UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
LABORATÓRIO DE IMUNOPATOLOGIA KEIZO ASAMI
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA APLICADA À SAÚDE

ADELMO CAVALCANTI ARAGÃO NETO

AVALIAÇÃO *IN VIVO* DO POTENCIAL PARA REPARO TECIDUAL UTILIZANDO HIDROGEL DE POLISSACARÍDEOS ASSOCIADO OU NÃO A LASERTERAPIA DE BAIXA INTENSIDADE

Recife
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde do Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Biologia Aplicada à Saúde.

Área de concentração: Biotecnologia.

Orientadora: Profª. Dra. Maria das Graças Carneiro da Cunha

Recife

2018

Catalogação na fonte:
Bibliotecária Claudina Queiroz, CRB4/1752

Aragão Neto, Adelmo Cavalcanti
Avaliação *in vivo* do potencial para reparo tecidual utilizando hidrogel de polissacarídeos associado ou não a laserterapia de baixa intensidade / Adelmo Cavalcanti Aragão Neto - 2018.
78 folhas: il., fig., tab.

Orientadora: Maria das Graças Carneiro da Cunha
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Biologia Aplicada à Saúde. Recife, 2018.

Inclui referências e anexos

1. Polissacarídeos 2. *Anacardium occidentale* L 3. Quitosana I. Cunha, Maria das Graças Carneiro da (orient.) II. Título

615.5 CDD (22.ed.) UFPE/CB-2019-090

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Dissertação/Tese apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde, do Centro de Biociências da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutor em Biologia Aplicada à Saúde.

Aprovado(a) em: 07/03/2018

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Aos meus pais, à minha esposa, à minha orientadora, aos amigos e colegas do laboratório de biotecnologia, e aos amigos e professores do programa de pós-graduação em biologia aplicada a saúde. Um forte abraço.

AGRADECIMENTOS

À Profª. Drª. Maria das Graças Carneiro da Cunha, pela orientação, cuidado, carinho, e pela grande paciência nesta jornada. – Muito obrigado por tudo. A senhora está no meu coração.

Aos amigos Prof. Dr. Paulo Soares, Profª. Drª. Priscilla Sales e Profª. Msc. Fernanda Andrade pela ajuda durante todas as fases da pesquisa, sem seu conhecimento e dedicação este trabalho não teria sido possível. – Vocês são amigos. O que precisarem estou as ordens.

Ao Prof. Dr. Luiz de Carvalho pelo apoio, orientação e estado de espírito contagiante. – Gosto muito de conversar com o senhor, mestre.

A todos os professores e funcionários do Departamento de Bioquímica e do Programa de pós-graduação em Biologia aplicada à Saúde, bem como do LIKA. – Muito obrigado por tudo.

À Profª. Drª. Maria Helena Madruga, médica veterinária, pela orientação e acolhimento no biotério do Laboratório de Imunopatologia Keizo Asami. – Muito obrigado mesmo, doutora.

À minha esposa, Thaís, pelo amor e carinho, e por me fazer tentar ser sempre uma pessoa melhor. – Amo você.

Aos meus pais, Adelmo e Nina, por tudo! – Amo vocês. - Não sei como pessoas tão simpáticas puderam ter um filho tão chato.

E finalmente, à FACEPE pelo apoio a pesquisa no estado de Pernambuco!

– Muito obrigado, pessoal!

“O maior erro que um homem pode cometer é sacrificar a sua saúde a qualquer outra vantagem.”

Arthur Schopenhauer

RESUMO

O objetivo do presente trabalho foi a avaliação do processo de cicatrização/ reparo tecidual em ratos Wistar. Foram avaliados ferimentos completos confeccionados na região dorsal dos animais, para a avaliação da cicatrização da pele, e defeitos críticos circulares na região de calvária, para o reparo ósseo, os quais foram tratados com um hidrogel a base dos polissacarídeos, policaju, extraído da goma do cajueiro (*Anacardium occidentale* L) e quitosana, sendo o mesmo denominado POLI-CHI. Foi empregado associado ou não a Laserterapia de baixa intensidade (LTBI) no espetro do vermelho (660 nm) para pele e no infravermelho (830 nm) para o osso. Para tal, foram utilizados 96 animais (60 para pele e 36 para o osso), machos, com idade entre 90 e 120 dias, os quais foram submetidos a procedimento cirúrgico para a confecção de ferida circular (Ø = 0,8 cm) na região dorsal torácica e defeito ósseo crítico com a utilização de trefina circular (Ø = 0,5 cm). Os animais foram divididos em 3 ou 4 subgrupos de acordo com o tratamento empregado: Controle (C), tratado com NaCl 0,1M; tratado com Hidrogel (H); tratado com LTBI (L), este subgrupo não foi utilizado para a avaliação do reparo ósso, e tratado com Hidrogel associado a LTBI (HL). Os mesmos foram submetidos a eutanásia com 03, 07 e 15 dias para pele e 15, 30 e 45 dias para osso. Com relação à avaliação da cicatrização de pele, os subgrupos H e HL apresentaram cicatrizes mais estéticas e contração da lesão significante em comparação ao C ($p < 0,05$) e maior presença de crosta fibrino-leucocitária, maior presença de fibras colágenas em H, HL e L, regressão da neoformação vascular em H e modulação em L e HL. Para o reparo ósseo, não foi identificada significância entre os subgrupos para a avaliação radiográfica, contudo, na avaliação da regressão do defeito realizada através da avaliação histológica, identificou-se significância entre HL e C para 15 e 45 dias e entre H e C para 45 dias, identificou-se também maior presença de neo-formação óssea para H e HL e neo-formação vascular. Tendo em vista o que foi apresentado, pode-se concluir que o POLI-CHI se apresenta como um promissor material para ser utilizado para o estimulo e modulação da formação tecidual, provavelmente estimulando a deposição de colágeno e ampliando a vasculatura local, essencial para o reparo de tecidos mineralizados.

Palavras-chave: Polissacarídeos. *Anacardium occidentale* L. Quitosana. Pele. Osso. Laser.

ABSTRACT

The aim of this study was to evaluate the tissue healing/ repair process in Wistar rats. Where evaluated complete surgical wounds in the dorsal thoracic region (for skin healing assessment) and critical size circular bone defects in the calvarium region (for bone assessment), which were treated using a polysaccharide hydrogel made using policaju, extracted from the *Anacardium occidentale* L. gum, and chitosan, being termed POLI-CHI. This treatment was made associating or not with the Low Level Laser Therapy (LLLT), in the red spectrum (660 nm) for skin and in the infrared spectrum (830 nm) for bone. Ninety-six animals were used (60 for skin and 36 for bone), male, age ranging from 90 to 120 days, they were subjected to surgical procedures to simulate a complete circular wound (Ø = 0,8 cm) in the thoracic dorsal region and a critical size defect in calvarium region (Ø = 0,5 cm) using a drill. The animals were distributed in 3 or 4 subgroups, according to the treatment, being: Control (C), treated using NaCl 0.1 M, Hidrogel (H), LLLT (L), this subgroup was not valuated for bone repair, and treated with hydrogel and LLLT (HL). The animals were euthanized after 3, 7, and 15 days for skin and at 15, 30, and 45 days for bone evaluation. Regarding wound healing, H and HL presented a more esthetic scar tissue and significant wound regression in comparison to C ($p < 0.05$), and yet a strong presence of Fibrin-leucocyte crust, strong presence of collagen fibers for H, HL and L, neovascular regression at H and modulation for L and HL. In the matter of bone repair, there was not significance between subgroups at the radiographic evaluation, although, in the defect's regression evaluation using histology measures was found that HL was significant to C at 15 and 45 days, and H to C at 45 days ($p < 0.05$). Also, was found a strong presence of bone and vascular new formation in H and HL. Observing the collected data, it's possible to conclude that that POLI-CHI presented is self as a valuable material to use for stimuli and modulation of tissue repair/ healing, probably by increasing the collagen deposition and increasing the local vase presence, essencial to a correct repair of mineralized tissue.

Key-Words: Polysaccharide. *Anacardium occidentale L.* Chitosan. Skin. Bone. Laser.

LISTA DE ILUSTRAÇÕES

REVISÃO DE LITERATURA

Artigo I

Artigo II

LISTA DE ABREVIATURAS E SIGLAS

CV	Canais de Volkmann
FC	Fatores de crescimento
FGF	Fator de crescimento de fibroblastos
FP	Folículos pilosos
GS	Glândulas sebácias
HD	Hipoderme
HILT	Laserterapia de alta intensidade
IL-1β	Iterleucina 1 β
IL-6	Interleucina 6
IL-8	Interleucina 8
LLLT	Laserterapia de baixa intensidade
M	Medula óssea
MCP-1	Proteína quimioatraente de macrófagos tipo 1
NF-κβ	Fator nuclear Kappa β
OC	Osso compacto
OT	Osso trabecular
P	Pele
PDGF	Fator de crescimento derivado de plaquetas
TN	Terminações nervosas livres
TNF-α	Fator de necrose tumoral alfa

SUMÁRIO

1 INTRODUÇÃO

Uma grande variedade de produtos terapêuticos ou não tem como fonte a flora e a fauna. Remédios, alimentos, fibras, óleos naturais e essenciais, cosméticos, e produtos químicos são alguns exemplos dos produtos que podem ser fabricados/obtidos a partir das inúmeras classes de compostos químicos extraídos das nossas espécies vegetais e animais. Os polissacarídeos representam uma das classes de maior relevância, uma vez que são polímeros naturais extraídos de plantas, algas, animais, fungos ou obtidos por via fermentativa, com uma ampla gama de aplicações, especialmente nas áreas alimentícia, biomédica, farmacêutica e cosmética.

Dentre diversos polissacarídeos com propriedades medicinais, o POLICAJU, extraído da goma do cajueiro *Anacardium occidentale L*., encontrado em países tropicais, tem apresentado resultados eficientes no processo de cicatrização de lesões cutâneas. O fácil acesso a este material natural, não tóxico, hidrofílico, biocompatível e biodegradável, o qual ainda apresenta interessante atividade biológica e boas propriedades reológicas são fatores que fazem com que seja viável o seu uso como matriz para imobilização e distribuição de drogas.

Com relação à facilidade de acesso e aplicabilidade, outro polissacarídeo que tem tido grande destaque é a quitosana, obtida por desacetilação da quitina, por via fermentativa, ou ainda encontrada naturalmente na parede celular de alguns fungos. Com relação às suas atividades biológicas, a mesma tem sido destacada por apresentar atividades antimicrobiana, antifúngica, anticâncer, hemostáticas, bem como atividade curativa e cicatrizante.

Dentre os diversos tipos de agentes curativos que ajudam a manter a umidade no local lesionado, podem-se destacar os hidrogéis. Os quais representam uma classe de sistema de liberação controlada de drogas que tem se destacado na entrega inteligente das mesmas. Os hidrogéis são definidos como uma rede polimérica reticulada capaz de absorver grande quantidade de água ou fluído biológico, sem se dissolverem, e têm sido utilizados em aplicações médicas e biológicas devido as suas características físico-químicas, podendo assim serem utilizados para conservar células, nutrientes, drogas ou proteínas.

Há evidências de que várias estratégias terapêuticas são capazes de modular eventos em todas as fases do processo de cicatrização de feridas cutâneas, dentre elas podemos destacar a laserterapia de baixa intensidade, do inglês Low Level Laser Therapy (LLLT). Aplicações da LLLT incluem o tratamento de feridas resultantes de traumas ou

lesões vasculares, restauração da função neural normal após a lesão, atenuação da dor e modulação do sistema imune. Combinações de terapias são muitas vezes necessárias para melhorar o efeito terapêutico através do sinergismo e para reduzir a dose ou frequência de cada um dos tratamentos de lesões, e, por conseguinte, reduzir o risco de efeitos adversos.

A busca por novas modalidades de tratamento para lesões teciduais é sempre uma constante, desta forma, visando a melhoria dos resultados, terapias combinadas, associando a Laserterapia com biomateriais vem se consolidando como forma de acelerar e melhorar a qualidade dos processos de reparo e cicatrização. Desta forma, o objetivo deste estudo foi avaliar o potencial de estimulo à cicatrização/ reparo tecidual (cutâneo e/ou ósseos) em ratos Wistar, tratados com hidrogel de policaju e quitosana associado ou não à LLLT.

2 OBJETIVOS

2.1 GERAL

Avaliar o potencial de estimulo a cicatrização/ reparo tecidual em ratos Wistar, tratados com hidrogel de policaju e quitosana associado ou não à Laserterapia de baixa intensidade.

2.2 ESPECÍFICOS

- Obter o hidrogel de policaju e quitosana (POLI-CHI);
- Realizar o tratamento tópico de lesões cutâneas experimentais em ratos Wistar utilizando POLI-CHI ou POLI-CHI associado à Laserterapia de baixa intensidade (LLLT);
- Acompanhar a evolução do processo de cicatrização (achados clínicos e mensuração de sua área) durante 14 dias;
- Acompanhar a cicatrização do ponto de vista histopatológico, através de biópsias no 3º, 7º e 14º dias do pós-operatório (Crosta fibrino-leucocitária, Colágeno, Necrose focal, Depósitos de fibrina, Exsudato neutrofílico, Edema, Exudato eosinofílico, Infiltrado mononuclear, Infiltração macrofágica, Granulomas, Neovascularização,Proliferação fibroblástica, e Fibrose);
- Realizar o tratamento de defeitos críticos ósseos realizados na calvária de ratos Wistar utilizando POLI-CHI ou POLI-CHI associado à LLLT;
- Acompanhar a evolução clínica dos animais durante os primeiros 15 dias;
- Analisar radiograficamente o potencial de reparo ósseo no 15^{o}, 30^{o} e 45^{o} dias após a confecção do defeito;
- Analisar através de softwares o reparo através da mensuração da distância entre os bordos do defeito crítico e cálculo de regressão.
- Acompanhar o reparo do ponto de vista histopatológico, através de biópsias no 15º, 30º e 45º dias do pós-operatório (Osso neo-formado, angiogênese, e deposição de colageno).

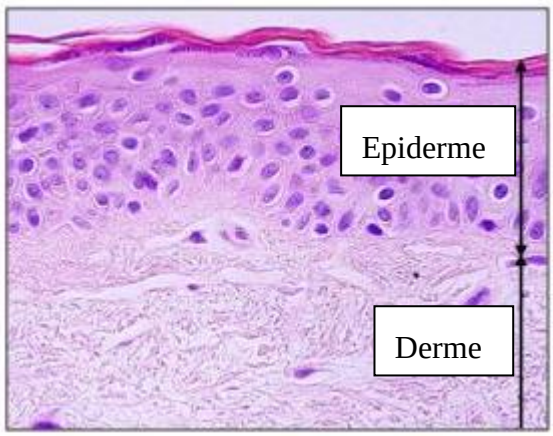
3 REVISÃO DE LITERATURA

3.1 ANATOMOFISIOLOGIA

3.1.1 TECIDO TEGUMENTAR (PELE)

A pele é o maior órgão externo do corpo humano, representando cerca de 15% do nosso peso corporal, possui múltiplas variações em relação a elasticidade, flexibilidade e resistência. É considerada o maior revestimento do corpo humano, visando, sobretudo, a proteção contra agentes externos inerentes ao ambiente. Divide-se em duas camadas: epiderme e derme, sendo suportadas pela hipoderme ou tecido subcutâneo (JINDAL, 2017) (Figura 1).

Figura 1. Fotomicrografia. Camadas da pele, evidenciando a epiderme e derme.



FONTE: Brohem et al. (2011).

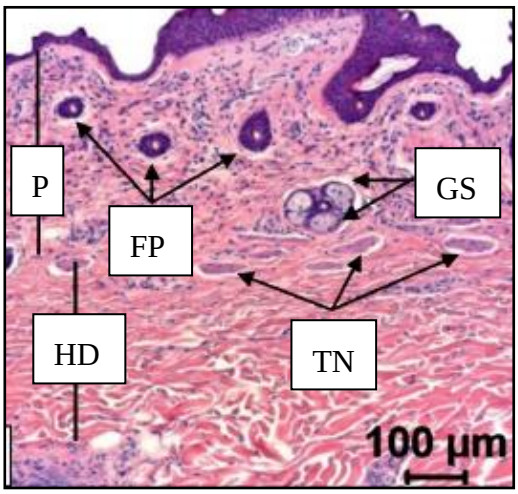
A primeira, mais externa, é formada basicamente por epitélio estratificado pavimentoso com espessura entre 0,05mm a 1,5 mm. É composta primariamente por três tipos distintos de células: melanócitos, responsáveis por sintetizar o pigmento melanina; células de Langerhans, elemento fundamental em reações imunes; e as células de Merkel, cuja função ainda não foi muito bem esclarecida pela ciência (HABIF, 2015).

A segunda camada, a derme é normalmente mais flexível e elástica. Contém grande quantidade de fibras protéicas, vasos sangüíneos, terminações nervosas, órgãos sensoriais e glândulas. Possui maior espessura na região dorsal do corpo em relação à ventral. Abaixo desta, existe a hipoderme que pode ser considerada como um tecido ou tela subcutânea, sendo composta principalmente por tecido conjuntivo frouxo e tecido adiposo. Desempenha as funções de isolamento térmico e fixação às estruturas

subjacentes (SAMPAIO e RIVITTI, 2014).

A pele possui os chamados anexos, como: unhas, pêlos, glândulas sudoríparas e sebáceas. As unhas são estruturas achatadas, elásticas, de textura córnea, aplicadas sobre a superfície dorsal das falanges distais. Os pêlos são encontrados em quase toda superfície do corpo. Variam muito em comprimento, espessura e cor. As glândulas sudoríparas consistem em um simples tubo cuja parte profunda constitui uma bolsa esférica ou oval chamada corpo da glândula, enquanto a porção superior ou ducto atravessa a derme e a epiderme, abrindo-se na superfície da pele por uma abertura afunilada. São muito abundantes na palma das mãos e planta dos pés (BONALUMI-FILHO, 2013). As glândulas sebáceas são órgãos glandulares pequenos e saculiformes alojados na derme, encontradas em abundância no couro cabeludo e na face. Cada glândula consiste de um simples ducto que emerge de um agrupamento ovalado ou em forma de garrafa (alvéolos), que são em geral de dois a cinco, podendo chegar, em alguns casos, até vinte. Cada alvéolo é composto de uma membrana basal transparente contendo certo número de células epiteliais (SAMPAIO e RIVITTI, 2014). Possui ainda inúmeras terminações nervosas livres. São sensíveis aos estímulos mecânicos, térmicos e especialmente aos dolorosos. São formadas por um axônio ramificado, envolto por células de Schwann, ambos envolvidos por uma membrana basal (JOHNSON e WOLFF, 2014) (Figura 2).

Figura 2. Fotomicrografia evidenciando a Pele (P), Hipoderme (HD) e anexos, como: Folículos pilosos (FP), Terminações nervosas livres (TN), e Glândulas sebáceas (GS).



Fonte: ISOLA et al., 2013.

Com relação a pigmentação da pele, um destaque especial deve ser dado aos melanócitos. Estes são células produtoras de melanina (responsáveis pela coloração da pele e a proteção celular contra a radiação solar), localizados em grande parte na epiderme. Em diversas circunstâncias nas quais ocorre lesão desse estrato a camada de melanócitos é depletada ocasionando assim a palidez característica de determinados tecidos reparados (SAMPAIO e RIVITTI, 2014).

3.1.2 CICATRIZAÇÃO DE FERIDAS

O processo de cicatrização ou reparo da pele envolve uma cascata ordenada de eventos, como: inflamação, proliferação (neoangiogênese, deposição de colágeno e reepitelização) e formação de tecido de remodelamento (WALLACE E BHIMJI, 2017). O objetivo destes eventos reparadores é impedir a invasão de patógenos e restabelecer a integridade dos tecidos danificados, reconstruindo assim a funcionalidade. Este processo envolve uma grande variedade de células (neutrófilos, macrófagos, fibroblastos), citocinas, quimiocinas, fatores de crescimento (FC) e espécies reativas de oxigênio (EROs) (LEAL, et al., 2017).

A inflamação caracteriza-se pelo aparecimento de dor, calor, rubor e edema. A injúria geralmente causa ruptura nos vasos sanguíneos, com extravasamento dos constituintes do sangue para a região lesada. O dano endotelial resulta na ativação de plaquetas e da cascata de coagulação, que leva à formação de uma camada de fibrina e em seguida, é formada uma matriz provisória para a migração celular (WANG et al., 2017).

Uma vez no local da ferida, neutrófilos e macrófagos ativados desencadeiam a liberação de mediadores inflamatórios, como citocinas, quimiocinas, EROs e enzimas proteoliticas. A produção de citocinas pró-inflamatórias como a interleucina 1β (IL-1β), interleucina 6 (IL-6) e o fator de necrose tumoral alfa (TNF-α) ocorre rapidamente após o trauma. Estes são importantes na ativação de células endoteliais e na expressão de moléculas de adesão, fato que contribui para o recrutamento e acúmulo de mais fagócitos na área inflamada (BERMAN et al., 2017). As quimiocinas, por sua vez, contribuem para a regulação da epitelização, angiogênese e remodelamento do tecido. As proteínas inflamatórias para macrófagos (MIP-1α, MIP-1β, MIP-2), proteína quimioatraente de macrófagos tipo 1 (MCP-1) e a interleucina 8 (IL-8) são exemplos de quimiocinas (PLIKUS et al., 2017).

A produção de mediadores inflamatórios é finamente regulada. Um dos pontos de regulação é ativação/inibição de fatores de transcrição como o fator nuclear Kappa β (NF-κβ). Este é formado por subunidades citoplasmáticas que se encontram na forma inativa. Quando ativado, O NF-κβ transloca para o núcleo e liga-se a região consenso de genes que expressam citocinas e enzimas oxidantes. A indução desta cascata de sinalização é necessária para que a resposta imune ocorra, entretanto, ela deve ser eficientemente desligada para evitar danos teciduais e retardo na cicatrização. Outro importante fator de transcrição envolvido no reparo tecidual é a proteína ativadora-1 (AP-1). Sua ativação e expressão é influenciada por interleucinas e o fator de crescimento transformante β (TGF-β) (YUAN et al., 2018).

O aumento na permeabilidade é um fator importante relacionado ao processo inflamatório da ferida. A permeabilidade elevada dos vasos permite a deposição de matriz rica em fibrina, a que é fundamental para a migração celular (WANG et al., 2017). A resposta migratória é um processo altamente regulado e envolve diversos mecanismos como a presença ou ausência de quimiocinas específicas, a habilidade da célula em migrar ao longo de um gradiente quimiostático, a interação entre receptor-ligante e a expressão de moléculas de adesão na superfície das células inflamatórias e nos vasos sanguíneos. Além desses fatores, a fluidez da membrana celular também modula a resposta quimiotática (WALLACE E BHIMJI,2017).

Na fase proliferativa do processo de cicatrização ocorre neoangiogênese, produção de colágeno jovem pelos fibroblastos e intensa migração celular, principalmente de queratinócitos, promovendo assim a reepitelização. Inicialmente, os macrófagos ativados liberam o fator de crescimento derivado de plaquetas (PDGF), TGF-β1 e fator de crescimento de fibroblastos (FGF), que estimulam a proliferação e a migração desses para a área lesada (STĘPNIEWSKI et al., 2017). Com o aumento do número de fibroblastos ativados, a matriz extracelular é substituída por tecido conjuntivo mais forte e elástico. Ao final da etapa proliferativa, o leito da ferida está totalmente preenchido por tecido de granulação (WANG et al., 2017).

A fase de remodelamento envolve etapas sucessivas de produção, lise e orientação das fibrilas de colágeno, aumentando a sua resistência pelo fato de que a organização das fibras acompanha as forças mecânicas a que o tecido está sujeito durante a atividade normal. Ao final desta etapa, os anexos da pele como folículos pilosos e glândulas sofrem regeneração limitada e a coloração da cicatriz permanece pálida, pois a regeneração dos melanócitos é deficiente e as cicatrizes são

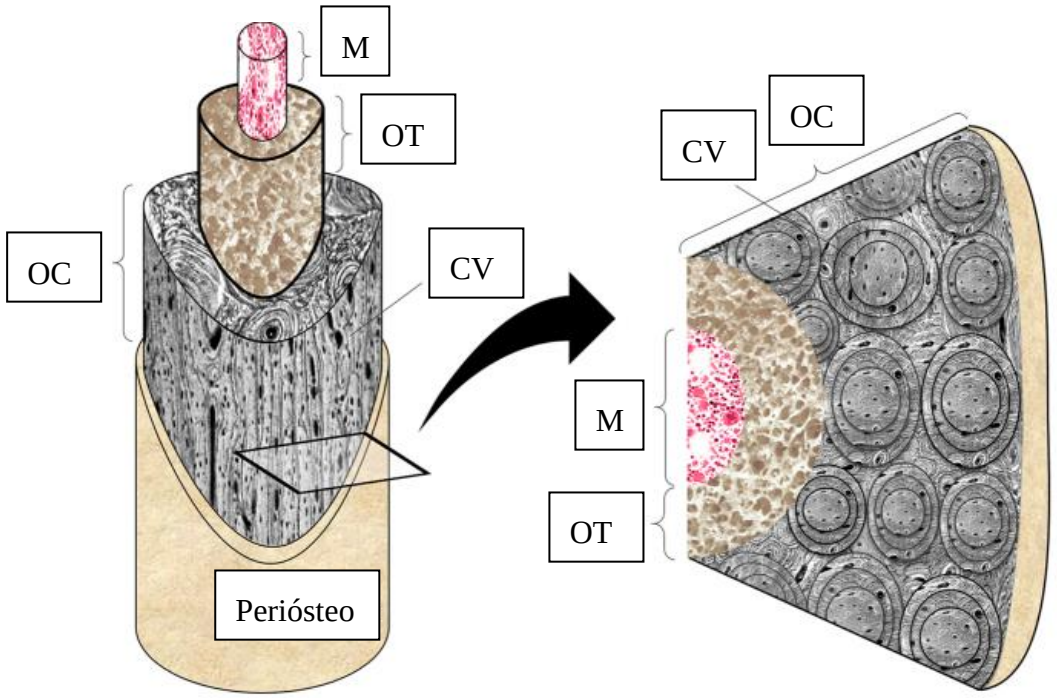
hipovascularizadas devido ao desaparecimento dos neocapilares. (STĘPNIEWSKI ET AL., 2017)

3.1.3 ANATOMOFISIOLOGIA DO TECIDO ÓSSEO

O tecido ósseo ao contrário da pele, a qual exerce a função primária de revestimento, caracteriza-se por ser um tecido conjuntivo especializado constituído por células e matriz extracelular. É distinto pelo processo de mineralização da matriz, produzindo um tecido extremamente duro e capaz de desempenhar a função de suporte, proteção de órgãos vitais e conformação do corpo (FLORENCIO-SILVA et al., 2015). Funciona também como depósito de cálcio, fósforo e outros íons, armazenando-os de maneira controlada, para manter constante sua concentração nos líquidos corporais (JUNQUEIRA e CARNEIRO, 2017). Sua estrutura complexa e dinâmica faz com que suas células estejam em constante manutenção da massa óssea através da remodelação, além de possuir uma inigualável capacidade de reparação, participando de um processo contínuo de remodelamento, no qual, pressões aplicadas sobre o tecido levam à sua reabsorção, enquanto a tração leva ao seu desenvolvimento (TRENTO et al., 2017).

O tecido ósseo se divide em compacto (cortical) e esponjo (trabecular). O cortical constitui-se por lamelas circunferências e concêntricas cercadas pelo periósteo que consiste de uma camada exterior fibrosa e uma camada interior formada por células osteoprogenitoras, fibroblastos e osteoblastos. Organiza-se em unidades chamadas ósteons ou sistemas de Havers. Vasos sanguíneos, linfáticos e nervos do periósteo penetram no osso compacto pelos canais perfurantes de Volkmann, irrigando o local (GARTNER, 2016). Já o tecido esponjoso não contém ósteons, e sim lamelas dispostas como uma trama irregular de finas colunas ósseas, as trabéculas. Os espaços macroscópicos entre as trabéculas são preenchidos por medula óssea vermelha, produtora de células sanguíneas. Esse tipo de tecido aparece no esqueleto axial e epífises dos ossos longos preenchendo os espaços entre as corticais (NEELAM, 2016) (Figura 3).

Figura 3. Tecido ósseo, evidenciando: Periósteo, Osso compacto (OC), Canais de Volkmann (CV), Osso Trabecular (OT), e Medula óssea (M).



FONTE: BIENZ E SAAD, 2015.

Com relação a composição da matriz óssea, esta constitui-se de água, proteínas fibrilares e minerais cristalizados. Bioquimicamente o osso forma-se por uma mistura de duas fases, a orgânica (50%) também chamada de matriz extracelular ou matriz osteóide e a fase inorgânica (50%) conhecida como fase mineral (JUNQUEIRA e CARNEIRO, 2017).

O componente orgânico compreende-se por fibras colágenas (95%) constituídas de colágeno do tipo I, proteoglicanos e glicoproteínas (osteocalcina, osteopontina e sialoproteína do osso). A calcificação ocorre somente em presença de fibras colágenas, os sais minerais começam a cristalizar no espaço microscópico entre as fibras de colágeno, quando este espaço está preenchido, os cristais minerais se acumulam ao redor das fibras de colágeno, essa combinação de sais cristalizados com fibras de colágeno caracteriza a dureza do osso (NEELAM, 2016).

A porção inorgânica do tecido ósseo (55%) constitui-se principalmente de cálcio e fósforo junto com outros componentes como bicarbonato, citrato, magnésio, sódio e potássio. Os dois primeiros existem basicamente na forma de cristais de hidroxiapatita [$Ca_{10}(PO_4)_6(OH)_2$]. Esses cristais encontram-se arrumados em um padrão organizado ao longo de fibras de colágeno tipo I (STĘPNIEWSKI ETAL., 2017).

Com relação aos componentes celulares, existem 4 células constituintes principais do tecido ósseo, sendo elas: as células osteogênicas ou osteoprogenitoras, sendo derivadas do mesênquima, localizam-se na camada interna do periósteo e no endósteo; osteoblastos, células mononucleadas de formato poliédrico, núcleo ovóide e citoplasma basofílico, responsáveis pela síntese da parte orgânica da matriz, produção de colágeno, formação de tecido osteóide e início da calcificação do mesmo; osteócitos, são os próprios osteoblastos, células maduras “aprisionadas” na matriz óssea, entretanto não são mais capazes de secretar ativamente componentes da matriz. Estas células comunicam-se entre si através de prolongamentos citoplasmáticos, mantendo as atividades celulares diárias do tecido ósseo, tais como trocas de nutrientes e metabólitos com o sangue, responsabiliza-se pelo intercâmbio de íons com a matriz óssea e o espaço extracelular; e osteoclastos, células gigantes e multinucleadas, com média de 10 a 15 núcleos, derivados da fusão de monócitos e células progenitoras hematopoiéticas, responsáveis pela reabsorção óssea, apresentando em seu citoplasma leve acidofilia, também liberam enzimas lisossômicas que degradam os componentes protético e mineral do tecido, formando depressões conhecidas de Howship. (JUNQUEIRA e CARNEIRO, 2017).

Durante o desenvolvimento embrionário a formação do tecido ósseo, também chamada de ossificação ou osteogênese, se dá de duas maneiras: ossificação intramembranosa e endocondral. A ossificação intramembranosa ocorre diretamente sobre ou no interior de uma membrana de tecido conjuntivo fibroso formado pela condensação de células mesenquimais. A substituição direta do mesênquima por tecido ósseo se resume na ossificação intramembranosa (JUNQUEIRA; CARNEIRO, 2017). Já a endocondral acontece dentro da cartilagem hialina. As células mesenquimais se transformam em condroblastos produzindo um “molde” de cartilagem do osso para posteriormente os osteoblastos substituírem essa cartilagem por osso (NETTER., 2014).

3.1.4 REMODELAMENTO E REPARO ÓSSEO

Embora aparentemente inertes, os ossos transformam-se durante toda a vida e quando lesados são capazes de se regenerar, fenômeno que demonstra sua permanente vitalidade. Essa homeostase é controlada por fatores mecânicos e humorais, locais e gerais (KATCHBURIAN e ARANA, 2017). Esse processo, conhecido como remodelamento ósseo caracteriza-se pela contínua substituição do tecido pelo

qual os osteoclastos “cavam” pequenos túneis, no tecido ósseo velho e em seguida os osteoblastos reconstroem com tecido novo (BRUNETTI et al., 2018).

Nos jovens, o desenvolvimento dos ossos é maior do que a reabsorção óssea, já que novos sistemas de Havers estão se desenvolvendo muito mais rápido do que os mais velhos, que estão sendo reabsorvidos. Mais tarde, na idade adulta, quando os discos epifisários se fecham e o crescimento ósseo é atingido, o desenvolvimento de osso novo é equilibrado com a reabsorção óssea (CHOI et al., 2018).

No adulto a formação e reabsorção de tecido ósseo permanecem em equilíbrio, ou seja, continuamente o tecido ósseo se remodela atendendo as forças aplicadas sobre ele (VASIKARAN S., 2018).

Nos casos em que a estrutura óssea é submetida a uma intensa força mecânica, é formada uma solução de continuidade na estrutura mineralizada, a fratura. Esta causa destruição da matriz óssea, morte das células, rachaduras no periósteo e no endósteo e possível deslocamento das extremidades (HUPP et al., 2015). Imediatamente após a lesão pelo rompimento de vasos sanguíneos no local, ocorre a formação de um coágulo e inicia assim a fase aguda do reparo. Este quadro hemorrágico desencadeia a formação do hematoma, geralmente nas primeiras oito horas após a lesão (SIZINIO, 2016).

Com a formação do hematoma interrompe-se o suprimento sanguíneo causando morte celular no local. Capilares sanguíneos crescem para dentro do coágulo, mastócitos, leucócitos e macrófagos migram para dentro da área, formando um tecido de granulação responsável pela liberação de fatores estimulantes do reparo tissular (HUPP et al., 2015). Após 48 horas da lesão, as células osteoprogenitoras se diferenciam em osteoblastos começando a produzir tecido osteóide junto ao tecido ósseo não vital, essas células começam a produzir as trabéculas de tecido ósseo esponjoso. Estas se unem as porções vivas e mortas dos fragmentos ósseos originais, formando então, o denominado calo ósseo. Finalmente este osso de aspecto trabeculado se remodela formando um osso lamelar duro. Restaura-se a cavidade medular, o contorno do osso e a sua estrutura interna. (NEELAM, 2016).

Os eventos envolvidos na consolidação de uma fratura óssea, de forma ordenada correspondem ao debridamento, remoção de remanescentes ósseos fragmentados; estabilização; e remodelagem do local fraturado. Esta pode ocorrer de forma primária, através de uma fixação rígida (os cotos são mantidos em posição pela

utilização de mini-placas de titânio parafusadas aos mesmos) ou de forma secundária, com a utilização de contenção externa como talas e casquete de gesso (HUPP, 2015).

3.2 LASERTERAPIA

3.2.1 LASER

A palavra Laser significa amplificação da luz por emissão estimulada de radiação e originou-se da abreviação de “Light Amplification by Stimulated Emission of Radiation” Este é constituído através de um meio ou material ativo que pode ser sólido, líquido, gasoso, semissólidos e este “influxo luminoso” é simplesmente uma manifestação de radiação eletromagnética em ondas. A geração desta dependente da excitação dos elétrons dos elementos constituintes do material ativo. Os elétrons que compõem os átomos ou moléculas do deste meio emitem luz (fótons) por meio de saltos de níveis de energia quando excitados e de acordo com o meio, são obtidos diversos comprimentos de onda na região do espectro visível e invisível (BAGNATO e PAOLITTO, 2014).

Dos materiais mais comumente utilizados como meios ativos na laserterapia, pode-se citar a mistura gasosa de Hélio e Neônio (He-Ne), o semicondutor diodo Arseneto de Gálio e Alumínio (Ga-Al-As), ou Alumínio-Gálio-Índio-Fósforo (Al-Ga-In-P). Essas misturas gasosas, quando expostas a elétrons excitados, produzem radiação na faixa entre 630 nm e 950 nm (PANDEY et al., 2016).

O laser adquire então, três características principais: monocromático (apresenta uma cor correspondente a um único comprimento de onda do espectro eletromagnético), colimado (a luz caminha na mesma direção, favorecendo a transmissão de uma grande quantidade de energia a um alvo) e coerente (todos os raios do laser apresentam coerência temporal e espacial) (BAGNATO; PAOLITTO, 2014).

3.2.2 LASER NOS SISTEMAS BIOLÓGICOS

No ano de 1965, Sinclair e Knoll criaram um equipamento de laser com efeito fotobioestimulante e adaptaram essa radiação à prática terapêutica em tecidos vivos (MESTER et al., 1985). Desta forma, a implementação de diferentes tipos de lasers em procedimentos na área da saúde possibilitou mudanças importantes nas intervenções médicas e odontológicas. Tais mudanças abrangem redução no tempo de cirurgia e no tempo de recuperação dos pacientes, redução das complicações pós-operatórias, edemas, maior controle das dores crônicas e facilitação da biomodulação na cicatrização de tecidos (COTLER et al., 2015).

Os lasers são classificados em alta potência e baixa potência. O laser de alta potência ou cirúrgico (Hight Intensity Laser Treatment – HILT) tem efeitos de ablação sendo indicado para procedimentos cirúrgicos como cortes, coagulação e cauterização. O laser de baixa intensidade ou de baixa potência (Low-level Laser Therapy – LLLT) é utilizado para fins terapêuticos e bioestimulantes, agindo principalmente como acelerador de processos cicatriciais (CAVALCANTI et al., 2011). A indicação dos tipos de laser é caracterizada pelos diferentes comprimentos de onda (determinantes da profundidade de penetração) e pela potência. Os lasers de baixa intensidade operam na faixa de 50 a 300 mW e por isso não produzem aquecimento nos tecidos vivos superior a $1^{o}C$.

Os efeitos terapêuticos do laser de baixa intensidade são atribuídos à capacidade da luz, nos comprimentos de onda principalmente vermelho ou infravermelho próximo, de modificar o metabolismo celular em consequência da absorção desta por fotorreceptores existentes nas células (HE et al., 2018). Em 1967, o médico húngaro Endre Mester demonstrou bons resultados terapêuticos com baixas densidades de energia ao publicar estudo que utilizou o laser de baixa intensidade He-Ne, para cicatrização de feridas (MESTER, 1967). Características como não invasividade, baixo custo e eficácia comprovada na cicatrização de tecidos e no controle de dor parecem favorecer o uso contínuo e cada vez mais frequente do laser como recurso terapêutico aos convencionais (COTLER et al., 2015).

A terapia por laser de baixa intensidade não se baseia no aquecimento, pois a energia dos fótons absorvida não é transformada em calor. Entretanto, fundamenta-se nos efeitos fotoquímicos, fotofísicos e fotobiológicos nas células e tecidos (PANDEY et al., 2016). De acordo com Karu (1987), o comprimento de onda, densidade de potência (intensidade), frequência de tratamento e, até mesmo, o tipo de lesão estão relacionados com o aumento da atividade celular, bem como a densidade de energia ou dose também influenciam estas reações.

Garcez et al. (2012) acrescentam que a interação entre laser e tecido biológico é dependente do comprimento de onda da luz e das propriedades ópticas dos tecidos, pois certos elementos teciduais como células, mitocôndrias e vasos podem provocar dispersão da luz. Parte do feixe de luz ao incidir sobre os tecidos irá penetrá-los e parte será refletido. Refração, espalhamento e transmissão são fenômenos que podem ocorrer quando a radiação eletromagnética for absorvida. Somente os fótons não refletidos, não absorvidos ou espalhados na mesma direção do feixe incidente são transmitidos pelos tecidos.

Os mecanismos de ação da luz após sua absorção são classificados em primários e secundários. os mecanismos primários atuam diretamente sobre as moléculas fotorreceptoras, ocorrem durante a irradiação e têm efeitos observados logo após a irradiação. Apesar de não terem sido completamente estabelecidos, uma série de hipóteses foi proposta para melhor elucidar os mecanismos primários, como: a geração de oxigênio singleto: espécies reativas de oxigênio são geradas por meio da absorção de fótons por porfirinas e flavoproteínas (MOSKVIN, 2017); a alteração das propriedades do estado excitado redox da citocromo C oxidase, esta torna-se eletronicamente excitada, que altera seu estado e promove aceleração de transferência de elétrons na cadeia respiratória (COTLER et al., 2017); a presença de óxido nítrico (NO), a absorção da luz pode reverter a inibição da citocromo c oxidase pelo óxido nítrico e aumentar a taxa respiratória (LEE et al., 2017); e o aumento da produção de ânions superóxidos: decorrente da ativação do fluxo de elétrons na cadeia respiratória (PANDEY et al., 2016).

Com relação aos mecanismos secundários das reações fotobiológicas, estes são responsáveis pela conexão entre a resposta à ação da luz pelos fotoaceitadores localizados na mitocôndria e os mecanismos de síntese de DNA e RNA localizados no núcleo. Uma complexa cascata de sinalização celular ou transdução e amplificação do sinal fotônico é ativada e está associada a mudanças na homeostasia celular, alterações no ATP, modulação da síntese de DNA e RNA, mudanças na permeabilidade de membrana, despolarização da membrana da célula e alcalinização do citoplasma (YIN et al., 2017). Estes mecanismos dependem de vários parâmetros como a dose de irradiação, o comprimento de onda, o modo de emissão (pulsado ou contínuo) e a intensidade da excitação. O estado geral redox e o pH da célula também influenciam a resposta celular à luz (DE BRITO VIEIRA et al., 2017).

Após a irradiação, através dos mecanismos secundários, ocorre o incremento de ATP mitocondrial promovendo muitos eventos que interferem no metabolismo celular. Em situações patológicas, o laser influencia o processo de troca iônica e potencializa o incremento de ATP. Além disso, alguns estudos mostram que o laser de baixa intensidade apresenta efeitos mais expressivos sobre órgãos e tecidos em certas condições patológicas, como, por exemplo, em situações de desordem funcional ou de injúria ao tecido (VAGHARDOOST et al., 2018). Células em estado de homeostasia sofrem pouca ou nenhuma influência da fototerapia e, portanto, o efeito da luz nem sempre pode ser detectado (PINHEIRO, 2009; MEYER et al., 2010).

3.2.3 LASER NO PROCESSO DE REPARO TECIDUAL

A utilização do laser de baixa intensidade com o objetivo de auxiliar o reparo tecidual é pesquisada desde 1963 (MESTER, 1967) e diversos estudos foram realizados evidenciando os efeitos do laser sobre a cicatrização de feridas e alívio de dor (ANDRADE et al., 2014; TSCHON et al., 2017; ARAGÃO-NETO et al., 2017; EBRAHIMI et al., 2018). A laserterapia de baixa intensidade é eficaz na cicatrização de feridas por atuar como fotobioestimulador além de modular o processo inflamatório e acelerar o processo de reparo tecidual (KARMISHOLT et al., 2018) Diversas modificações histológicas são observadas nas feridas tratadas com laser terapêutico, e incluem a redução da quantidade de infiltrado inflamatório, aumento na formação de tecido de granulação, aumento na proliferação fibroblástica e síntese de componentes da matriz extracelular, especialmente colágeno, maior neovascularização e epitelização precoce. (VAGHARDOOST et al., 2018)

Com relação ainda ao processo de cicatrização, de acordo com alguns estudos, a aplicação precoce do laser sobre feridas mostrou-se capaz de acelerar o fechamento das mesmas, com efeitos nas fases inflamatória e proliferativa. Além disso, o laser estimula um processo de cicatrização com maior organização das fibras colágenas o que influencia até mesmo o aspecto estético da cicatriz (ARAGÃO-NETO et al., 2017; KARMISHOLT et al., 2018).

Os parâmetros de irradiação do laser que devem ser fornecidos em qualquer estudo experimental ou clínico são: comprimento de onda em nanômetros (nm), potência do aparelho em miliwatts (mW), densidade de potência em mW/cm^2, tempo de tratamento em segundos (s), energia administrada em Joules (J) e a densidade de energia (dose) em J/cm^2 para pequenos animais ou pesquisa em cultura de células, tamanho do ponto de saída do feixe da luz ou área do spot em cm^2, energia acumulada entregue em todas as sessões em Joules, aplicação com ou sem contato com a pele (distância em cm), modo de emissão contínuo ou pulsado. (GARCEZ et al. 2012)

A potência de saída média do equipamento é usada para efetuar o cálculo da densidade de energia a ser administrada no tecido. A potência é a quantidade de energia associada aos fótons que atingem o tecido por unidade de tempo e é expressa em Watts (W). Já a densidade de potência é definida como a potência de saída da luz por unidade de área, normalmente é dada em mW/cm^2 e permite avaliar a possibilidade de dano térmico. Refere-se à quantidade de potência óptica por unidade de área na superfície do tecido, mas não considera a radiação absorvida ou espalhada. O cálculo desta grandeza

física é realizado considerando-se a área do spot ou área da seção transversal do feixe. A densidade de potência é inversamente proporcional à área do spot (GARCEZ et al., 2012).

Densidade de energia ou dose ou ainda fluência é a grandeza definida pela quantidade de energia fornecida em uma determinada área, portanto, estabelece os efeitos fotobiológicos de estimulação, inibição ou não manifestação dos efeitos terapêuticos. Esse parâmetro mistura o conceito de “medicamento” e “dose” em uma única expressão e ignora a irradiância. Usar Joules como expressão da dose é potencialmente não confiável, uma vez que pressupõe reciprocidade. Na visão de alguns pesquisadores a maneira mais segura de prescrever a laserterapia de baixa potência é definir os parâmetros de irradiação e posteriormente definir o período de irradiação(ões) como dose(s) (HUANG et al., 2009).

O tempo é uma variável importante na obtenção de bons resultados. Na laserterapia de baixa intensidade, o estímulo pode ser o tempo de aplicação ou a densidade de potência. Portanto, quando a irradiação for insuficiente, não haverá resposta, mas, quando a irradiação alcança o limite necessário para provocar a ação biológica, a bioestimulação ocorre, já Irradiações muito superiores ao necessário desencadeariam bioinibição (GARCEZ et al., 2012).

3.3 POLISSACARÍDEOS

3.3.1 POLICAJU

Os polissacarídeos, polímeros hidrofílicos naturais de cadeia longa linear ou ramificada, constituídos de monossacarídeos, são atóxicos, biocompatíveis, biodegradáveis e de fácil solubilização, podendo formar hidrogéis ou cristais líquidos em solução (SOARES et al., 2014). O polissacarídeo POLICAJU extraído da goma do cajueiro *Anacardium occidentale L.*, encontrado em países tropicais, tem apresentado resultados eficientes no processo de cicatrização de lesões cutâneas (SCHIRATO et al., 2006; MOREIRA et al., 2015; SILVA et al., 2016; ARAGÃO-NETO et al., 2017; SOUZA-FILHO et al., 2018) (Figura 4).

Figura 4. Cajueiro (*Anacardium occidentale L.)*

Fonte: ARAUJO, 2010.

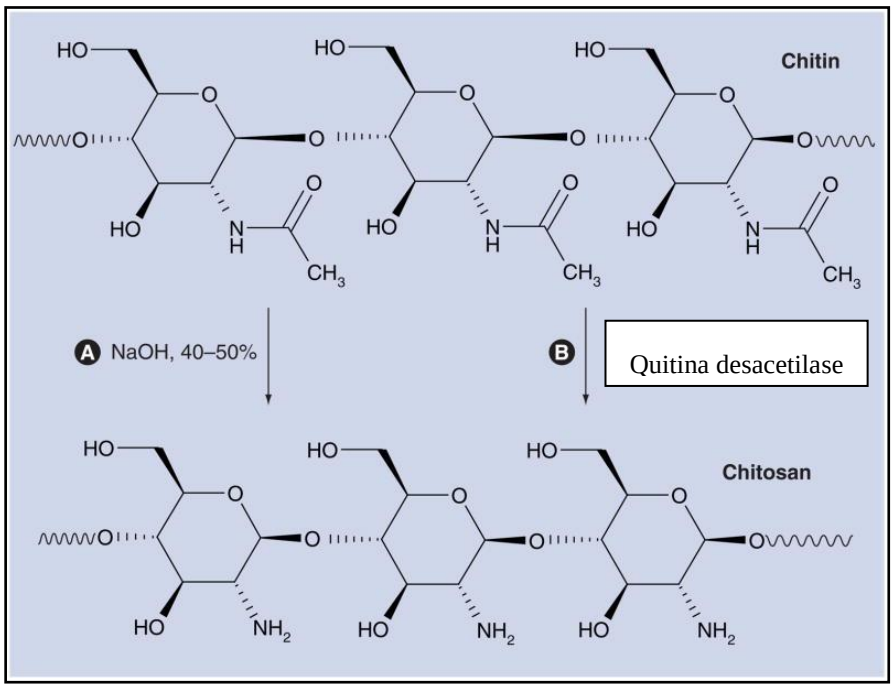
O policaju é um polissacarídeo ácido complexo (arabinogalactana ácida) com massa molecular de 1,6 x 105 Da, composto por uma cadeia principal formada por unidades de D-Galp unidas por ligações glicosídicas β - (1→3) substituídos em O-6, tendo como resíduos terminais a arabinose, raminose, ácido glucurônico, ácido 4-Ometilglucurônico, xilose, glicose e manose (DE PAULA E RODRIGUES, 1995; MENESTRINA et al., 1998). Este polissacarídeo tem sido relatado como potencial constituinte de filmes e espessantes (CARNEIRO-DA-CUNHA et al., 2009; SOUZA et al., 2010; MOREIRA et al., 2015; SILVA et al., 2016), além disso, outros estudos confirmaram a atividade antidiarreica (ARAÚJO et al. 2015), gastroprotetora (CARVALHO et al., 2015), anti-inflamatória (SOUZA-FILHO et al., 2018) e cicatrizante (SCHIRATO et al., 2006; MOREIRA et al., 2015; SILVA et al., 2016; ARAGÃO-NETO et al., 2017; SOUZA-FILHO et al., 2018). O fácil acesso a este material de baixo custo, não tóxico, hidrofílico, biocompatível e biodegradável, o qual ainda apresenta interessante atividade biológica e boas propriedades reológicas são fatores que fazem com que seja viável o seu uso como matriz para imobilização e distribuição de drogas (SOARES et al., 2014).

3.3.2 QUITOSANA

A quitosana, polissacarídeo derivado da quitina, obtida por desacetilação da mesma, seja por via alcalina ou enzimática, pode ser também encontrada naturalmente em alguns fungos (MUZZARELLI et al., 2012; 2013), tem sido investigada pela

comunidade científica em aplicações biomédicas e terapêuticas, por possuir propriedades curativas (ARAGÃO-NETO et al., 2017; RANIBAR E YUSEF, 2018), bem como atividade antimicrobiana (ARAIN et al., 2013; ALEANIZY et al., 2018). (Figura 5)

Figura 5. Desacetilação da quitina para a quitosana. (A) – alcalina e (B) – enzimática.



Fonte: TIANHONG et al., 2011.

Com relação às atividades biológicas, a quitosana provoca inibição do crescimento de micro-organismos, uma vez que em contato com os fluidos fisiológicos, seus grupos amínicos são protonados e ligam-se aos micro-organismos, resultando na aglutinação das células microbianas e inibição do seu crescimento, estando este mecanismo intimamente relacionado às suas propriedades físico-químicas e às características da membrana do micro-organismo, (SIMONCIC e TOMSIC, 2010).

3.4 HIDROGEIS

Os hidrogéis são definidos como uma rede polimérica tridimensional capaz de absorver grande quantidade de água ou fluído biológico. Quimicamente são baseados em polímeros hidrofílicos intercruzados para prevenir a sua dissolução em água, podendo assim ser utilizados para conservar células, nutrientes, drogas ou proteínas. Em um ambiente aquoso, os grupos hidrofílicos da rede polimérica são hidratados causando inchaço e gerando a estrutura em "rede" e a forma do hidrogel. Esse termo implica no

intercruzamento químico ou físico entre os grupamentos ativos dos polímeros em composição. Além disso, os hidrogéis podem ser formulados em uma variedade de formas físicas, incluindo filmes e revestimentos comestíveis, sendo micro ou nanoparticulados (WHITE et al., 2016; IKAI et al., 2017).

Reológicamente, as soluções aquosas de polímeros hidrofílicos em concentrações baixas ou moderadas normalmente apresentam um comportamento newtoniano. Por outro lado, uma vez que ligações cruzadas entre as diferentes cadeias de polímeros são introduzidas, as "redes" assim obtidas mostram um comportamento visco-elástico e, por vezes, um comportamento puramente elástico (XU et al., 2017).

Em geral, os hidrogéis são biocompatíveis, sendo a biocompatibilidade promovida pelo seu alto teor de água e as semelhanças físico-químicas que possuem com a matriz extracelular nativa de tecidos orgânicos, tanto em composição, quanto mecanicamente (GEEVER et al., 2008).

A biodegradabilidade da matriz polimérica pode ser projetada através de vias enzimáticas, além de vias hidrolítica ou ambiental como por exemplo, pH, temperatura ou campo elétrico, no entanto, a degradação nem sempre é desejável, dependendo do tempo de liberação e local de entrega da biomolécula (TAURIN et al., 2017).

Devido à sua capacidade de absorção de água, os hidrogéis possuem ampla aplicação em diferentes áreas biotecnológicas, como por exemplo, são utilizados como materiais para lentes de contato; separação de biomoléculas ou células; matrizes para a imobilização de células; como dispositivos para a liberação controlada de compostos bioativos (MIRONIHARPAZ et al., 2012); em práticas clínicas da medicina experimental para a engenharia e regeneração de tecidos (ZHU e MARCHANT, 2011)

A natureza elástica dos hidrogéis hidratados inchados permite minimizar a irritação dos tecidos circundantes após implantação. A baixa tensão interfacial entre a superfície do hidrogel e do fluido corporal minimiza a adsorção de proteína e adesão celular, o que reduz as chances de uma reação imunológica negativa. Além disso, os hidrogéis possuem várias características que os tornam excelentes veículos de entrega de drogas (HUYNH et al., 2018).

Além das capacidades supracitadas, os hidrogéis são relativamente deformáveis e desta forma podem se adaptar à superfície a qual são aplicados. Neste último contexto, as propriedades de muco ou bioadesividade de alguns hidrogéis podem ser vantajosas para imobilizá-los no local da aplicação, mesmo que a superfície tópica não seja horizontal (TAURIN et al., 2017).

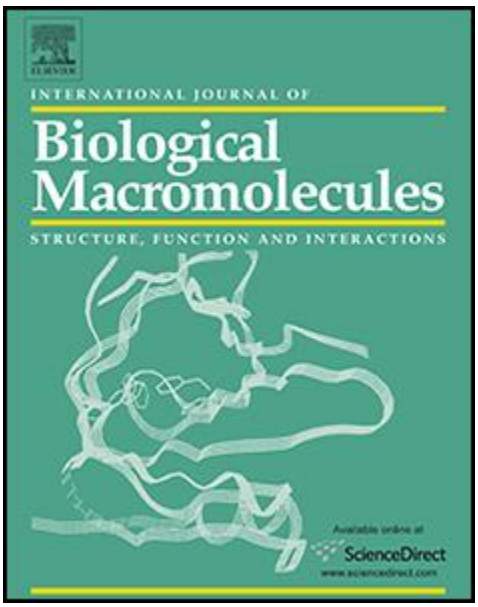
Com relação as potencialidades de modalidades terapêuticas combinadas envolvendo hidrogéis, Aragão-Neto (2017) e colaboradores realizaram uma avaliação quali-quantitativa do reparo tecidual *in vivo*, utilizando Ratos Wistar. O objetivo foi avaliar a possibilidade de uma reação sinérgica envolvendo um hidrogel confeccionado a partir dos polissacarídeos policaju (goma do cajueiro Anacardium Occidentale) e a quitosana (obtida da desacetilação da quitina) com a laserterapia de baixa intensidade (LLLT) no espectro do vermelho (660 nm). Os resultados obtidos indicaram um processo cicatricial significante com relação a regressão / cicatrização de lesões cirurgicamente confeccionadas na região dorsal, estas foram tratadas utilizando o hidrogel associado ou não a LLLT. Esse estudo indicou a pertinência da aplicação de terapias envolvendo modalidades distintas para acelerar o reparo tecidual.

RESULTADOS

ARTIGO I

Combined therapy using low level laser and chitosan-policaju hydrogel for wound healing

Artigo publicado na revista International Journal of Biological Macromolecules



Fator de Impacto: 2.8

International Journal of Biological Macromolecules 95 (2017) 268–272

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Short communication

Combined therapy using low level laser and chitosan-policaju hydrogel for wound healing

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ARTICLE INFO

Article history:
Received 6 September 2016
Received in revised form 3 November 2016
Accepted 7 November 2016
Available online 13 November 2016

Keywords:
Polysaccharides
Anacardium occidentale L.
Chitosan
Cutaneous dressing

ABSTRACT

We have evaluated the effect of POLI-CHI hydrogel based on policaju (POLI) from cashew tree (*Anacardium occidentale* L.) gum and chitosan (CHI), associated or not with Low level laser therapy (LLLT), in wound healing. Sixty male Wistar rats were assigned into four groups: POLI-CHI hydrogel (H); LLLT (L); POLI-CHI with LLLT (HL) and saline control (C). Macroscopic evaluations were carried out using clinical observations and area measurements, as well as microscopic analysis by histological criteria. H and HL presented more esthetical scar tissue and larger wound contraction compared to C. Histopathological analyzes showed: stronger presence of fibrin-leukocyte crust in L and HL at day 3; stronger collagen presence in H, L and HL; weak presence of focal necrosis at 7 and 14 days in H; weak neutrophilic exudate in H, L and HL; regression of the vascular neoformation at 7 days in H, and modulation of the same in L and HL. These results demonstrated that POLI-CHI contributed to more efficient healing process and modulation of the inflammation; furthermore, the combined use with LLLT subtle potentiated this process.

1. Introduction

Skin wounds heal in four stages: hemostatic, inflammatory, proliferative and remodelative [1]. Polysaccharides have been used in wound healing. Policaju obtained from cashew tree (*Anacardium occidentale* L.) gum has showed potential application in wound healing [2]. Chitosan, a polysaccharide derived from the chitin by de-acetylation, also has presented biocompatibility, biodegradability, low toxicity, hemostatic, healing properties, and antimicrobial activity [3,4]. Both polysaccharides can form hydrogels or crystals in solutions [5].

Hydrogels are three-dimensional polymer nets capable of absorbing large amounts of water or biological fluid, being used to preserve cells, nutrients, drugs or proteins, and also represent a drug delivery system class [6]. Due to its physicochemical similarities with the extracellular matrix the hydrogels are generally biocompatible [7]. The combination of policaju (POLI) and chitosan (CHI) termed POLI-CHI has been previously characterized by our lab [8]. Evaluating hydrogels using different proportions the most attractive was that composed of POLI:CHI, 1:4. The FT-IR analyses confirmed the existence of physical interactions between the polysaccharides involved and rheological measurements showed an increase in complex viscosity with the increase of chitosan content.

Low level laser therapy (LLLT) acts causing several biological effects, such as: increasing proliferation and activation of lymphocytes, increasing the phagocytosis on macrophages; and the secretion of growth factors in fibroblasts, enhancing the uptake of fibrin and collagen through emission of radiation by stimulating the most external electric field [9].

The combination of laser therapy and sodium alginate/chitosan-based hydrogel film improved burn healing, apparently by modulating the epithelisation, blood vessels formation and collagenization processes [10].

The aim of this study was to evaluate the healing of skin wounds induced in Wistar rats treated with the POLI-CHI hydrogel combined or not with LLLT.

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http://dx.doi.org/10.1016/j.ijbiomac.2016.11.019
0141-8130/

2. Materials and methods

2.1. Materials

Polysaccharide from cashew tree (*Anacardium occidentale* L.) gum (POLI), collected from the south coast of Pernambuco, Brazil, was obtained according to Souza [11]. The chitosan (CHI) (deacetylation > 75%) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Hydrogel preparation

The POLI-CHI hydrogel was made of policaju (POLI) and chitosan (CHI) in a ratio of 1:4 according to Soares [8]. Briefly, 50 mL of stock solutions of 10% (w/v) of policaju and 1% (w/v) of chitosan in 1% lactic acid (v/v) were prepared in advance. In a separated beaker, 15 mL of chitosan solution plus 200 μL of 0.1 M $CaCl_2$ was added and kept under stirring in Ultra-Turrax (IKA, USA) at 7000 rpm for 20 min. Then, using a 27 G syringe and a flow of 1 mL/min, was added 5 mL of policaju solution. The mixture was left under stirring (7000 rpm) for 20 min. The pH was adjusted to 5.0 with 1 M NaOH solution and called pre-gel. The pre-gel solution was distributed in petri dishes and kept in an oven at 40 °C for 16 h for drying and polymerization. The thin film obtained was hydrated with distilled water and termed POLI-CHI hydrogel, which was stored under refrigeration at 4 °C.

2.3. Animals and treatment groups

Sixty male Wistar rats (*Rattus norvegicus*), 90-120 day-old, weighing 250–300 g were anesthetized intraperitoneally with 2% (w/v) of xylazine hydrochloride and 10% (w/v) ketamine hydrochloride at 1:1 ratio. A circular surgical wound (Ø = 0.8 cm) were made in the skin of the dorsal region of each animal using a biopsy punch and a scalpel blade No. 15. After surgery the animals were randomly divided into four groups, according to treatment (n = 15): (H) 0.1 mL of POLI-CHI hydrogel; (L) LLLT; (HL) 0.1 mL of POLI-CHI hydrogel plus LLLT and (C) 0.1 mL of 0.9% (w/v) NaCl as Control. The irradiation was carried out in a punctually way starting from the center of the wound at 2 mm from the skin using Therapy XT (DMC medical, USA). The parameters used were: λ = 660 nm, A = 1 cm2, ED = 4 J/cm2, P = 100 mW, F = 50 Hz. This treatment was carried out after surgery and at a 48 h interval until the euthanasia time. All animal procedures were in accordance with the Colégio Brasileiro de Experimentação Animal (COBEA) and the Animal Ethical Committee/UFPE No. 23076.050933/2012–10.

2.4. Macroscopic evaluation

The specimens were clinical daily evaluated according to the presence of the following criteria: edema, hyperemia, presence of exudate, crust, detachment and epithelialization. Images were generate using a photographic camera (Alpha 3000 K/B – SONY) and a tripod (Viv-Tr75 – Vivitar). The wound area image was processed using the ImageJ software (version 1.45) and the area (pixels) was applied in the contraction of the wound formula: [(initial area – area on the day of measurement)/initial area] × 100 = percentage of contraction on the day of measurement [12].

2.5. Euthanasia and histological processing

Five animals from each group were sacrificed after 3, 7 and 14 days following the surgical procedure using lethal doses of sodium thiopental (200 mg kg^{-1}). Skin fragments were collected with a wide margin (± 1 cm) from the original lesion and storaged in 10% (v/v) formalin [13]. The histological specimens were included in paraffin and after microtome cut, the sections were stained using hematoxylin-eosin (HE), for cellular observation, and picrosirius (PS) for collagen fibers.

2.6. Light microscopic evaluation

The microscopy slides were analyzed according to presence and intensity (absent, weak, moderate, and strongly present) of the following histological findings: Fibrin-leukocyte crust, Collagen, Focal necrosis, Fibrin deposits, Neutrophilic exudates, Edema, Eosinophilic exudates, Mononuclear infiltrate, Macrophage infiltrate, Granuloma, Neovascularization, Fibroblast proliferation and Fibrosis.

2.7. Statistical analysis

The statistical evaluation was carried out using the analysis of variance (ANOVA) method and Bonferroni's multiple comparison test. The statistical significance was 5% ($p < 0.05$) and the software used for data entry and processing was the Graphpad Prism for Windows, version 5.0 from Graphpad Software, Inc.

3. Results and discussion

3.1. Macroscopic evaluation

The clinical findings are shown in Fig. 1. Edema, Hyperemia, Exudate and Crust are present from day 1 to 3 after surgery in all groups, although was found a thicker Crust without Exudate in L and HL groups in comparison to other ones. From day 4 to 6, all groups still present Crust being Edema and Hyperemia absents. At day 6, the HL group started to lose its crust (Detatchment). From day 7 to 9, all groups presented a similar pattern with Crust, Detachment and Reepithelization. From day 10 to 12 presented Detachment and Reepithelization, and from day 13 to 14, just reepithelization.

These results demonstrated that in all experimental groups the scar tissue after the surgical procedure was much reduced compared to control, wich was more evident at day 14. They are in accordance with Moravvaej [14] and Avci [15] studying the reduction of hypertrophic scars in human patients under LLLT. In this study H group presented the same pattern than HL group, which may suggest a hydrogel benefit. This advantage has been reported in rosacea skin disease treatment by sulfated anionic polysaccharide [18].

In this study soon after the surgical procedures, the animals of L and HL groups started to feed and drink, while in other groups it was only after 12 h. This indicates that the LLLT may act as an analgesic factor corroboranting the findings of Pozza [16]. According to Soon & Acton [17] animals subjected to stress and pain had a poor wound healing process.

The wound contraction expressed in percentage is displayed in Fig. 2. At day 3, the H group presented statistically higher arithmetical mean (56.21 ± 4.31) followed by HL (48.86 ± 11.53), compared to control (28.57 ± 14.59), being H also significant in comparison to L (33.68 ± 7.43).

Observing day 7, H (84.22 ± 3.51), L (69.99 ± 9.52) and HL (84.46 ± 4.42) were statistically significant in comparison to control (39.83 ± 14.58). At 14 days after surgery there was not significance in comparison to control (H: 94.15 ± 1.17; HL: 96.13 ± 1.46; L: 94.97 ± 1.63; C: 84.09 ± 3.86). The experimental groups presented a similar trend for repair. These findings corroborate with studies with chitosan [19,20], policaju [2,21] and LLLT [15,22] as healing agents.

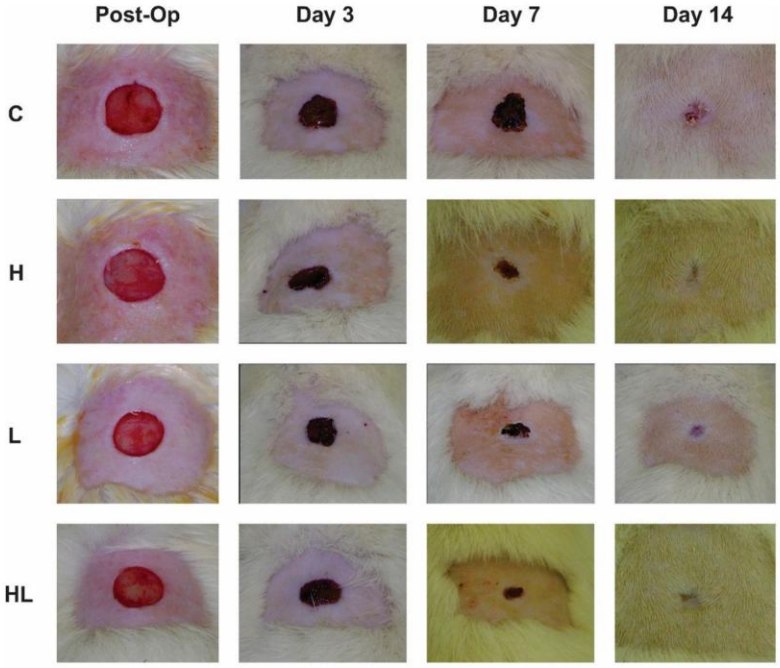

Fig. 1. Macroscopic aspects of the induced lesions by the time of evaluation, using for treatment: (C) Control 0.1 M NaCl, (H) POLI-CHI, (L) LLLT, (HL) POLI-CHI + LLLT.

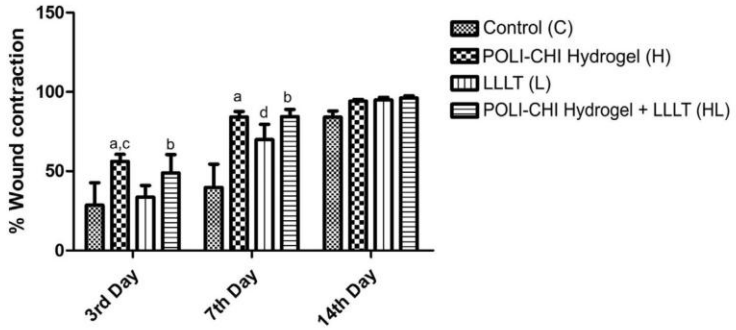

Fig. 2. Wound contraction percentage by time. Comparison of the Arithmetical Mean (AM) and Standard deviation (SD) between experimental groups and control using the method of analysis of variance (ANOVA) and Bonferroni's multiple comparisons test. There was found statistical significance between group H and C (a) at day 3 and 7; between group HL and C (b) at day 3 and 7, between H and L at day 3 (c) and between L and C at day 7 (d) ($p < 0.05$).

3.2. Microscopic evaluation

The main objective of this analysis was to delineate a histological overview of the specimens and to compare the different findings in a temporal way (Fig. 3). The fibrin-leukocyte crust is responsible for keeping the wound environment humid and protected. At day 3, crust was stronger in L and HL groups in comparison to H and C groups, but at the 7th day happened an inversion, presenting the HL group with a weak presence. This may suggest, as occurred in the clinical evaluation, an early detachment of the crust from the borders, being so a hint of acceleration of the wound healing process. At the 14th day, there was not fibrin-leukocyte crust in any evaluated specimens. This early detachment for LLLT was found by Pinheiro [22].

The Collagen presence was similar in all groups at day 3, and moderately present at day 7 in all treated groups (H, L and HL), diverging from C group that keep unaltered. At day 14 all groups presented strong collagen presence. The data suggested early collagen formation in all treated groups, which may imply a benefit of the repair process. Another aspect that emerged was the thickening of collagen arrangement in experimental groups in comparison to C group. This may be due to an increase in collagen fiber maturation aided by the healing agents [23,24].

Evaluating focal necrosis, at the day 3, the HL group presented a minor amount (weak) in comparison to H, L and C (moderate).

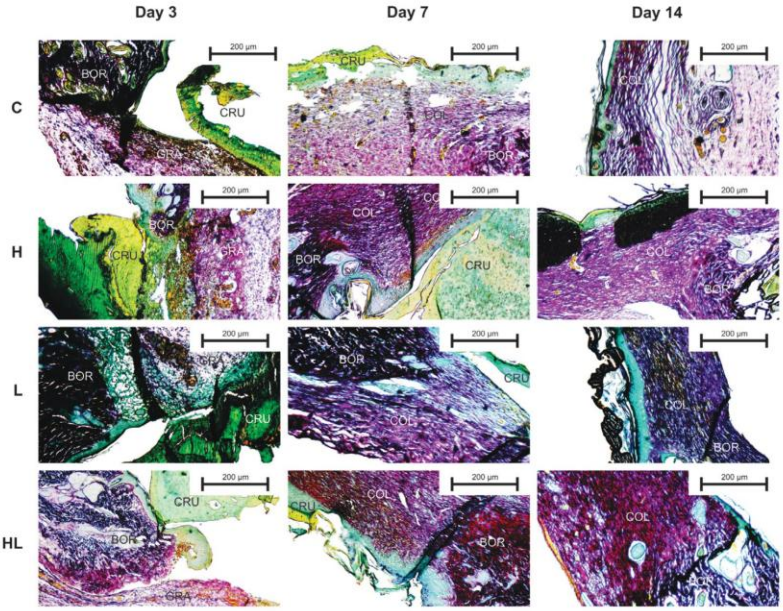

Fig. 3. Light microscopy of stained specimens (Picrosirius PS) by group and time, where: ANE Dermal Annexes; BOR Mature Border; COL Collagen Fibers; CRU Fibrin Leukocyte Crust; GRA Granulation tissue.

At day 7 the H group does not present any sign of focal necrosis differing from L, HL and C groups (weak). This regression pattern in H group may suggest an improvement in the healing process. The necrotic loci phagocytosis may be increased by the POLI-CHI hydrogel. On the other hand, LLLT may reduce the phagocytosis, once the focal necrosis persisted in the new formed matrix. This finding goes against the study of Neves [25] working with necrosis areas in rat's skin flaps.

All groups presented Macrophage infiltrated in a weak form at day 3, being the H group lightly superior (weak to moderate). At the 7th day, H and HL groups presented in an almost absent way, and in C group no presence was found. The macrophage presence is fundamental to the repair process degrading and removing components of damaged connective tissue, such as collagen, elastin, and proteoglycans. They also secrete chemotactic factors that attract other inflammatory cells to the site of wound and produce prostaglandins, which act as potent vasodilators, affecting the permeability of micro vessels [26].

Edema was moderately found in HL group; weak in H, L and C groups at day 3 and almost absent in H and HL and weak to moderate in C groups at day 7. At the 14th day it was completely absent in H, L and HL and still weakly present in C. This may indicate an elongating of the wound repairing process in C, which may suggest a modulation of inflammatory process, as indicated by Sezer [27] concerning a chitosan hydrogel, and Lima [28] regarding LLLT.

Evaluating Neovascularization, at day 3, was found minimal variation between H, HL and C groups, highlighting the L that showed moderately present. At the 7th day, occurred a major variation in C group that showed it strongly present, while in H group the presence was moderated, and in L and HL was found in a weak form. It may suggest that the vasculature regression after the inflammatory stage was stimulated in treated groups, which may be the contributive factor for the tissue maturation, emphasizing the H group, once it presented improvement in the neovascular formation in the early stage and regression at the proliferative stage, suggesting a modulatory effect.

Concerning hydrogel used, the proportion of POLI:CHI, 1:4 was selected based in a previous study [8] in order to avoid the instability of the matrix caused by increasing of POLI concentration, reducing its elasticity and potentiating viscosity.

Finnaly, the light microscopic evaluation at the 14th day showed cure of wound in all groups but it is important to highligh that the contraction presented in H group was the important factor for the validation of the POLI-CHI hydrogel healing properties.

4. Conclusions

The results showed that the POLI-CHI hydrogel contributed for a most effective wound healing and modulation of the inflammatory process. The combined use of POLI-CHI hydrogel with LLLT showed better wound contraction, larger collagen presence, minor focal necrosis and early epithelization

Acknowledgments

A.C.A.N. and P.A.G.S. are recipient of a scholarship from the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), respectively. M.G.C.C. and M.T.S.C. express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research grants and fellowship.

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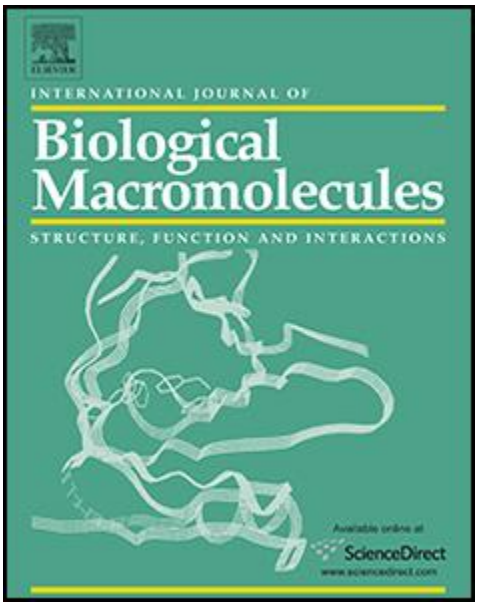
ARTIGO II

Evaluation of bone repair using chitosan-policaju hydrogel combined with low level laser therapy in a rat calvarium critical-defect: a radiographic and histological overview

Artigo a ser submetido a revista International Journal of Biological Macromolecules



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Evaluation of bone repair using chitosan-policaju hydrogel combined with low level laser therapy in a rat calvarium critical-defect: a radiographic and histological overview.

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Abstract

We have evaluated the effect of POLI-CHI hydrogel based on policaju (POLI) from cashew tree (*Anacardium occidentale L.*) gum and chitosan (CHI), associated or not with Low level laser therapy (LLLT), in calvarium critical size defects. Thirty-six male Wistar rats were assigned into three groups: POLI-CHI hydrogel (H); POLI-CHI with LLLT (HL) and saline control (C). They were subjected to euthanasia at 15, 30 and 45 days after surgery. The specimens were subjected to radiographic evaluations (to obtain radiodensity) and histological (qualitative and quantitative) to obtain a bone modulation profile of the proposed treatments. In the matter of radiographic evaluation, there was not significance between subgroups, although, in the defect's regression using histology measures was found that HL was significant to C at 15 and 45 days, and H to C at 45 days ($p < 0.05$). Also, was found a strong presence of bone and vascular new formation in H and HL. Observing collected data, it's possible to conclude that that POLI-CHI presented is self as a valuable material to use for stimuli and modulation of bone repair, probably by increasing the collagen deposition and increasing the local vase presence, essential to a correct repair of mineralized tissue.

Key-words: Hydrogel, Chitosan, Policaju, Laser, Bone.

Introduction

Autogenous bone graft is a commonly used method to repair bone defects, but it is criticized for its donor site morbidity and the volume that is harvested is limited [1]. In this context, allograft bone is also often used in clinic as a substitute, although also faces risks of recipient rejection and disease transmission [2]. To avoid the disadvantages of tissue-based bone graft, synthetic biocompatible scaffolds and other materials have been applied in tissue engineering for bone regeneration, such as laponite bioceramics [3], Hydroxyapatite [4], borosilicate bioactive glass [5], collagen sponge [6], and polysaccharide hydrogels [7].

Hydrogels are based on hydrophilic polymers that, when cross-linked, do not dissolve. In an aqueous environment, the hydrophilic groups of the polymer chains are hydrated generating a “network” structure. The term network involves chemical or physical crosslinkage between the active groups of the polymer [8]. Due to its physicochemical similarities with the extracellular matrix, hydrogels are generally biocompatible [9]. The combination of polysaccharides as policaju (POLI) and chitosan (CHI) termed POLI-CHI has been previously characterized and studied by our lab [8,10], evaluating hydrogels using different proportions, being the most usable that composed of POLI:CHI, 1:4. The FT-IR analyses confirmed the existence of physical interactions between the polysaccharides involved and rheological measurements showed an increase in complex viscosity with the increase of chitosan content.

In recent years, the use hydrogels as bone healing or repairing agents is increasing, in Nafee [11] a chitosan based one was evaluated for its capacity to alendronate delivery, had both biodegradability and biocompatibility and presented site-specific, time-controlled and intra-articular delivery properties. Following the same premise, a hydroxyphenyl propionic acid hydrogel was tested aided by a delivering bioactive calcium accumulating peptide. The study was promisor, creating an environment that enhanced bone repair [12]. Regarding an intra-articular evaluation, Rieger [13] accessed a hydrogel of Chitosan and hyaluronic acid on subchondral bone during osteoarthritis in a rabbit model, enhancing microarchitectural parameters and leading to mineral density changes, and to subchondral bone loss.

Among non-chirurgical treatments, can be highlighted the Low Level Laser Therapy (LLLT) which acts causing several biological effects, such as: increasing proliferation and

activation of lymphocytes, increasing the phagocytosis on macrophages; and the secretion of growth factors in fibroblasts, enhancing the uptake of fibrin and collagen through emission of radiation by stimulating the most external electric field [14]. Previous in vitro and in vivo researches have also examined potential of LLLT to accelerate bone healing after a trauma or defect. The expression profile of both angio-genic and inflammatory genes seems to be modulated by the laser therapy [15]. LLLT also appears to stimulate osteoblast proliferation, collagen deposition, and early bone maturation, leading to bone neoformation [16].

Concerning conjugated treatments for bone repair, Oliveira [6] evaluated a collagen sponge scaffold implantation associated with LLLT on repairing critical sized bone defects, obtaining a synergic effect. The used hydrogel in this study, the POLI-CHI hydrogel was previously analyzed in vivo regarding its potential to aid the heal of skin wounds associated or not with LLLT with significant results [10], so the aim of this paper was to access the application in calvarium critical-sized defects in Wistar rats.

Material and method

Materials

Polysaccharide from cashew tree (*Anacardium occidentale L*.) gum (POLI), collected from the south coast of Pernambuco, Brazil, was obtained according to Souza [17]. The chitosan (CHI) (deacetylation > 75%) was purchased from Sigma–Aldrich Chemical Co. (St.Louis, MO, USA). All other chemicals were of analytical grade.

Hydrogel preparation

The POLI-CHI hydrogel was made of policaju (POLI) and chitosan(CHI) in a ratio of 1:4 according to Soares [8]. Briefly, 50 mL of stock solutions of 10% (w/v) of policaju and 1% (w/v) of chitosan in 1% lac-tic acid (v/v) were prepared in advance. In a separated beaker, 15 mL of chitosan solution plus 200 L of 0.1 M $CaCl^2$ was added and kept under stirring in Ultra-Turrax (IKA, USA) at 7000 rpm for 20 min. Then using a 27 G syringe and a flow of 1 mL/min, was added 5 mL of policaju solution. The mixture was left under stirring (7000 rpm) for 20 min. The pH was adjusted to 5.0 with 1 M NaOH solution and called pre-gel. The pre-gel solution was distributed in petri dishes and kept in an oven at 40◦C for 16 h for drying and polymerization. The thin film obtained was hydrated with distilled water and termed POLI-CHI hydrogel, which was stored under refrigeration at 4◦C.

Animals and treatment groups

Thirty-six male rats of the Wistar strain (*Rattus norvegicus*) [90-120 day-old, weighing 250-300 g] were submitted to experimental surgical procedures, being anesthetized intraperitoneally with 2 % (w/v) of xylazine hydrochloride and 10 % (w/v) ketamine hydrochloride at 1:1 ratio. The antisepsis of cranial region was made using 1 % (w/v) povidone-iodine and 0.9 % (w/v) NaCl sterile solutions. A full thickness circular parietal bone defect (Ø = 0.5 cm) was made in calvarium region of each animal using a drill according to the critical defect methodology developed by Bosch [18]. The tissue divulsion previously to the lesion was performed using a blade 15 and Metzenbaum and Iris scissors. After the surgery they were randomly divided into three groups (n = 12) according to the treatment: (C) Control, 0.1 ml of 0.9% (w/v) NaCl; (H) POLI-CHI hydrogel and (HL) POLI-CHI hydrogel and LLLT irradiation. After the surgical procedures they were placed in isolated cages. The light-dark cycle was of 12 h, beginning the brightly one at 6 h am. The environment temperature was set at 23 ± 1 ^{o}C and the water and food (ration) was *ad libitum*. All animal procedures were in accordance with the Colégio Brasileiro de Experimentação Animal (COBEA) and the Animal Ethical Committee of the Universidade Federal de Pernambuco approved the experimental protocol no n. 23076.024154/2015-1. The groups H and HL received an application of 0.1 ml of hydrogel in the defect area, and laser irradiation if it applies, and the group C received saline solution. In the matter of the laser irradiation, the animals that were subjected to LLLT (group HL) were irradiated in a punctually way starting from the center of the defect with Therapy XT (DMC medical, USA). The irradiation was carried out after surgery and at a 48 h during the first two weeks. The irradiation parameters used were: λ = 830 nm, A = 1 cm2; ED = 4 J/cm2, P = 100 mW, F = 50 Hz, delivering a total of 16 J. [19].

Macroscopic and behavioral evaluation

After the surgical procedures until 15 days, animals were clinical evaluated daily according to the presence of the following criteria: Edema, Hyperemia, Exudate, Crust, Detachment and Reepithelialization in the suture area. Were also evaluated the start of the feeding and drink process and was collected every atypical behavior.

Radiographic evaluating

The specimens were subjected to perpendicular incidence radiographies (70 KVp, 10mA e 0.3 s – Dabi Atlante, Ribeirão Preto, SP, Brazil) and the generated files were analyzed

using the ImageJ software ver 1.51 (National Institute of Health, USA), being the defect area selected and identified mean (± standard deviation) of the radiological density ranging from 0 (Black) to 255 (white).

Euthanasia and histological processing

Four animals from each group were sacrificed after 15, 30 and 45 days after the surgical procedure. They were subjected to lethal doses of sodium tiopental (200 $mg.Kg^{-1}$), and bone fragments are collected with a wide margin (complete calvarium) and kept in 10 % (v/v) formalin. The histological specimens were subjected to formic acid at 5 % for decalcification and included in paraffin and after microtome cut at the center of defect, the sections were stained using hematoxylin-eosine (HE), for cellular and bone observation, and picrosirius (PS) for collagen fibers.

Histological evaluating

The microscopy slides were analyzed according to presence and intensity (absent, weak, moderate, and strongly present) of the following histological findings: Neovascularization, New bone formation and Collagen. The slides were digitally processed using Dino-Eye Microscope Eye – Model AM4023X(R4) (ANMO – Taiwan), and the Image-Pro PLUS software ver 6.0 software (Media Cybernetics – USA). The distance between the defect borders was measured (pixels) and was applied in the regression of the wound formula: [(initial distance – distance on the day of measurement)/ initial distance x 100], obtaining the percentage of regression on the day of measurement [10].

Statistical analysis

The statistical evaluation was carried out using the analysis of variance (ANOVA) method and Bonferroni's multiple comparison test. The statistical significance was 5 % ($p < 0.05$) and the software used for data entry and processing was the Graphpad Prism for Windows, version 5.0 from Graphpad Software, Inc.

Results and Discussion

Macroscopic and behavioral evaluation

After the completion of the calvarium bone defect and suture procedures (Fig. 1), animals were diary evaluated regarding its behavior and the closed wound healing process. Edema, Hyperemia, Exudate and Crust are present from day 1 to 3 after surgery in all

groups, although was found a thicker Crust without Exudate in H and HL groups in comparison to C. From day 4 to 7, all groups still present Crust being Edema and Hyperemia absents. At day 8, the H and HL groups started to lose its crust (Detatchment). From day 9 to 12, all groups presented a similar pattern with Detachment. From day 12 to 15 there was not visible Crust left. The early Detachment of wounds is found in others studies that evaluated LLLT [10, 19]. The repilation of the sutured area was also observed initiating from day 5 in HL subgroup and from day 7 in C. This goes in accordance with other studies that evaluated the LLLT regarding hair and fur grow [20,21]. Concerning reepithelization, once the skin lesion was subjected to primary closure (suture) it was not evidently observed.

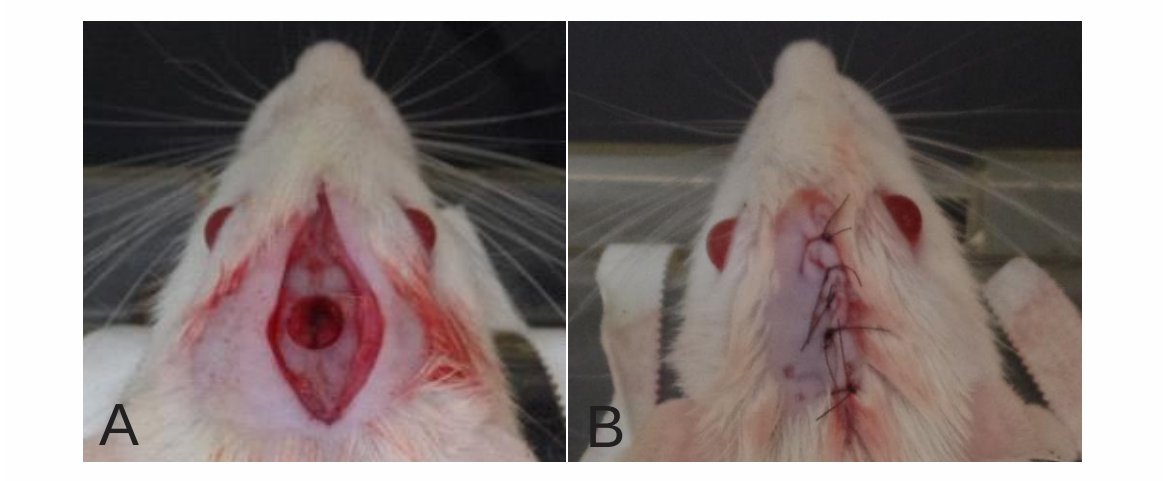

Fig 1. Bone defect. A - A full thickness circular parietal bone defect (Ø = 0.5 cm) was made in the calvarium region of each specimen. B- Suture of periosteum and skin.

In the matter of animal behavior, specimens from H and HL subgroups started to feed and drink few hours after surgery and in C it occurs only after 14h. Some LLLT studies has demonstrated a trend for analgesic and anti-inflammatory properties [22,23], as well as chitosan regarding anti-inflammatory and antioxidant [24,25], and POLI-CAJU [26]. This way, is plausible to infer an effect on pain modulation. The result was also observed in an early study that evaluated the same hydrogel conducted by this group [10] and according to Soon& Acton [27] animals subjected to stress and pain had a poor healing process.

One aspect of used methodology was the option for separated control specimens. In original methodology used by Bosch [18], authors preconized the use of pariate specimens (containing experiment and control in the same animal, using different surgical defects).

This choice was due to the possibility of the control defect contamination once hydrogel had the possibility to dislocate from its original site. So, maintaining one defect for animal avoids this possibility. Concerning the chosen animal model, according to Spicer [28] it offers a reliably, reproducible and analogs to clinical condition method that allows for evaluation of biomaterial and bone tissue engineering approaches within a non-load-bearing orthotopic site. In our study, any other area or bone used may present a more prevalent variation in results, primarily caused by trauma (biting or licking) of the sutured site.

Radiographic evaluation

Evaluating radiodensity pre euthanasia, where obtained the following: at day 15, H group presented the higher arithmetical mean (23.273 ± 0.972) followed by HL (22.012 ± 0.995) and C (21,400 ± 2,163); at day 30, was perceived a higher radiodensity in HL group (27.944 ± 1.565), followed closely by H (27.681 ± 2.269) and then C (26.034 ± 3.239); and at day 45, H presented again a higher mean (30.366 ± 1.990), followed by HL (30.252 ± 0.795) and C (29.303 ± 3.023). There was not found significance between subgroups ($p < 0.05$) (Fig. 2).

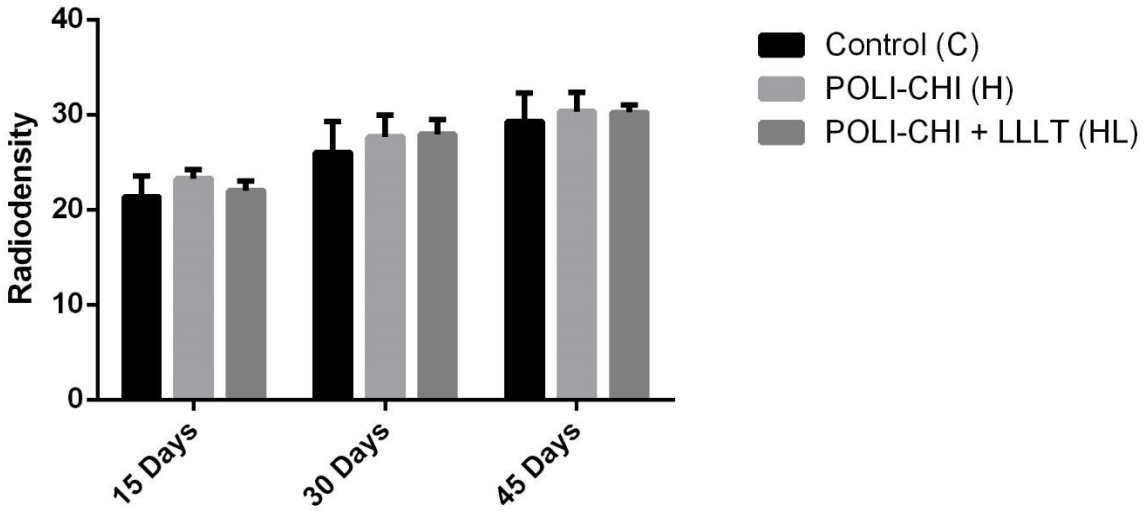

Fig 2. Radiodensity evaluation by time. Comparison of the radiodensity mean and between experimental groups and control. There was not significance between subgroups ($p < 0.05$).

The radiographic evaluation offers a potential to access the new formed bone and its density without animal euthanasia, yet its only suggest, being necessary other methods to

correct infers a trustful result. In our study the radiographic evaluation did not present significant results regarding experimental subgroups in comparison to control, although it indicate a trend to gradual increase of radiodensity. In a study carried out by Spin-Neto [29] using a similar calvaria model, they found significant results regarding low molecular weight chitosan and high molecular weight in comparison to control at 15 days, although did not present significant results at 60 days, being this way inconclusive using radiodensity measures, being this method dependent of completion by other evaluative methods, such as histological data.

Histological evaluation

The main objective of this analysis was to delineate a histological overview of the specimens and to compare the different findings in a temporal way as well as evaluate the defect's diameter reduction comparing the experimental subgroups with control (Fig. 3).

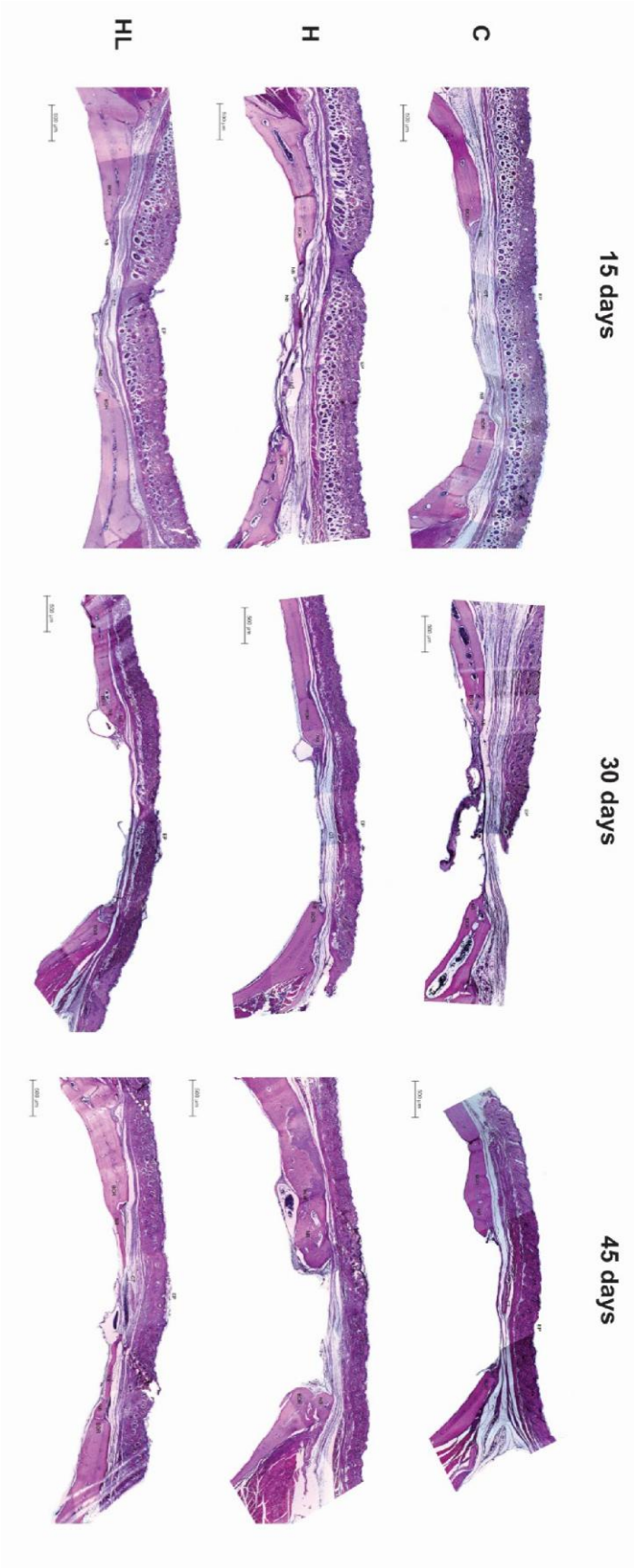

Figure 3. Histological overview. Subgroups by time of euthanasia, highlighting defect area and borders.

The neovascularization process post bone trauma is a primary requirement to obtain a quality bone repair. In our study, at day 15, in HL and H it was strongly present and in C moderate. At 30 days, it was moderate to weak in every subgroup and at 45 weak (Fig 4A). This decrease of neovascularization is caused by maturation and consequently vascular regression. Some LLLT studies indicate a positive effect regarding new vessel formation [30,31] and vascular endothelial growing factor – VEGF [32] which corroborate our findings regarding HL subgroup. Also, concerning chitosan use, a study that evaluated calcitonin gene-related peptide (CGRP)/chitosan-strontium cement identified a significantly upregulated expression of VEGF gene [33].

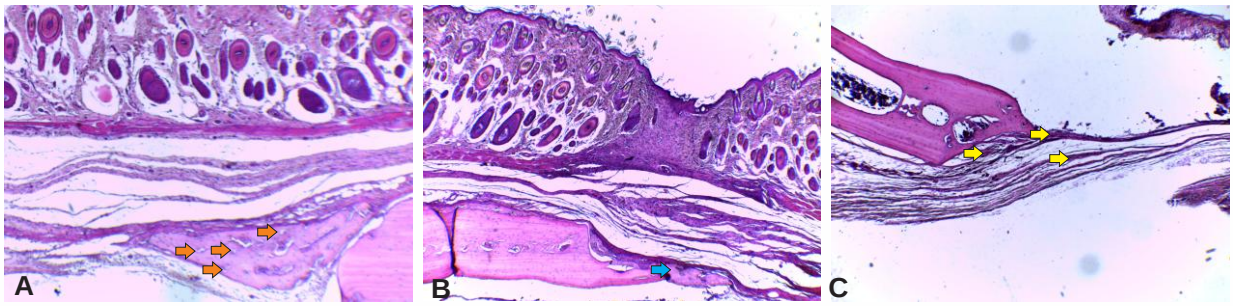

Figure 4. Histological evaluation. A – Neovascularization – orange arrows (HE, 40x, H at 15 days), B – New bone formation – blue arrow (HE, 40x, HL at 15 days), C – Collagen fibers – yellow arrows (HE, 40x, C at 30 days).

In the matter of New bone formation (Fig 4B), HL group presented it strongly present at all evaluated times, followed by H presenting strongly to moderate, primarily around the defect borders, and C presented it moderately. LLLT was evaluated, aided or not by other products, for bone formation in several studies [16,19,33,34,35], this way aiming to reduce the use of live specimens our study did not selected a LLLT solo subgroup. Our evaluation intended to compare a possible synergic effect of different modality treatments. A study investigated the effect of chitosan (Ch) porous 3D scaffolds embedded with resolvin D1 (RvD1), an endogenous pro-resolving lipid mediator, obtaining significant results *in vivo* bone healing [36], other research conducted by Spin-neto [29] obtained poor results, although the selected model was set at 8 mm diameter critical defect, which according to Bosch [18] cannot be closed without a osteoconductive material, such as bioceramic. In the matter of POLICAJU use, there was not found studies that evaluated the

polyssacharide regarding bone repair. A single research evaluating bone maintenance was identified, indicating a trend for bone or antiinflammation modulation effect [26].

The deposition of collagen fibers is indispensable to a correct and effective tissue healing. The arrange of collagen fibers in the early stages of bone formation is responsible to guide the later calcification process. In this matter, at 15 days, all subgroups presented it strongly. At 30 days, strongly to moderate, and at 45 days moderate at C and moderate to weak to H and HL, indicating a path to bone formation (Fig. 4C). These findings go against the study of Bölükbaşı [37] which evaluated in vitro the potential to osteoblast and formation and collagen deposition after LLLT. In our studies was found a similar pattern between subgroups, although neovascular formation previously described is responsible for the viability of bone tissue, avoiding this way a fibrous or cartilaginous formation repair.

Regarding defect's diameter reduction in percentage, at day 15, HL group presented higher arithmetical mean (41,197 ± 1.369) followed by H (34.783 ± 0.750) and C (30.219 ± 0,802), being HL significant to C. At day 30, H presented a superior value (30.043 ± 0,805), followed by C (27.295 ± 1.225) and HL (26.984 ± 2.930). At day 45, HL group presented higher mean (55.714 ± 7.161), followed by H (48.711 ± 4.131) and C (40.010 ± 2.811), being H and HL significant to C ($p < 0.05$) (Fig. 5).

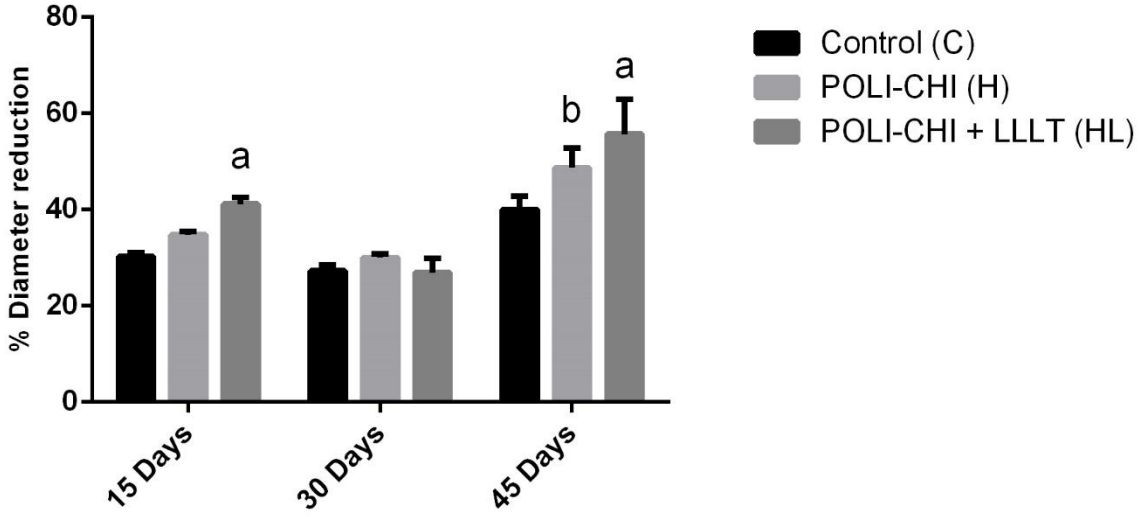

Figure 5. Diameter reduction expressed in percentage. HL subgroup was significant to C at 15 and 45 days (a) and H was significant to C at 45 days (b). ($p < 0.05$)

The experimental groups presented a similar trend for bone formation, highlighting the HL subgroup. These findings corroborate with studies with LLLT [34,35], chitosan [38,29] and policaju [26] as bone modulation agents, and yet may be suppose a combining effect of the LLLT and POLI-CHI based on the results. The question regarding subgroups at 30 days, and its poor results may be due to several factors, including bone formation around the borders and not within defect, this way avoiding the closure, and situational questions related to specimens, such as stress and others. Other factor that demands further discussion is the difference between radiographic and histologic evaluation. The radiographic evaluation indicated a trend for density and is obtained by the media of the selected area, do not differentiating the presence of bone spiculae or delicate structures. The border bone formation probably was responsible for similar results regarding subgroups.

Conclusions

The results showed that the POLI-CHI hydrogel contributed for bone formation and the combined use of POLI-CHI hydrogel with LLLT showed yet better results in neovascularization and the possibility for defect's closure.

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5. CONCLUSÕES

Os resultados obtidos permitem concluir que:

Artigo I

- O tratamento utilizando o hidrogel POLI-CHI contribuiu para a cicatrização bem como na modulação do processo inflamatório.
- Os animais apresentaram cicatrizes mais estéticas, maior regressão da área da lesão, maior formação de colageno, menor presença de necrose focal, menor presença de exudato neutrofílico, menor presença de edema, e regressão da neoformação vascular.
- O tratamento utilizando o Hidrogel POLI-CHI associado à LLLT atuou potencializando o reparo desencadeado pelo hidrogel, bem como induzindo um maior conforto pós-operatório com sinalização de atividade analgésica e aceleração dos estágios cicatriciais.
- Tendo em vista o que foi exposto, o Hidrogel POLI-CHI acrescido ou não da LLLT atuou de forma a estimular a cicatrização, e havendo a possibilidade do tratamento combinado, este deve ser indicado por atuar de forma sinérgica.

Artigo II

- Não foi identificada reação de corpo estranho ou processo inflamatório crônico com a implantação do biomaterial;
- O hidrogel POLI-CHI contribuiu para a neo-formação óssea em região de calvária submetida a confecção de defeito crítico;
- O uso combinado do POLI-CHI associado a LTBI melhorou o processo, ampliando o potencial para neo-formação vascular e a possibilidade para um possível fechamento do defeito.

E finalmente, o POLI-CHI se apresenta como um promissor biomaterial para ser utilizado tanto como adjuvante nos processos de cicatrização cutânea como um material com potencial para uso no preenchimento de defeitos ósseos.

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ANEXO A – Normas do periódico

INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES

Structure, Function and Interactions

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

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Ofício nº 85/15

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Processo nº 23076.024154/2015-01

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado "**Avaliação do potencial para reparo ósseo de efeitos críticos confeccionados na calvária de ratos wistar com a utilização de hidrogel de polissacarídeos associados á hidroxiapatita",**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Biotério do Departamento de Nutrição; Animal Rato heterogênico; Linhagem; Wistar; idade; 90-120 dias; peso; 250-300g; Nº total de animais a ser utilizado; 216

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1801584