendonca and Coutinho-Netto, 2009), and

the presence of platelets in the Control group suggests that this group showed delayed remodeling phase.

Conclusion

It can be concluded that treatment with Xiloglucana membranes with or without Cramoll 1,4 wounds in diabetic rats is effective. These results extend the biotechnological potential of Cramoll 1,4 and Xiloglucana, making these interesting compounds for the pharmaceutical industry.

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Figure 1: Clinical evaluation of the wounds of diabetic mice. **A** – Control Group; **B** – Cramoll Group; **C** – Xylo Group; **D** - Xylo + Cramoll Group. T0 demonstrates the production of the wounds in the experimental groups; T2 demonstrates edema and hyperemia in **A** and **B**, and the formation of the primary crust in all experimental groups; in T7 there was observed the presence of secondary crust and detachment in all groups, but **B** presented reepithelialization and the contraction of the wound is most evident; in T12, it is observed that the secondary crust fell, there was reepithelialization and wound retraction in all experimental groups, but both reepithelialization and retraction were more pronounced in the treated groups.

Figure 2: Percentage of wound contraction after 2, 7 and 12 days of treatment. Means followed by the same letter do not differ significantly from each other by two-way ANOVA test. Statistically significant differences $p < 0.05$.

Figure 3: Histopathological analysis of the wounds of diabetic mice with two days of treatment (T2). **A** - Control Group; **B** - Cramoll Group; **C** - Xylo Group; **D** - Xylo + Cramoll Group. Important to observe an intense inflammatory infiltrate in Xylo Group, a moderate infiltrate in Xylo + Cramoll Group, and a floppy vascular granulation tissue in Cramoll Group. H.E. staining CR - crust; TV - vascular granulation tissue.

Figure 4: Histopathological analysis of the wounds of diabetic mice with seven days of treatment (T7). A - Control Group; B - Cramoll Group; C - Xylo Group; D - Xylo + Cramoll Group. Important to observe an acute inflammatory infiltrate in the Control group; early reepithelialization in Control, Cramoll and Xylo + Cramoll groups; presence of keratin in the groups Cramoll and Xylo + Cramoll. H.E. staining CR - crust; TFV - fibrovascular granulation tissue; EP - Epithelium; Q - Keratin.

Figure 5: Histopathological analysis of the wounds of diabetic mice with twelve days of treatment (T12). A - Control Group; B - Cramoll Group; C - Xylo Group; D - Xylo + Cramoll Group. Important to observe the presence of a mature fibrous tissue in groups Cramoll, Xylo and Xylo + Cramoll. H.E. staining TFV - Fabric fibrovascular; TF - fibrous tissue; EP - Epithelium; Q - Keratin.

Table 1: Hematological parameters of diabetic mice after 2, 7 and 12 days of treatment. A – Control Group; B – Cramoll Group; C – Xylo Group; D - Xylo + Cramoll Group. Mean ± SD (n = 5). Means followed by the same letter do not differ significantly from each other by two-way ANOVA test. Statistically significant differences $p < 0.05$.

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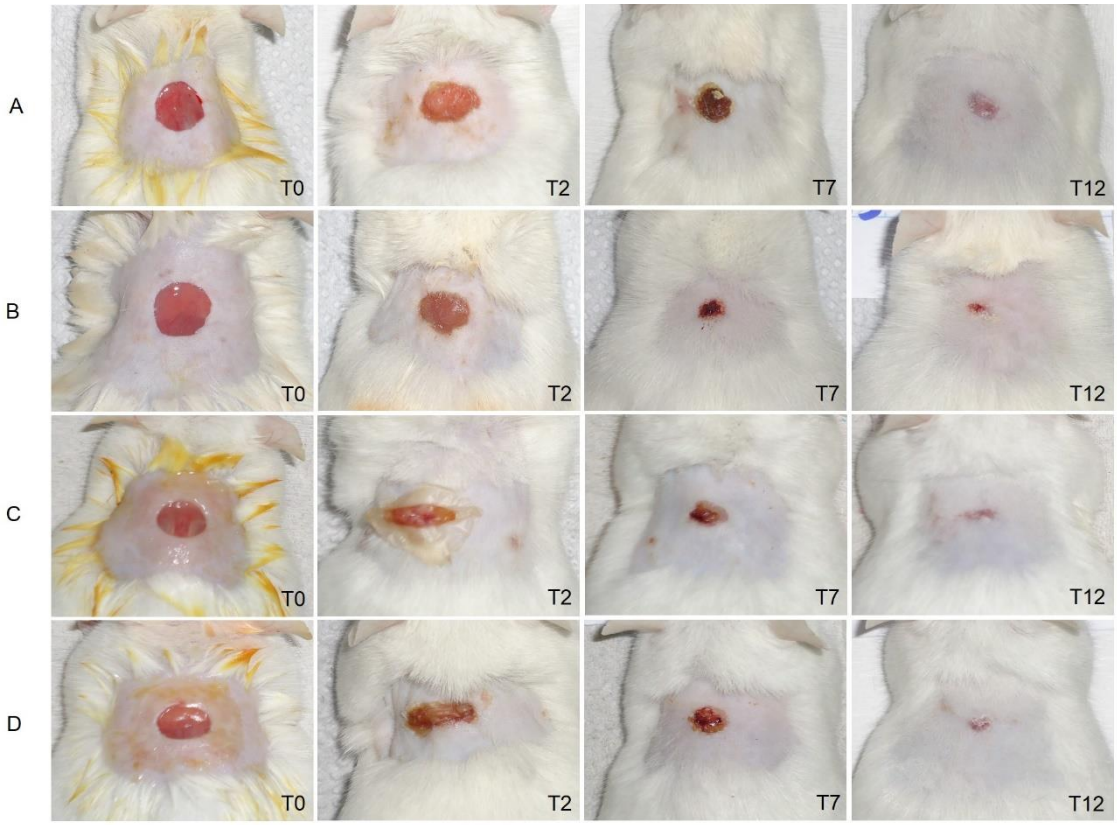

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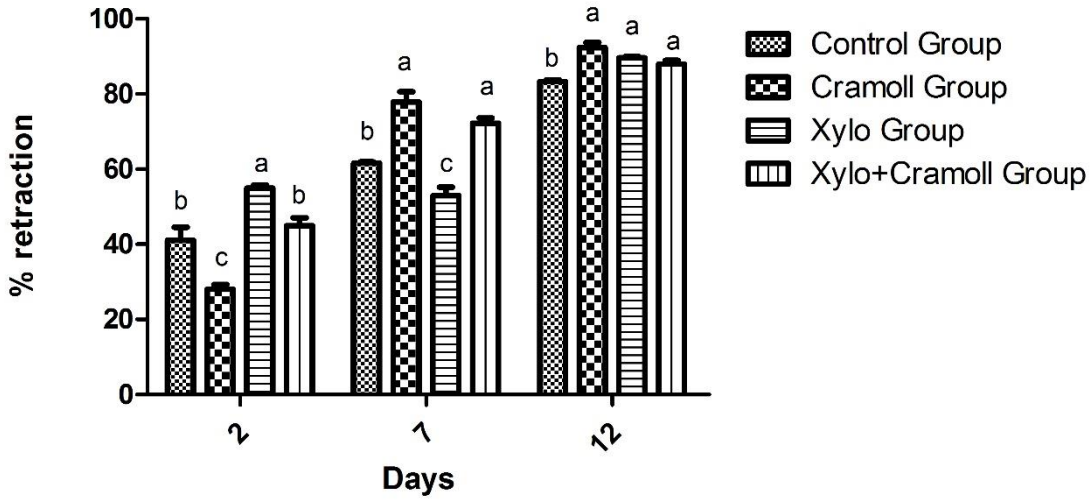

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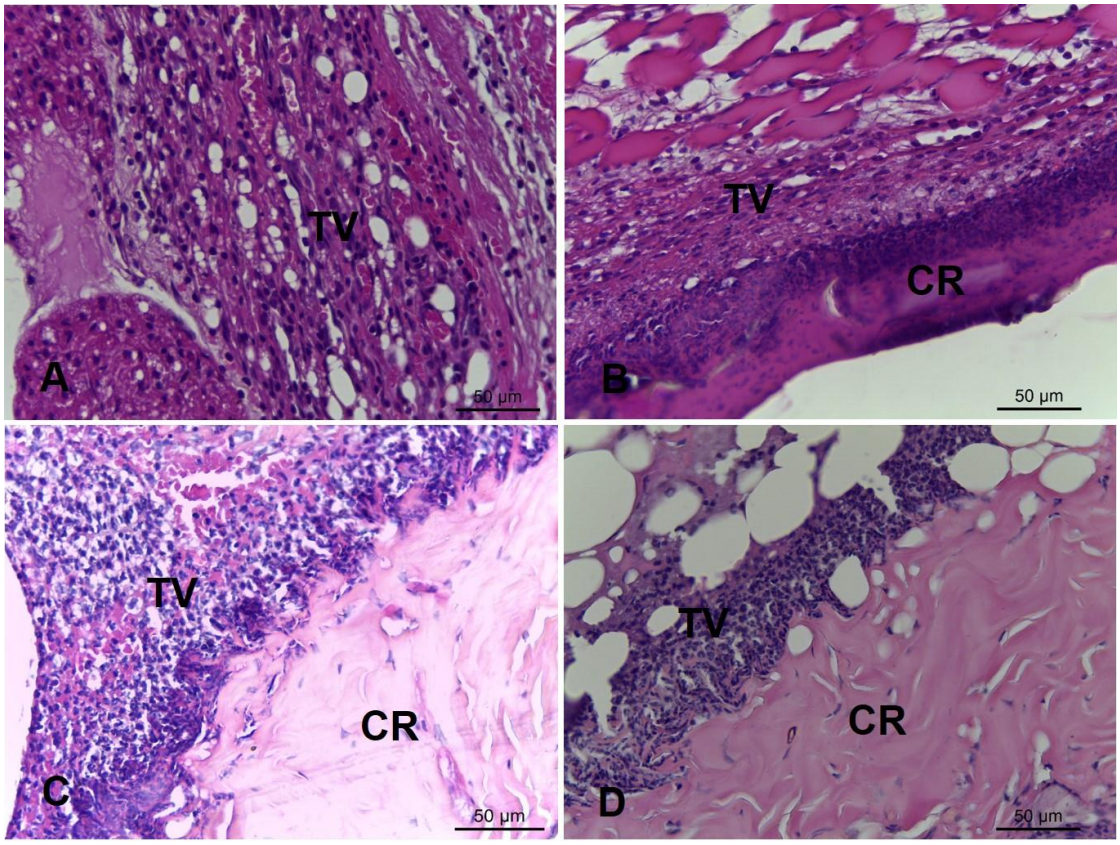

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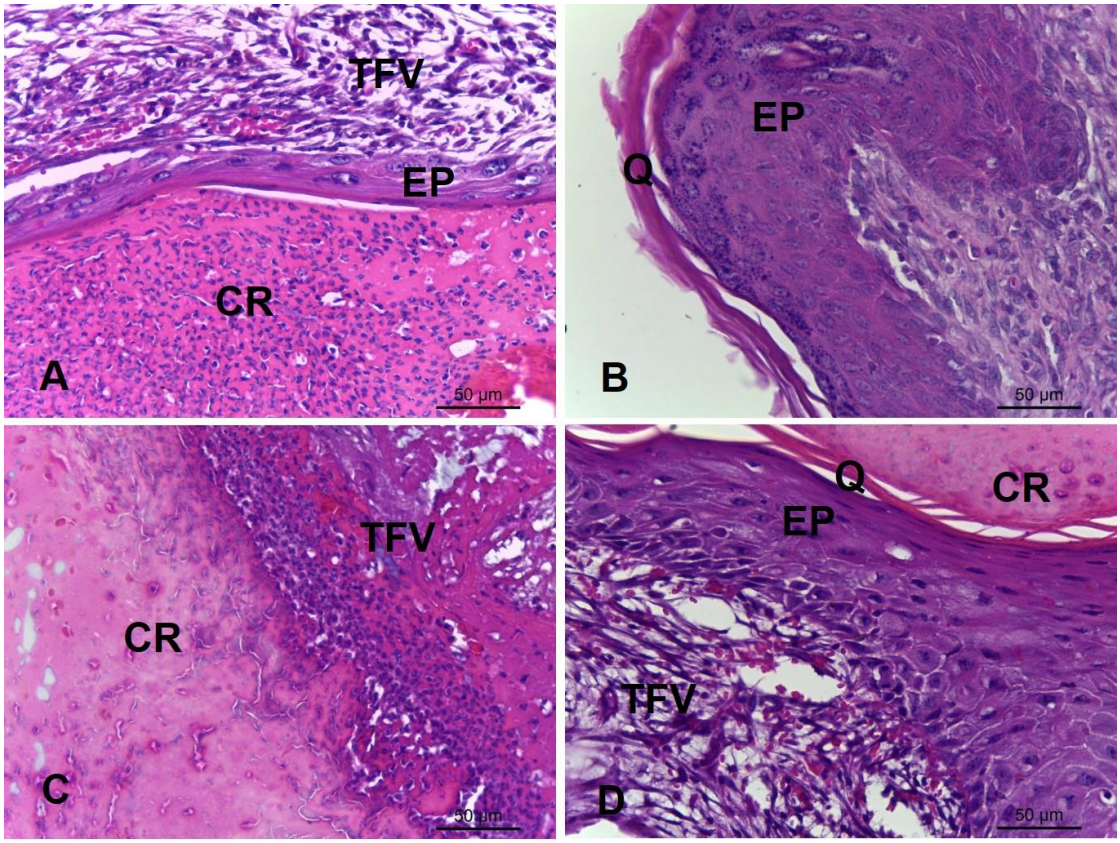

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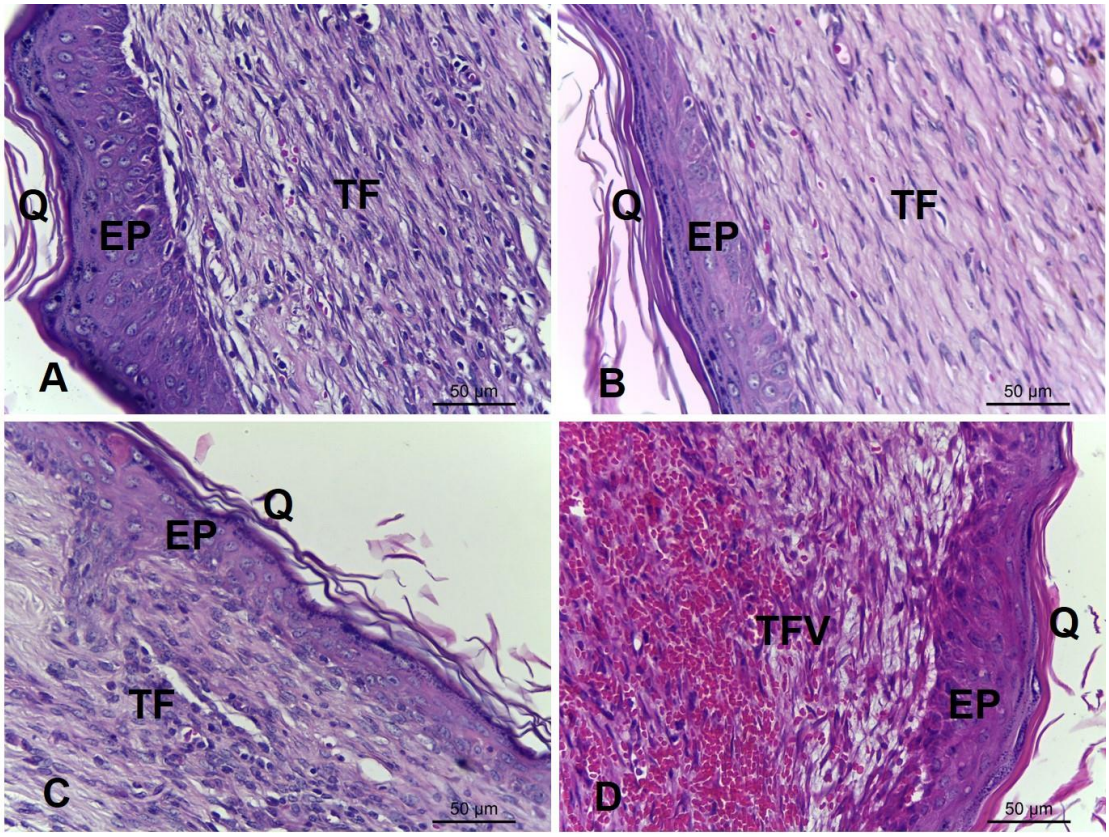

Tabela 1

Parameters	2th day				7th day				12th day			
	A	B	C	D	A	B	C	D	A	B	C	D
Erythrogram Erythrocytes mil/mm3	9,204±0,88	8,276±1,03	8,622±1,35	8,498±1,11	8,758±0,99	10,388±0,33	8,018±0,99	8,274±1,12	9,624±0,25	9,208±0,45	8,774±0,02	9,028±0,21
Hemoglobin g/dL	14,1±1,50a	12,94±1,85a	13,74±1,86a	13,3±1,69a	14,04±0,77ab	14,04±0,77a	12,74±1,47b	12,86±2,02b	14,36±0,28a	14,26±0,05a	13,8±0,27a	15,24±0,49a
Hematocrit %	47,74±3,97a	44,06±5,12a	44,86±7,88a	45,32±5,06a	45,08±5,43a	49,72±1,82a	44,52±5,52a	44,54±7,04a	48,42±2,70a	46,46±1,38a	48,18±1,47a	49,06±0,16a
Plateletcount Platelets mil/mm³	795,8±103,76a	865,2±138,29ab	1041,2±111,18ab	1122,8±167,78b	906,4±409,74a	922,2±34,38a	1423,8±33,48b	1271,2±220,06b	1117±145,88a	743,6±193,05b	816,2±152,26ab	532,6±332,84b
WBC Total Leukocytes mm³	6,228±1,79ab	10,816±2,65a	4,294±4,74b	6,284±4,19ab	6,108±0,83a	15,23±0,46b	7,114±2,98a	10,052±5,82a	9,432±0,23a	10,716±3,47a	7,192±2,01a	9,436±0,42a
Neutrophils %	26,2±22,64a	6,6±2,07b	15,8±1,09ab	19,8±7,42ab	33±24,64a	9±0,70b	16,8±5,71b	9,6±3,20b	9,4±1,51a	7,6±1,94a	12,4±2,19a	16,8±2,04a
Eosinophils %	0±0a	0±0a	0±0a	0±0a	0,6±0,89a	0,4±0,54a	0±0a	0±0a	0,4±0,54a	0,4±0,54a	0,4±0,89a	0,2±0,44a
Basophils %	0,2±0,44a	0,4±0,89a	0±0a	0±0a	0,4±0,54a	0±0a	0,2±0,44a	0,2±0,44a	0±0a	0±0a	0±0a	0±0a
Lymphocytes %	69,6±21,47a	77,6±32,81a	82,2±2,04a	75,4±7,36a	73,4±20,91a	90,2±1,09a	76,6±6,02a	89±3,67a	86,8±1,64a	90±2,73a	82,4±7,66a	76±1,73a
Monocytes %	3,2±1,64a	1,4±0,89ab	0,4±0,54b	0±0b	1,6±0,54a	1,4±0,54a	2,1±2,04a	1±1,22a	2,2±1,78a	0,6±0,54a	0,4±0,89a	0,8±1,30a

*Means followed by the same letter do not differ significantly from each other by two-way ANOVA test.

6. Anexos (Normas da Revista)

INTERNATIONAL JOURNAL OF PHARMACEUTICS

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