Universidade Federal de Uberlândia
Instituto de Genética e Bioquímica
Pós-Graduação em Genética e Bioquímica

EFEITOS DA ANGIOTENSINA-(1-7) NA INFLAMAÇÃO E REPARO TECIDUAL

Aluna: Simone Ramos Deconte
Orientador: Professor Dr. Fábio de Oliveira
Co-orientadora: Professora Dra. Fernanda de Assis Araújo

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_____

Professor Dr. Fábio de Oliveira
(Orientador)

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SUMÁRIO

APRESENTAÇÃO

Após o nascimento, a resposta inflamatória é uma consequência inevitável da lesão tecidual. Em uma variedade de condições patológicas (artrite reumatoide, psoríase, aterosclerose, diabetes, doença de Crohn, câncer, aderências), o processo inflamatório é persistente e contribui para a manutenção e agravamento destas doenças. Estudos experimentais estabeleceram o dogma de que a inflamação é essencial para o estabelecimento da homeostase cutânea após lesão. Nos últimos anos, aumentou o número de estudos associando linhagens específicas de células inflamatórias como responsáveis por uma série de citocinas relacionadas ao reparo tecidual. Recentemente, este dogma foi contestado, e estudos têm levantado dúvidas sobre a validade do pré-requisito essencial da inflamação para reparação tecidual eficiente. De fato, em modelos experimentais de reparo, foi demonstrado que a inflamação retarda a cura resultando em aumento da formação de cicatrizes. Além disso, a inflamação crônica, uma característica marcante da ferida que não cura, predispõe ao desenvolvimento de câncer. Assim, uma compreensão mais detalhada dos mecanismos que controlam a resposta inflamatória e como, durante o reparo, a inflamação direciona para o processo de cura serve como um marco na terapia de reparo de tecidos patológicos. Nesse sentido, a pesquisa científica tem centrado esforços com o objetivo de identificar agentes terapêuticos com propriedades anti-inflamatórias e/ou anti-fibrogênicas que possam ser utilizados no combate e/ou prevenção de vários processos patológicos onde estes componentes co-existem. Nesse trabalho, avaliamos os efeitos da Angiotensina-(1-7), um peptídeo derivado da Angiotensina II, na inflamação crônica induzida por implantes sintéticos e na cicatrização de feridas excisionais em camundongos. Nossos resultados revelaram que esse peptídeo foi capaz de reduzir a resposta inflamatória e aumentar a angiogênese e a deposição de colágeno local. Esses resultados levaram a uma melhora na cicatrização da ferida e demonstram que esse peptídeo pode ser um possível agente terapêutico em doenças em que a inflamação crônica e o reparo encontram-se alterados.

Conforme as normas definidas pelo Programa de Pós-Graduação em Genética e Bioquímica da UFU, os resultados referentes a esta tese são apresentados em três capítulos sob a forma de referencial teórico (Capítulo I) e manuscritos (Capítulo II e III).

CAPÍTULO 1: FUNDAMENTAÇÃO TEÓRICA

1. INTRODUÇÃO

1.1. Inflamação

A inflamação é uma resposta imune essencial ao organismo e que permite sua sobrevivência durante a infecção ou lesão além de manter a homeostase dos tecidos sob uma variedade de condições nocivas. Apesar de essencial e benéfica, essa resposta leva à diminuição transitória da função tecidual, o que por sua vez, pode contribuir para a patogênese de várias doenças crônicas [1]. As interações entre as células do sistema imune inato, sistema imune adaptativo e os mediadores inflamatórios são responsáveis por coordenar uma série de mecanismos efetores da inflamação aguda e crônica. Essa série coordenada de mecanismos efetores da inflamação pode contribuir para a lesão do tecido, o estresse oxidativo, o remodelamento da matriz extracelular, a angiogênese e a fibrose em diversos tecidos alvos [2].

De modo geral, a resposta inflamatória aguda, desencadeada por infecção ou lesão tecidual, envolve a liberação de componentes sanguíneos (plasma e leucócitos) para o sítio infeccioso ou lesivo. Essa resposta é desencadeada por receptores da resposta imune inata, como os receptores *Toll-like* (TLRs) e NOD ((*nucleotide-bindingoligomerization-domainprotein)-like*). Esse reconhecimento inicial é mediado por macrófagos e mastócitos residentes, levando a produção de uma variedade de mediadores inflamatórios incluindo citocinas, quimiocinas, aminas vasoativas, eicosanoides e produtos de cascatas proteolíticas[3]. O efeito imediato desses mediadores leva a alterações locais que causam vasodilatação, extravasamento de líquidos, adesão endotelial e recrutamento de células inflamatórias, as quais são normalmente restritas aos vasos sanguíneos. As células endoteliais ativadas mediam o extravasamento de leucócitos enquanto previne a saída de eritrócitos. Essa seletividade ocorre por uma interação de selectinas endoteliais com integrinas e receptores de quimiocinas nos leucócitos, os quais ocorrem na superfície endotelial bem como no espaço extracelular em que proteínas

plasmáticas recentemente depositadas formam uma matriz provisória para a ligação das integrinas dos leucócitos [4].

Os neutrófilos são as primeiras células a deixarem os vasos sanguíneos, sendo predominantes nas primeiras 24 horas após o início do processo. Mediadores químicos, como a quimiocina CXCL-1 (Quimiocina motivo CXC ligante 1), atuam no recrutamento de neutrófilos os quais, uma vez ativados, secretam o conteúdo de seus grânulos, contendo principalmente, a enzima mieloperoxidase (MPO), que auxilia na destruição de patógenos [5]. Uma série de citocinas e quimiocinas pró-inflamatórias, incluindo a família das interleucinas (IL), fator de necrose tumoral-α (TNF-α) e interferon (IFN) são extensivamente produzidos por vários tipos celulares do sítio inflamatório, ligando e ativando receptores acoplados à proteína G nas células imunes[6, 7]. A ativação destes receptores desencadeia uma cascata de sinais intracelulares que alteram a morfologia e migração celular.

Os monócitos começam a migrar dos vasos 18 a 24 horas depois de iniciada a diapedese, que se acumulam rapidamente e então, os macrófagos passam a ser as células predominantes após 48 horas [8-10]. Monócitos atraídos por quimiocinas como a CCL-2/JE/MCP-1 (proteína quimiotática de monócitos-1) migram para o sítio inflamatório onde se diferenciam em células dendríticas e macrófagos M1 (que possuem propriedades pró-inflamatórias, com capacidade de mediar respostas de defesa contra uma variedade de micro-organismos) [11]. Os macrófagos irão produzir uma série de fatores de crescimento e citocinas responsáveis por uma ampla variedade de respostas em vários tipos celulares incluindo células endoteliais (ECs), células epiteliais e células de origem mesenquimal [8]. Os neutrófilos e macrófagos M1 de fase inflamatória predominam nas primeiras 6 a 24 horas, a partir daí, após realizarem sua ação no local, os neutrófilos entram em apoptose. Em seguida, os macrófagos promovem a fagocitose de neutrófilos apoptóticos (eferocitose), promovendo uma alteração no fenótipo do macrófago com perfil M1 para macrófago com perfil M2 de fase resolutiva (aqueles com papel na síntese de mediadores anti-inflamatórios/pró-resolutivos, importantes no processo de reparo tecidual) em 24 horas a 72 horas [12, 13].

O reparo acontece simultaneamente ao processo de resolução da resposta inflamatória, o qual os macrófagos M2 secretam grandes quantidades

de mediadores pró-resolutivos, como o fator de crescimento transformante beta (TGF-β) que estimula a deposição de colágeno. As citocinas pró-angiogênicas, como o fator de crescimento do endotélio vascular (VEGF) e o fator de crescimento fibroblástico (FGF), também são importantes no processo de reparo, induzindo a formação de novos vasos sanguíneos (angiogênese) [14]. Na medida em que, os mediadores pró-resolutivos sobrepõem aos mediadores pró-inflamatórios, favorece o reparo tecidual e consequentemente o retorno da homeostase tecidual [13]. O processo de reparo tecidual é regulado por diversos mediadores químicos e coordenado por interações das células com elementos da matriz extracelular, como por exemplo, o colágeno.

Esta resposta inflamatória é regulada por um balanço entre fatores pró e anti-inflamatórios que coexistem no sítio lesivo. Se o agente desencadeador da inflamação não é eliminado pela resposta inflamatória aguda ou persiste por alguma outra razão, a fase de resolução pode não ser induzida de forma eficaz e uma inflamação crônica se estabelece [15]. A inflamação crônica é uma resposta inflamatória com duração prolongada (semanas, meses ou anos), sendo caracterizada, principalmente, por infiltrado de células mononucleares (macrófagos, linfócitos e plasmócitos), destruição tecidual (induzida pelos produtos das células inflamatórias), angiogênese e fibrose (deposição de colágeno) [16-18]. Essa condição pode ser causada por infecções crônicas, dano tecidual irreparável, alergênicos persistentes e partículas estranhas ou endógenas indigeríveis. Além disso, um grande número de doenças inflamatórias crônicas não tem seu agente desencadeador bem definido e não são necessariamente desencadeadas por infecção ou dano tecidual como nos casos de obesidade, diabetes tipo 2, aterosclerose, doenças neurodegenerativas e câncer. Nesses casos, a inflamação crônica parece desenvolver um ciclo vicioso que conecta a inflamação e o processo patológico em si. Um exemplo é o que acontece em pacientes obesos em que a obesidade pode desencadear uma inflamação crônica que pode estimular, pelo menos em parte, a resistência à insulina e a diabetes, características da obesidade[19]. Sendo assim, passagem de uma potente resposta inflamatória para o processo de reparo é um ponto crítico promovido por sinais anti-inflamatórios locais, os quais são rápidos e reversíveis[20]. Sempre que moléculas pró-inflamatórias excederem às anti-inflamatórias, a inflamação

torna-se exacerbada. Este desequilíbrio resulta no aumento da produção de proteases, proteoglicanos, mediadores lipídicos que, concomitantemente, reforçam o processo[21].

1.2. Reparo tecidual

Existem quatro respostas básicas que podem ocorrer após uma lesão: 1- regeneração, um processo que ocorre quando há perda da estrutura e função, mas o organismo tem capacidade de restabelecer a estrutura do tecido exatamente ao que era antes da lesão; 2- cicatrização deficiente, quando há deposição insuficiente de tecido conectivo e matriz enfraquecida; 3-cicatrização excessiva, quando ocorre deposição exacerbada de tecido conjuntivo que resulta em alteração da estrutura tecidual e, assim, perda da função (como as fibroses e aderências); 4-reparo normal, quando há restabelecimento do equilíbrio entre a formação da cicatriz e o remodelamento da cicatriz [22].

O reparo após uma lesão é um fenômeno evolutivamente conservado nos organismos vivos. Nos organismos unicelulares está restrita à presença de enzimas responsáveis pela recuperação de elementos estruturais (como os constituintes do citoesqueleto, membranas e paredes celulares) e de moléculas de alta complexidade (como proteínas de elevada complexidade estrutural, RNAs e o DNA). Em organismos mais complexos, o reparo de tecidos pode ocorrer de duas formas que envolvem diversas cascatas de sinalização inter e intracelulares [23]. Este é um processo complexo e dinâmico que resulta na restauração da arquitetura tecidual e da função após a lesão [24].

O processo de cicatrização começa imediatamente após a lesão, de forma eficiente e organizada, caracterizada por quatro fases distintas, mas sobrepostas: hemostasia, inflamação, proliferação e remodelamento. A hemostasia é ativada pelo contado de componentes do sangue e das plaquetas com o colágeno, ativando assim, a cascata de coagulação. As plaquetas também liberam citocinas e fatores de crescimento como PDGF e TGF-β que

resultam em quimiotaxia de leucócitos, migração e proliferação celular e ativação de mais células inflamatórias.

A inflamação, conforme descrita anteriormente apresenta uma fase inicial caracterizada pela liberação de mediadores inflamatórios, alterações vasculares e pelo infiltrado predominante de neutrófilos nas primeiras 24 horas e por uma fase tardia em que, após aproximadamente 48 horas do início da lesão, ocorre predominância de macrófagos no sítio lesivo. A presença de macrófagos é um sinal de que a fase inflamatória está próxima de acabar e que a fase proliferativa está iniciando [25].

Na fase proliferativa, os fibroblastos são as células predominantes. Esta célula de origem mesenquimal é responsável por produzir a nova matriz necessária para restaurar a estrutura e função do tecido lesado. A liberação de interleucinas, FGF, TNF-α e TGF-β por plaquetas e células inflamatórias (macrófagos e linfócitos T), são de grande importância para a fase proliferativa, no entanto, o TGF-β tem um papel central nesta fase, pois é considerado o principal regulador das funções dos fibroblastos [26]. O TGF-β aumenta a transcrição de genes para colágeno, proteoglicanos e fibronectina e assim, aumenta a produção de proteínas de matriz. Ao mesmo tempo o TGF-β diminui a secreção de proteases responsáveis pela degradação da matriz e também estimula o inibidor de protease, inibidor tecidual de metaloprotease (TIMP) [27].

Fibrina e fibronectina são componentes iniciais da matriz extracelular. Posteriormente, os fibroblastos sintetizam glicosaminoglicanos, proteoglicanos, colágeno e outras proteínas formando o tecido de granulação [28]. Após o pico da atividade proliferativa, os fibroblastos se desdiferenciam em um fenótipo contrátil, o miofibroblasto[29], uma célula que voltou na escala de diferenciação e apresenta características de células mesenquimais e fibroblasto, a qual continua proliferando e sintetizando colágeno [28]. O colágeno depositado inicialmente é mais fino que o colágeno de um tecido sem lesão e está orientado paralelamente à pele. Com o passar do tempo, o colágeno inicial é reabsorvido e um colágeno mais espesso é depositado e organizado paralelamente às linhas de tensão. Estas alterações são acompanhadas também com um aumento da força tensora da ferida indicando uma correlação positiva com a espessura/orientação do colágeno e força tensora e determinam a fase de remodelamento [30].

À medida que o tecido de granulação, decorrente da proliferação de fibroblastos e células endoteliais vasculares, rico em colágeno preenche a área lesada, o número de células inflamatórias diminui e os fibroblastos e as células endoteliais continuam a sintetizar fatores de crescimento da matriz extracelular. Os macrófagos desempenham um papel central neste processo, englobando células apoptóticas de forma rápida e eficiente, intermediando a morte de células do estroma, parênquima e células imunológicas[31]. Devido à alta atividade metabólica no sítio lesivo, há um aumento da demanda por oxigênio e nutrientes. Fatores locais no microambiente da lesão, como pH reduzido, redução da tensão de oxigênio e aumento de lactato, atuam sobre as células epidérmicas, fibroblastos, macrófagos e células endoteliais vasculares, promovendo a liberação de fatores como VEGF, bFGF e TGF-β necessários para induzir a angiogênese (formação de novos vasos sanguíneos) [32, 33]. A resposta angiogênica é essencial para uma reparação tecidual normal, pois além de fornecer nutrientes e células inflamatórias para o tecido lesado, os novos vasos sanguíneos facilitam a remoção do material residual e auxiliam no desenvolvimento do tecido de granulação [34]. Em condições inflamatórias crônicas, as células endoteliais ativadas por citocinas e fatores de crescimento recrutam ativamente células imunes da circulação para o tecido e participam da angiogênese para dar suporte à contínua demanda de oxigênio e nutrientes pelas células inflamatórias.

1.2.1. Cicatrização de feridas

Ferida pode ser definida como qualquer ruptura da integridade da pele, bem como membranas mucosas ou outras estruturas do corpo, podendo ter diferentes profundidades, tamanhos, formas e ser causada por razões distintas [41].

A cicatrização de feridas cutâneas é um processo de reparo tecidual altamente dinâmico que envolve uma complexa sequência de eventos celulares e bioquímicos. Após um estágio inicial inflamatório, caracterizado pela infiltração de neutrófilos e de macrófagos, segue-se à formação de um tecido

fibroproliferativo rico em colágeno e vasos sanguíneos neoformados e, finalmente, a fase de maturação envolve epitelização, resolução da inflamação e remodelação da derme [35].

O recrutamento de leucócitos inflamatórios para uma ferida, além de ser uma aquisição evolutiva dos mamíferos para evitar a invasão de micro-organismos, é também um componente crítico durante o reparo tecidual devido ao amplo espectro de mediadores liberados. No entanto, ainda não existe um consenso se a resposta inflamatória dificulta ou acelera a cicatrização de feridas e se afeta a qualidade do reparo tecidual [36]. Por exemplo, embriões podem reparar feridas na ausência de resposta inflamatória e sem formação de cicatriz [37]. Além disso, estudos sugerem que neutrófilos inibem o processo de reparo tecidual [38]. Isto pode ser demonstrado em feridas crônicas nas quais a resolução da inflamação é ineficiente [39].

Por outro lado, a neovascularização é também fundamental para o processo de cicatrização, pois o crescimento de novos vasos sanguíneos fornece suprimentos de oxigênio e nutrientes adequados para o tecido em cicatrização, além de facilitar o acesso de leucócitos e a remoção de material residual[36]. Entretanto, o crescimento excessivo e/ou persistente de novos vasos sanguíneos está associado à cronificação do processo inflamatório e ao desenvolvimento de fibrose [40]. Observa-se, então, a necessidade de se entender melhor a inter-relação e os mecanismos que regulam a ativação de células inflamatórias e endoteliais em processos de cicatrização.

Evolutivamente, a cicatrização de uma ferida incide em eventos celulares e moleculares que interagem para que ocorra a repavimentação e a reconstrução do tecido, na tentativa de manter a estrutura anatômica e a função normal da região [41]. Para atingir esse objetivo, o processo de cicatrização envolve três fases que se sobrepõem no tempo e espaço: 1- inflamação, 2- formação de tecido de granulação com deposição de matriz extracelular e 3- remodelamento do tecido [36].

Após um estágio inicial inflamatório, caracterizado pela infiltração de neutrófilos e de macrófagos, segue-se à formação de um tecido fibroproliferativo rico em colágeno e vasos sanguíneos neoformados e, finalmente, a fase de maturação envolve epitelização, resolução da inflamação e remodelamento da derme [42]. Devido à liberação de mediadores químicos

produzidos principalmente por macrófagos, a migração e ativação de fibroblastos são intensificadas. Estes fibroblastos migram da região das bordas para o centro da ferida, produzindo colágeno e causando uma substituição da matriz extracelular por tecido conjuntivo mais forte e elástico [43]. Este processo é chamado de fibroplasia e é controlado especialmente por citocinas da família do TGF-β. Para que o processo de fibroplasia seja eficiente é necessária a ocorrência em paralelo da formação de novos vasos sanguíneos, ou seja, é necessária a neovascularização da região [23].

A formação do tecido de granulação é iniciada com a fibroplasia[44], sendo composto por macrófagos, fibroblastos e vasos neoformados que são suportados por uma matriz frouxa de fibronectina (rede de sustentação da migração, traça o caminho para os leucócitos), ácido hialurônico e colágeno [44]. A neovascularização é essencial neste estágio, uma vez que permite a troca de gases e a nutrição das células metabolicamente ativas. VEGF-A é o principal fator solúvel regulador da formação de novos vasos sanguíneos durante o desenvolvimento do tecido cicatricial [45].

A migração e ativação de macrófagos e fibroblastos para a região da ferida, somada à presença de vasos neoformados, permitem que os componentes da nova matriz extracelular passem a ser localmente produzidos principalmente por estas células. Os fibroblastos passam a depositar grandes quantidades de fibronectina que, embora seja substrato que desempenha outras funções, basicamente serve para a fixação da própria célula. Outra substância produzida em grande quantidade neste segundo estágio é o ácido hialurônico. Estas duas substâncias predominam na matriz durante as primeiras fases do reparo, pois esta combinação propicia um microambiente eficiente para a movimentação das células, necessárias nesta etapa [23]. A própria natureza anatômica da ferida proporciona um estímulo para a migração e proliferação de fibroblastos, células epiteliais e queratinócitos a partir das suas margens, fenômeno este denominado "efeitos de vizinhança livre" [46].

Conforme o leito da ferida é preenchido com o tecido de granulação, a quantidade de macrófagos e fibroblastos vai sendo reduzida. A ação dos fibroblastos especializados (miofibroblastos) promove contração da ferida, diminuindo assim a área de superfície de lesão [28].

A reepitelização da ferida também ocorre nesta fase. Quando o tecido de granulação chega ao nível da epiderme, os queratinócitos tornam-se células hiperproliferativas e migratórias, fornecendo um substrato adequado para a evolução do processo mitótico destas células. Além disso, estas células produzem e secretam componentes da matriz extracelular e polipeptídeos sinalizadores, ao mesmo tempo em que seu citoesqueleto é alterado para a produção de queratina [43].

Uma vez que o processo de cicatrização vai avançando, o leito da ferida fica totalmente preenchido pelo tecido de granulação, o qual vai sendo enriquecido com mais fibras de colágeno adquire a aparência de uma massa fibrótica, característica da cicatriz. A acentuação na deposição do colágeno e o desaparecimento da maioria das células (observa-se a apoptose de fibroblastos e células endoteliais) conduzem à formação final da cicatriz. A resolução completa de uma ferida somente pode ser considerada depois de concluída a maturação e remodelamento da matriz extracelular [23]. A fase de remodelamento se inicia pouco tempo após o surgimento da ferida, persistindo por um grande tempo após o seu fechamento. Uma cicatriz cutânea completamente madura possui apenas 70% da resistência da pele normal [47].

De acordo com Clark (1993) [48], as primeiras células que sofrem apoptose são as células endoteliais, seguidas dos fibroblastos, levando gradualmente a uma cicatriz acelular. Nos meses seguintes à formação do tecido de granulação, a matriz extracelular sofre uma contínua e lenta transformação. O processo de remodelamento da cicatriz, portanto, envolve etapas sucessivas de produção, digestão e orientação das fibras de colágeno [43].

Ao final desta etapa de remodelamento da cicatriz, anexos da pele, como glândulas e folículos pilosos, sofrem regeneração limitada e a coloração da cicatriz empalidece devido à deficiente regeneração dos melanócitos[49] e as cicatrizes são hipovascularizadas devido ao desaparecimento dos neocapilares [23].

O reparo é uma consequência do ambiente e do perfil de inflamação que foi desencadeado. O reparo anormal de feridas resulta de desordens no remodelamento do tecido de granulação e pode levar a uma cicatrização hipertrófica ou fibrose. Além disso, quando o reparo ocorre de forma

inapropriada, desencadeia-se uma resposta inflamatória crônica a qual pode levar ao retardo da cicatrização e isso representa um problema direto para o indivíduo, causando desconforto físico e/ou psicológico e demora na reabilitação, podendo até causar amputação de membros e morte por septicemia nos casos mais extremos. Portanto, existe um grande interesse no estudo de moléculas/fármacos, os quais possam modular o processo de cicatrização de feridas.

1.3. Sistema Renina Angiotensina (SRA)

O Sistema Renina Angiotensina (SRA) é um sistema hormonal composto por várias enzimas, peptídeos ativos e inativos, que juntos desempenham importante papel na fisiologia cardiovascular por regular a pressão arterial (PA) e manter o balanço hidroeletrolítico. Classicamente, o angiotensinogênio produzido no fígado, é hidrolisado pela renina, liberada pelas células justaglomerulares renais, e produz um decapeptídeo denominado angiotensina I (Ang-I), a qual é convertida pela enzima conversora de angiotensina (ECA) em angiotensina II (Ang-II) um octapeptídeo biologicamente ativo [50].

A Ang-II exerce inúmeros efeitos biológicos através da ativação de seu receptor AT1, dentre as quais destacam-se: vasoconstrição, estímulo ao mecanismo da sede, liberação de aldosterona e vasopressina, aumento da reabsorção de sódio e água nos túbulos renais, hipertrofia celular, fibrose, inotropismo e cronotropismo positivos, formação de radicais de superóxido, ativação do sistema nervoso autônomo simpático, secreção de endotelinas, dentre outros [51]. A Ang-II também pode se ligar ao seu outro receptor AT2 e exercer efeitos opostos aos do receptor AT1. A ativação de AT2 promove diferenciação celular, reparo tecidual, apoptose, vasodilatação, inibição do crescimento e da proliferação celular [52]. Entretanto, a identificação de novos peptídeos biologicamente ativos, a descoberta de novas enzimas para o metabolismo de angiotensinas, novos receptores angiotensinérgicos, de interações receptor-receptor e novas funções do SRA e da atuação local deste

sistema, independentemente de sua secreção hormonal, têm modificado a visão clássica do SRA [51, 53].

A Angiotensina-(1-7) (Ang-(1-7)) é um metabólito do SRA que, por muito tempo foi considerado inativo [54]. Esse conceito começou a se modificar a partir dos estudos de Schiavone e colaboradores (1988)[55] evidenciando que a Ang-(1-7) exercia um efeito semelhante ao da Ang II na liberação *in vitro* de vasopressina em culturas de extrato de neuro-hipófise. Desde então, diversos estudos têm demonstrado efeitos de grande importância e que produziram alterações conceituais de fundamental importância relacionadas a Ang-(1-7). Dentre eles destacam-se a descoberta da ECA2 (enzima conversora de angiotensina 2) como a enzima responsável pela formação da Ang-(1-7) e a identificação do seu receptor endógeno denominado Mas [56, 57].

A ativação do receptor Mas pela Ang-(1-7) tem efeitos antagônicos àqueles promovidos pela interação da Ang II com o receptor AT1, como: vasodilatação, antiarritmogênico, redução de fibrose, redução da proliferação celular, redução da hipertrofia, entre outros [51, 58]. Desse modo, o conceito de SRA atualmente é de um sistema complexo e bidirecional que atua por meio de dois eixos antagônicos: o clássico ECA-Ang II-receptor AT1 e o contrarregulador ECA2-Ang-(1-7)-receptor Mas [51].

As funções da Ang-(1-7) ainda estão sendo estudadas, mas existem evidências que este peptídeo funcione como um antagonista da angiotensina II e exerça também propriedades antifibróticas, antiproliferativas, atuando beneficamente na inflamação e nos processos fibróticos [51, 59-61], além de apresentar alterações nos níveis de citocinas pró- e anti-inflamatórias [62]. Entretanto, muitos resultados ainda são controversos e os mecanismos envolvidos ainda não são completamente caracterizados.

1.3.1. Angiotensina-(1-7) x Inflamação

Devido à escassez de estudos sobre os componentes do eixo ECA2-Ang-(1-7)-Mas [63], muitas vezes é necessário inferir nos efeitos potenciais da Ang-(1-7) a partir da avaliação do eixo clássico do SRA ou do bloqueio desse

eixo por meio de inibidores da enzima conversora de angiotensina (iECAs) ou antagonistas de seus receptores AT1 [64-66].

Estudos têm demonstrado um importante papel para a Ang II nos processos inflamatórios [64, 65]. Além disso, alguns componentes do SRA têm sido detectados em células inflamatórias. Um exemplo é o estudo de Silver e colaboradores (2004) [67] mostrando que os mastócitos produzem renina sendo, portanto, um sítio extrarrenal de síntese desta importante enzima formadora de Ang I. Outra evidência foi a verificação de que os monócitos contêm alguns constituintes do SRA, cuja expressão é aumentada durante a diferenciação destes em macrófagos, importante passo no processo de inflamação [63]. Células dendríticas também são moduladas por componentes do SRA, como demonstrado por Nahmod e colaboradores (2003) [68]. Nesse estudo a diferenciação das células dendríticas mostrou-se, pelo menos em parte, dependente dos receptores AT1, uma vez que antagonistas desses receptores (losartan, candesartan ou irbesartan) foram capazes de inibir o processo.

O papel de Ang II como modulador de fatores de transcrição para proteínas inflamatórias também já foi observado, como por exemplo, a estimulação do fator de transcrição pró-inflamatório NF-κB em células mononucleares e monócitos pela Ang II. A inibição do NF-κB resulta em redução da lesão induzida pela Ang II em camundongos transgênicos que superexpressam Ang II [69, 70]. A Ang II regula algumas citocinas inflamatórias, tais como o TNF-α e a IL-1 e a IL-6, que, por sua vez, são capazes de regular a Ang II [71].

Alvarez e colaboradores (2004) [72] verificaram que a exposição à Ang II induzia adesão dos leucócitos nas arteríolas da microcirculação mesentérica por meio de ativação do receptor AT1. Nesse mesmo estudo, foi observado que a Ang II estimulou a expressão de moléculas de adesão de células endoteliais (CAMs), tanto em vênulas quanto em artérias. Outros estudos destacam a participação da Ang II no recrutamento de leucócitos através da formação e liberação de quimiocinas CC (MCP-1/CCL2, MCP-3/CCL7), da ativação de células T(RANTES/CCL5 e MIP-1α/CCL3) e da liberação de quimiocinas CXC (CINC/KC e MIP-2) [64]. Além disso, a Ang II induziu a secreção de IL-8, MCP-1, RANTES e MCP-3 via ativação de receptor AT1 em

culturas de células humanas e aumentou a expressão de receptor CXCR2 nesses tipos celulares [73]. Nabah e colaboradores (2004) [64] demonstraram que o bloqueio do receptor CXCR2 inibiu a síntese e liberação de quimiocinas do tipo CC induzidas por Ang II. Verificaram ainda, que o bloqueio do receptor CXCR2 inibiu o recrutamento de células mononucleares para a cavidade peritoneal, sugerindo o potencial terapêutico do bloqueio tanto de AT1 quanto de CXCR2 para prevenir disfunções, tais como a formação de lesões ateroscleróticas.

Guo e colaboradores (2008) [74], mostraram utilizando culturas de macrófagos que a superexpressão de ECA2 evitou o aumento da expressão de MCP-1 induzida por Ang II. Esses autores sugerem que um dos mecanismos para essa proteção seria a produção de Ang-(1-7), uma vez que o uso de A779, antagonista do receptor Mas, reverteu o efeito protetor da superexpressão da ECA2 em cultura de macrófagos.

A ação pró-inflamatória de componentes do SRA já foi verificada em doenças pulmonares. O estudo de Weber (1997) [75], em fibrose pulmonar, mostrou a participação da Ang II na conversão de fibroblasto em miofibroblasto, com consequente acúmulo de colágeno, a partir de estímulo à expressão de TGFβ. A inibição da ECA ou o bloqueio do receptor AT1 em modelos experimentais de fibrose pulmonar induzida por bleomicina atenuaram a apoptose epitelial, fibrose intersticial e deposição de colágeno [76].

Oudit e colaboradores (2007) [77] mostraram que animais com deleção do gene para ECA2 apresentaram piora da função cardíaca, aumento das citocinas inflamatórias (IL-1, IL-6, MCP-1) e do número de neutrófilos total em um modelo de cardiomiopatia em camundongos. A utilização de antagonista do receptor AT1 nesses animais minimizou os efeitos observados [77].

1.4. Modelo de implantes sintéticos de poliéster-poliuretano

A implantação cirúrgica de matrizes sintéticas no espaço subcutâneo de roedores tem possibilitado o estudo de vários processos associados à inflamação e reparo tecidual. Este modelo de implantação subcutânea de matrizes esponjosas em animais foi descrito inicialmente por Grindlay & Waugh

(1951) [78] e modificado por Andrade e colaboradores, em 1987[79]. Neste estudo, esponjas de poliéster canuladas centralmente com tubos de polietileno eram utilizadas como matriz para o crescimento dos vasos sanguíneos e a avaliação da neovascularização era feita através da técnica do clearance do xenônio radioativo (133Xe) aplicado localmente. Apesar de ter sido desenvolvido inicialmente como um modelo de angiogênese, o modelo de implantes também já foi utilizado para alojar células tumorais para o estudo da angiogênese tumoral [80]. Atualmente, é caracterizado como um modelo de angiogênese inflamatória, inflamação aguda ou crônica e de aderências [81-83].

A matriz implantada induz uma reação inflamatória tipo corpo estranho, com a formação de tecido de granulação rico em células inflamatórias, novos vasos sanguíneos e matriz extracelular, sendo envolvido por uma cápsula fibrosa [78, 84]. A utilização de implantes de esponjas permite o estudo do infiltrado inflamatório, da angiogênese e da deposição da matriz extracelular; permite também a análise bioquímica dos fluidos coletados, bem como o efeito de drogas sobre o processo [84, 85] e investigação de várias características morfofuncionais tanto em condições normais como em patológicas [81].

2. OBJETIVOS

Tendo em vista estudos envolvendo a Ang-(1-7) em modelos inflamatórios e de reparo tecidual tanto *in vitro* quanto *in vivo*, e ainda, os efeitos da angiotensina II sobre a inflamação e fibrose, uma vez que a Ang-(1-7) apresenta, na maioria dos casos estudados, efeitos antagônicos aos da angiotensina II; Este trabalho teve como objetivo o estudo dos efeitos da Ang-(1-7) em modelo de inflamação crônica induzida por implantes sintéticos de poliéster-poliuretano e em modelo de cicatrização de feridas excisionais em camundongos.

REFERÊNCIAS

1. Okin, D. and R. Medzhitov, *Evolution of inflammatory diseases.* Curr Biol, 2012. **22**(17): p. R733-40.
2. Libby, P., *Inflammatory mechanisms: the molecular basis of inflammation and disease.* Nutr Rev, 2007. **65**(12 Pt 2): p. S140-6.
3. Barton, G.M., *A calculated response: control of inflammation by the innate immune system.* J Clin Invest, 2008. **118**(2): p. 413-20.
4. Pober, J.S. and W.C. Sessa, *Evolving functions of endothelial cells in inflammation.* Nat Rev Immunol, 2007. **7**(10): p. 803-15.
5. Burg, N.D. and M.H. Pillinger, *The neutrophil: function and regulation in innate and humoral immunity.* Clin Immunol, 2001. **99**(1): p. 7-17.
6. Charo, I.F. and R.M. Ransohoff, *The many roles of chemokines and chemokine receptors in inflammation.* N Engl J Med, 2006. **354**(6): p. 610-21.
7. Charo, I.F. and M.B. Taubman, *Chemokines in the pathogenesis of vascular disease.* Circ Res, 2004. **95**(9): p. 858-66.
8. de Visser, K.E., A. Eichten, and L.M. Coussens, *Paradoxical roles of the immune system during cancer development.* Nat Rev Cancer, 2006. **6**(1): p. 24-37.
9. Filho, G.B.B., *Patologia*. 7a. ed, ed. 7a. 2006, Rio de Janeiro: Guanabara Koogan.
10. Moore, M.M., et al., *Inflammation and cancer: causes and consequences.* Clin Pharmacol Ther, 2010. **87**(4): p. 504-8.
11. Hasko, G. and P. Pacher, *Regulation of macrophage function by adenosine.* Arterioscler Thromb Vasc Biol, 2012. **32**(4): p. 865-9.
12. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
13. Ortega-Gomez, A., M. Perretti, and O. Soehnlein, *Resolution of inflammation: an integrated view.* EMBO Mol Med, 2013. **5**(5): p. 661-74.
14. Schultz, G.S. and A. Wysocki, *Interactions between extracellular matrix and growth factors in wound healing.* Wound Repair Regen, 2009. **17**(2): p. 153-62.
15. Medzhitov, R., *Inflammation 2010: new adventures of an old flame.* Cell. **140**(6): p. 771-6.
16. Szekanecz, Z. and A.E. Koch, *Vascular endothelium and immune responses: implications for inflammation and angiogenesis.* Rheum Dis Clin North Am, 2004. **30**(1): p. 97-114.
17. Ferguson, M.E., et al., *Results of intravascular stent placement for fibrosing mediastinitis.* Congenit Heart Dis, 2010. **5**(2): p. 124-33.
18. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease.* Nature, 2013. **496**(7446): p. 445-55.
19. Hotamisligil, G.S., *Inflammation and metabolic disorders.* Nature, 2006. **444**(7121): p. 860-7.
20. Tracey, K.J., *The inflammatory reflex.* Nature, 2002. **420**(6917): p. 853-9.
21. Mrowietz, U. and W.H. Boehncke, *Leukocyte adhesion: a suitable target for anti-inflammatory drugs.* Curr Pharm Des, 2006. **12**(22): p. 2825-31.

22. van Zuijlen, P.P., et al., *Scar assessment tools: implications for current research.* Plast Reconstr Surg, 2002. **109**(3): p. 1108-22.
23. Balbino, C.A.P., L.M.; Curi, R., *Mecanismos envolvidos na cicatrização: uma revisão.* Rev Bras Ciências Farmacêuticas, 2005. **41**(1): p. 27-51.
24. Lazarus, G.S., et al., *Definitions and guidelines for assessment of wounds and evaluation of healing.* Arch Dermatol, 1994. **130**(4): p. 489-93.
25. Diegelmann, R.F. and M.C. Evans, *Wound healing: an overview of acute, fibrotic and delayed healing.* Front Biosci, 2004. **9**: p. 283-9.
26. Roberts, A.B. and M.B. Sporn, *Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta).* Growth Factors, 1993. **8**(1): p. 1-9.
27. Hall, M.C., et al., *The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1.* J Biol Chem, 2003. **278**(12): p. 10304-13.
28. Vorstenbosch, J., et al., *Transgenic mice overexpressing CD109 in the epidermis display decreased inflammation and granulation tissue and improved collagen architecture during wound healing.* Wound Repair Regen, 2013. **21**(2): p. 235-46.
29. Hinz, B., *Formation and function of the myofibroblast during tissue repair.* J Invest Dermatol, 2007. **127**(3): p. 526-37.
30. Hendricks, T., et al., *Inhibition of basal and TGF beta-induced fibroblast collagen synthesis by antineoplastic agents. Implications for wound healing.* Br J Cancer, 1993. **67**(3): p. 545-50.
31. Duffield, J.S., *The inflammatory macrophage: a story of Jekyll and Hyde.* Clin Sci (Lond), 2003. **104**(1): p. 27-38.
32. Hunt, T.K., et al., *Studies on inflammation and wound healing: angiogenesis and collagen synthesis stimulated in vivo by resident and activated wound macrophages.* Surgery, 1984. **96**(1): p. 48-54.
33. Tonnesen, M.G., X. Feng, and R.A. Clark, *Angiogenesis in wound healing.* J Investig Dermatol Symp Proc, 2000. **5**(1): p. 40-6.
34. Nissen, N.N., et al., *Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing.* Am J Pathol, 1998. **152**(6): p. 1445-52.
35. Shenoy, V., et al., *The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension.* Am J Respir Crit Care Med, 1997. **182**(8): p. 1065-72.
36. Eming, S.A., et al., *Regulation of angiogenesis: wound healing as a model.* Prog Histochem Cytochem, 2007. **42**(3): p. 115-70.
37. Stramer, B.M., R. Mori, and P. Martin, *The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair.* J Invest Dermatol, 2007. **127**(5): p. 1009-17.
38. Dovi, J.V., L.K. He, and L.A. DiPietro, *Accelerated wound closure in neutrophil-depleted mice.* J Leukoc Biol, 2003. **73**(4): p. 448-55.
39. Pierce, G.F., *Inflammation in nonhealing diabetic wounds: the space-time continuum does matter.* Am J Pathol, 2001. **159**(2): p. 399-403.

40. Costa, C., J. Incio, and R. Soares, *Angiogenesis and chronic inflammation: cause or consequence?* Angiogenesis, 2007. **10**(3): p. 149-66.
41. Gurtner, G.C., et al., *Wound repair and regeneration.* Nature, 2008. **453**(7193): p. 314-21.
42. Shaw, T.J. and P. Martin, *Wound repair at a glance.* J Cell Sci, 2009. **122**(Pt 18): p. 3209-13.
43. Hatanaka, E.C., R., *Ácidos graxos e cicatrização: uma revisão. .* Rev Bras Farmacologia, 2007. **88**(2): p. 53-58.
44. Shimizu, N., et al., *Development of a functional wound dressing composed of hyaluronic acid spongy sheet containing bioactive components: evaluation of wound healing potential in animal tests.* J Biomater Sci Polym Ed, 2014: p. 1-14.
45. Howdieshell, T.R., et al., *Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation.* J Surg Res, 2001. **96**(2): p. 173-82.
46. Montesano, R. and L. Orci, *Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing.* Proc Natl Acad Sci U S A, 1988. **85**(13): p. 4894-7.
47. Beanes, S.R., et al., *Skin repair and scar formation: the central role of TGF-beta.* Expert Rev Mol Med, 2003. **5**(8): p. 1-22.
48. Clark, R.A., *Biology of dermal wound repair.* Dermatol Clin, 1993. **11**(4): p. 647-66.
49. Johnston, D.E., *Wound healing in skin.* Vet Clin North Am Small Anim Pract, 1990. **20**(1): p. 1-25.
50. Ackerly, J.A., A.F. Moore, and M.J. Peach, *Demonstration of different contractile mechanisms for angiotensin II and des-Asp1-angiotensin II in rabbit aortic strips.* Proc Natl Acad Sci U S A, 1977. **74**(12): p. 5725-8.
51. Santos, R.A., A.J. Ferreira, and E.S.A.C. Simoes, *Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis.* Exp Physiol, 2008. **93**(5): p. 519-27.
52. Carey, R.M., *Angiotensin type-2 receptors and cardiovascular function: are angiotensin type-2 receptors protective?* Curr Opin Cardiol, 2005. **20**(4): p. 264-9.
53. Ferrario, C.M., et al., *Counterregulatory actions of angiotensin-(1-7).* Hypertension, 1997. **30**(3 Pt 2): p. 535-41.
54. Greene, L.J., et al., *Brain endo-oligopeptidase B: a post-proline cleaving enzyme that inactivates angiotensin I and II.* Hypertension, 1982. **4**(2): p. 178-84.
55. Schiavone, M.T., et al., *Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide.* Proc Natl Acad Sci U S A, 1988. **85**(11): p. 4095-8.
56. Santos, R.A., et al., *Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8258-63.
57. Donoghue, M., et al., *A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9.* Circ Res, 2000. **87**(5): p. E1-9.

58. Tallant, E.A., D.I. Diz, and C.M. Ferrario, *State-of-the-Art lecture. Antiproliferative actions of angiotensin-(1-7) in vascular smooth muscle.* Hypertension, 1999. **34**(4 Pt 2): p. 950-7.
59. Pan, C.H., C.H. Wen, and C.S. Lin, *Interplay of angiotensin II and angiotensin(1-7) in the regulation of matrix metalloproteinases of human cardiocytes.* Exp Physiol, 2008. **93**(5): p. 599-612.
60. Esteban, V., et al., *Angiotensin-(1-7) and the g protein-coupled receptor MAS are key players in renal inflammation.* PLoS One, 2009. **4**(4): p. e5406.
61. Cook, K.L., et al., *Angiotensin-(1-7) reduces fibrosis in orthotopic breast tumors.* Cancer Res, 2010. **70**(21): p. 8319-28.
62. Shenoy, V., et al., *The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension.* Am J Respir Crit Care Med, 2010. **182**(8): p. 1065-72.
63. Nabah, Y.N., et al., *Angiotensin II induces neutrophil accumulation in vivo through generation and release of CXC chemokines.* Circulation, 2004. **110**(23): p. 3581-6.
64. Abu Nabah, Y.N., et al., *CXCR2 blockade impairs angiotensin II-induced CC chemokine synthesis and mononuclear leukocyte infiltration.* Arterioscler Thromb Vasc Biol, 2007. **27**(11): p. 2370-6.
65. Hagiwara, S., et al., *Effects of an angiotensin-converting enzyme inhibitor on the inflammatory response in in vivo and in vitro models.* Crit Care Med, 2009. **37**(2): p. 626-33.
66. Silver, R.B., et al., *Mast cells: a unique source of renin.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13607-12.
67. da Silveira, K.D., et al., *Anti-inflammatory effects of the activation of the angiotensin-(1-7) receptor, MAS, in experimental models of arthritis.* J Immunol, 2010. **185**(9): p. 5569-76.
68. Nahmod, K.A., et al., *Control of dendritic cell differentiation by angiotensin II.* FASEB J, 2003. **17**(3): p. 491-3.
69. Theuer, J., et al., *Angiotensin II induced inflammation in the kidney and in the heart of double transgenic rats.* BMC Cardiovasc Disord, 2002. **2**: p. 3.
70. Chiba, T. and K. Umegaki, *Pivotal roles of monocytes/macrophages in stroke.* Mediators Inflamm, 2013. **2013**: p. 759103.
71. Wassmann, S., et al., *Interleukin-6 induces oxidative stress and endothelial dysfunction by overexpression of the angiotensin II type 1 receptor.* Circ Res, 2004. **94**(4): p. 534-41.
72. Alvarez, A., et al., *Direct evidence of leukocyte adhesion in arterioles by angiotensin II.* Blood, 2004. **104**(2): p. 402-8.
73. Jacobi, J., et al., *Exogenous superoxide mediates pro-oxidative, proinflammatory, and procoagulatory changes in primary endothelial cell cultures.* Free Radic Biol Med, 2005. **39**(9): p. 1238-48.
74. Guo, Y.J., et al., *ACE2 overexpression inhibits angiotensin II-induced monocyte chemoattractant protein-1 expression in macrophages.* Arch Med Res, 2008. **39**(2): p. 149-54.
75. Weber, K.T., *Fibrosis, a common pathway to organ failure: angiotensin II and tissue repair.* Semin Nephrol, 1997. **17**(5): p. 467-91.

76. Li, J., Y.P. Zhang, and R.S. Kirsner, *Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix.* Microsc Res Tech, 2003. **60**(1): p. 107-14.
77. Oudit, G.Y., et al., *Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice.* Cardiovasc Res, 2007. **75**(1): p. 29-39.
78. Grindlay, J.H. and J.M. Waugh, *Plastic sponge which acts as a framework for living tissue; experimental studies and preliminary report of use to reinforce abdominal aneurysms.* AMA Arch Surg, 1951. **63**(3): p. 288-97.
79. Andrade, S.P., T.P. Fan, and G.P. Lewis, *Quantitative in-vivo studies on angiogenesis in a rat sponge model.* Br J Exp Pathol, 1987. **68**(6): p. 755-66.
80. Andrade, S.P., et al., *Effects of platelet activating factor (PAF) and other vasoconstrictors on a model of angiogenesis in the mouse.* Int J Exp Pathol, 1992. **73**(4): p. 503-13.
81. Araújo, F.A., *Avaliação dos efeitos de estatinas na angiogênese inflamatória em camundongos*, in *Department of Physiology and Biophysics*. 2009, Universidade Federal de Minas Gerais: Belo Horizonte. p. 153.
82. Araujo, F.A., et al., *Atorvastatin inhibits inflammatory angiogenesis in mice through down regulation of VEGF, TNF-alpha and TGF-beta1.* Biomed Pharmacother, 2010. **64**(1): p. 29-34.
83. Guabiraba, R., et al., *Blockade of cannabinoid receptors reduces inflammation, leukocyte accumulation and neovascularization in a model of sponge-induced inflammatory angiogenesis.* Inflamm Res, 2013. **62**(8): p. 811-21.
84. Mendes, J.B., et al., *Host response to sponge implants differs between subcutaneous and intraperitoneal sites in mice.* J Biomed Mater Res B Appl Biomater, 2007. **83**(2): p. 408-15.
85. Andrade, S.P., et al., *Sponge-induced angiogenesis in mice and the pharmacological reactivity of the neovasculature quantitated by a fluorimetric method.* Microvasc Res, 1997. **54**(3): p. 253-61.
86. Meng, W., et al., *Autocrine and Paracrine Function of Angiotensin 1-7 in Tissue Repair During Hypertension.* Am J Hypertens, 2014.
87. Simoes e Silva, A.C., et al., *ACE2, angiotensin-(1-7) and Mas receptor axis in inflammation and fibrosis.* Br J Pharmacol, 2013. **169**(3): p. 477-92.
88. Machado, R.D., R.A. Santos, and S.P. Andrade, *Opposing actions of angiotensins on angiogenesis.* Life Sci, 2000. **66**(1): p. 67-76.
89. Machado, R.D., R.A. Santos, and S.P. Andrade, *Mechanisms of angiotensin-(1-7)-induced inhibition of angiogenesis.* Am J Physiol Regul Integr Comp Physiol, 2001. **280**(4): p. R994-R1000.
90. Phillips, R.J., et al., *Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis.* J Clin Invest, 2004. **114**(3): p. 438-46.
91. Campos, P.P., Y.S. Bakhle, and S.P. Andrade, *Mechanisms of wound healing responses in lupus-prone New Zealand White mouse strain.* Wound Repair Regen, 2008. **16**(3): p. 416-24.

92. Araujo, F.A., et al., *Implant-induced intraperitoneal inflammatory angiogenesis is attenuated by fluvastatin.* Clin Exp Pharmacol Physiol, 2011. **38**(4): p. 262-8.
93. Barcelos, L.S., et al., *Production and in vivo effects of chemokines CXCL1-3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice.* Inflamm Res, 2004. **53**(10): p. 576-84.
94. Puchtler, H., et al., *Methacarn (methanol-Carnoy) fixation. Practical and theoretical considerations.* Histochemie, 1970. **21**(2): p. 97-116.
95. Leite, S.N., et al., *Experimental models of malnutrition and its effect on skin trophism.* An Bras Dermatol, 2011. **86**(4): p. 681-8.
96. Pereira, N.B., et al., *Apoptosis, mast cell degranulation and collagen breakdown in the pathogenesis of loxoscelism in subcutaneously implanted sponges.* Toxicon, 2014.
97. Puchtler, H., F.S. Waldrop, and L.S. Valentine, *Polarization microscopic studies of connective tissue stained with picro-sirius red FBA.* Beitr Pathol, 1973. **150**(2): p. 174-87.
98. Junqueira, L.C., G. Bignolas, and R.R. Brentani, *Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections.* Histochem J, 1979. **11**(4): p. 447-55.
99. Deconte, S.R., et al., *Alterations of antioxidant biomarkers and type I collagen deposition in the parotid gland of streptozotocin-induced diabetic rats.* Arch Oral Biol, 2011. **56**(8): p. 744-51.
100. Deryugina, E.I. and J.P. Quigley, *Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: contrasting, overlapping and compensatory functions.* Biochim Biophys Acta, 2010. **1803**(1): p. 103-20.
101. Moura, S.A., et al., *Local drug delivery system: inhibition of inflammatory angiogenesis in a murine sponge model by dexamethasone-loaded polyurethane implants.* J Pharm Sci, 2011. **100**(7): p. 2886-95.
102. Belo, A.V., et al., *Murine chemokine CXCL2/KC is a surrogate marker for angiogenic activity in the inflammatory granulation tissue.* Microcirculation, 2005. **12**(7): p. 597-606.
103. Ferreira, M.A., et al., *Tumor growth, angiogenesis and inflammation in mice lacking receptors for platelet activating factor (PAF).* Life Sci, 2007. **81**(3): p. 210-7.
104. Barcelos, L.S., et al., *Role of the chemokines CCL3/MIP-1 alpha and CCL5/RANTES in sponge-induced inflammatory angiogenesis in mice.* Microvasc Res, 2009. **78**(2): p. 148-54.
105. Saraswati, S. and S.S. Agarwal, *Strychnine inhibits inflammatory angiogenesis in mice via down regulation of VEGF, TNF-alpha and TGF-beta.* Microvasc Res, 2013. **87**: p. 7-13.
106. Machado, R.D., et al., *Vasodilator effect of angiotensin-(1-7) in mature and sponge-induced neovasculature.* Regul Pept, 2002. **107**(1-3): p. 105-13.
107. Mirabelli, P., et al., *Early effects of dexamethasone and anti-VEGF therapy in an inflammatory corneal neovascularization model.* Exp Eye Res, 2014. **125C**: p. 118-127.
108. Araujo, F.A., et al., *3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (fluvastatin) decreases inflammatory angiogenesis in mice.* APMIS, 2012. **121**(5): p. 422-30.

109. Teixeira, A.S. and S.P. Andrade, *Glucose-induced inhibition of angiogenesis in the rat sponge granuloma is prevented by aminoguanidine.* Life Sci, 1999. **64**(8): p. 655-62.
110. Benter, I.F., D.I. Diz, and C.M. Ferrario, *Cardiovascular actions of angiotensin(1-7).* Peptides, 1993. **14**(4): p. 679-84.
111. Fontes, M.A., et al., *Evidence that angiotensin-(1-7) plays a role in the central control of blood pressure at the ventro-lateral medulla acting through specific receptors.* Brain Res, 1994. **665**(1): p. 175-80.
112. Santos, R.A., et al., *Characterization of a new angiotensin antagonist selective for angiotensin-(1-7): evidence that the actions of angiotensin-(1-7) are mediated by specific angiotensin receptors.* Brain Res Bull, 1994. **35**(4): p. 293-8.
113. Paula, R.D., et al., *Angiotensin-(1-7) potentiates the hypotensive effect of bradykinin in conscious rats.* Hypertension, 1995. **26**(6 Pt 2): p. 1154-9.
114. Marshall, J.S., *Mast-cell responses to pathogens.* Nat Rev Immunol, 2004. **4**(10): p. 787-99.
115. Jung, M., et al., *Mast cells produce novel shorter forms of perlecan that contain functional endorepellin: a role in angiogenesis and wound healing.* J Biol Chem, 2013. **288**(5): p. 3289-304.
116. Lu, D.Y., et al., *Mast cell leukemia: An extremely rare disease.* J Chin Med Assoc, 2014.
117. Arizmendi, N., et al., *Rac2 is involved in bleomycin-induced lung inflammation leading to pulmonary fibrosis.* Respir Res, 2014. **15**: p. 71.
118. Ammendola, M., et al., *Mast cells density positive to tryptase correlates with angiogenesis in pancreatic ductal adenocarcinoma patients having undergone surgery.* Gastroenterol Res Pract, 2014. **2014**: p. 951957.
119. Lu, J., et al., *Tryptase inhibitor APC 366 prevents hepatic fibrosis by inhibiting collagen synthesis induced by tryptase/protease-activated receptor 2 interactions in hepatic stellate cells.* Int Immunopharmacol, 2014. **20**(2): p. 352-7.
120. Marcus, Y., et al., *Angiotensin 1-7 as means to prevent the metabolic syndrome: lessons from the fructose-fed rat model.* Diabetes, 2013. **62**(4): p. 1121-30.
121. El-Hashim, A.Z., et al., *Angiotensin-(1-7) inhibits allergic inflammation, via the MAS1 receptor, through suppression of ERK1/2- and NF-kappaB-dependent pathways.* Br J Pharmacol, 2012. **166**(6): p. 1964-76.
122. Feltenberger, J.D., et al., *Oral formulation of angiotensin-(1-7) improves lipid metabolism and prevents high-fat diet-induced hepatic steatosis and inflammation in mice.* Hypertension, 2013. **62**(2): p. 324-30.

CAPÍTULO 2: MANUSCRITO 1

Title:

DUAL EFFECTS OF ANG-(1-7) ON THE DEVELOPMENT OF THE FIBROVASCULAR TISSUE INDUCED BY IMPLANTATION OF SYNTHETIC MATRIX IMPLANTATION IN MICE

Authors:

Simone Ramos Deconte[1,2,4], Puebla Cassini Vieira[2,3],Robson Augusto Souza Santos[3,4], Tatiana Carla Tomiosso[2],Fábio de Oliveira[1,2,4], Lucíola da Silva Barcelos[3,4], Silvia Passos Andrade[3,4], Fernanda de Assis Araújo[2,4].

Affiliation:

[1]Institute Genetics and Biochemistry (INGEB),[2]Institute of Biomedical Sciences (ICBIM), Federal University of Uberlândia (UFU), Uberlândia - MG, Brazil.[3]Department of Physiology and Biophysics, Federal University of Minas Gerais (UFU), Belo Horizonte - MG, Brazil.[4]National Institute in Science and Technology in Nanobiopharmaceutics (NanoBiofar), Belo Horizonte -MG, Brazil.

Corresponding author:

Fernanda de Assis Araújo

E-mail address: folaraujo@gmail.com

Current address:

Instituto de Ciências Biomédicas (ICBIM) – Área de Ciências Fisiológicas (ARFIS), Universidade Federal de Uberlândia (UFU).

Av. Pará 1720 - Bloco 2A, piso superior – sala 120,

CEP 38400-902,

Uberlândia-MG, Brazil.

Tel.: +55 34 3218-2200

Resumo

Angiotensina-(1-7) (Ang- (1-7)), é um heptapeptídeo bioativo com propriedades antiangiogênicas, antifibróticas e antiproliferativas. No entanto, os efeitos deste peptídeo no desenvolvimento de tecido fibroproliferativo induzido por matriz de esponja sintética ainda não foram completamente caracterizados. *Metodologia:* camundongos machos *swiss* (7-8 semanas) receberam como implantes discos de esponja de poliéster-poliuretano para indução de tecido fibrovascular. O tratamento com Ang-(1-7) (30 ng) foi realizado por administração diária intraimplante. Para determinação da angiogênese foi avaliado o conteúdo de hemoglobina (Hb), número de vasos sanguíneos e níveis de citocinas pró-angiogênicas (VEGF e bFGF). Para os fatores inflamatórios foram dosadas mieloperoxidase-MPO, N-acetil -β-D-glucosaminidase-NAG, níveis de citocinas pró-inflamatórias -TNF-α, CXCL1 / KC e CCL2). A fibrogênese foi analisada de acordo com a deposição de colágeno e níveis de TGFβ-1. Todas as análises foram analisadas a partir dos implantes coletados nos dias 1, 4, 7, 9 e 14 pós-implantação. *Resultados:* Ang- (1-7) exerceu duplo efeito no desenvolvimento da neovascularização dos implantes de esponja, avaliado pela medição do conteúdo de Hb e do número de vasos. Nos dias 4 e 7 o peptídeo inibiu os aumentos desses parâmetros quando comparados com o grupo não tratado. Do dia 9 em diante, o péptideo aumentou os níveis de Hb (42%), número de vaso (46%), FGF (24%) e nível de VEGF (29%) em relação ao grupo controle. Esse padrão também foi observado para o marcador inflamatório e a produção de citoquinas. O tratamento com a Ang- (1- 7) foi capaz de diminuir CXCL1 (48%), TNF-α (25%), NAG (58%), CC L2 (32%) do dia 1 e MPO (27%) a partir do dia 3 em relação ao grupo controle. Este padrão mudou em relação ao reparo do tecido em implantes de esponja. O colágeno solúvel total (105%), o teor de colágeno (37%) e o TGF-β1 (50%) aumentaram após 7 dias de tratamento com Ang- (1-7). Durante esses 14 dias, processos fibróticos e de remodelação tecidual foram envolvidos, como mostrado pelo aumento de mastócitos em relação ao grupo controle (32%) ($p < 0,05$). *Conclusão:* após 14 dias de tratamento, o efeito inibitório da Ang- (1-7) na atenuação da inflamação e promoção da angiogênese e componentes fibrogênicos do tecido fibrovascular induzido pela matriz sintética se estende ao alcance das ações da Ang- (1-7) podendo indicar potencial terapêutico no controle de doenças.

Palavras-chave: Angiotensina- (1-7), angiogênese, inflamação, fibrose, implantes, esponja

Abstract

Background: (Ang-(1-7)), a bioactive heptapeptide formed from the fragmentation of angiotensin I and acts as an antagonist of angiotensin II and carries antiangiogenic, antifibrotic, antiproliferative properties. However, the effects of this peptide on the development of fibroproliferative tissue induced by synthetic sponge matrix has not been fully characterized. *Methods:* Male Swiss mice (7–8 weeks) were implanted subcutaneously with polyether-polyurethane sponge discs for induction of the fibrovascular tissue. Ang-(1-7) treatment (30ng) was carried out by intraimplant daily administration. Implants collected 1, 4, 7, 9 and 14 days postimplantation were analyzed for determination of angiogenesis (hemoglobin (Hb), blood vessels number and levels of pro-angiogenic cytokines (VEGF and bFGF), inflammation (myeloperoxidase- MPO and N-acetyl-β-D-glucosaminidase-NAG activities and levels of pro-inflammatory cytokines -TNF-α, CXCL1/KC and CCL2) and fibrogenesis (collagen deposition and TGFβ-1 levels). *Results*: Ang-(1-7) exerted dual effects of the development of neovascularization in the sponge implants as assessed by measuring Hb content and number of vessels. At days 4 and 7 the peptide inhibited increases of these parameters when compared with the non-treated group. From day 9 onwards, the peptide increased hemoglobin (42%), vessel number (46%), FGF (24%) and VEGF level (29%) relative to the control group. This pattern was also observed for the inflammatory marker and cytokine production. The Ang-(1-7) treatment was able to decreased CXCL1 (48%), TNF-α (25%), NAG (58%), CCL2 (32%) from day 1 and MPO (27%) from day 3 relative to the control group. This pattern changed relative to tissue repair in sponge implants. Total soluble collagen (105%), collagen content (37%) and TGF-β1(50%) were increased after 7 days of treatment with Ang-(1-7). During these 14 days there was involvement of processes of fibrosis and tissue remodeling, as shown by mast cells number that were greater than control group (32%)($p<0.05$).*Conclusion:* After 14 days of treatment the inhibitory effect of Ang-(1-7) to attenuate inflammation and promote angiogenesis and fibrogenic components of the fibrovascular tissue induced by the synthetic matrix extends the range of actions of the Ang-(1-7) and may indicate its therapeutic potential in controlling diseases.

Keywords: Angiotensin-(1-7), angiogenesis, inflammation, fibrosis, implants, sponge disc

Introduction

The renin-angiotensin system (RAS) plays an integral role in maintaining vascular tone, salt and water homeostasis, and cardiac function in humans [86]. Chronic activation of RAS causes hypertension, accompanied by cardiac, renal, and vascular injury/remodeling that eventually leads to failures, such as renal, heart failure, and coronary disease [86]. Recent advances have improved our understanding of the renin-angiotensin system [87]. Angiotensin-(1-7) (Ang-(1-7)), a bioactive heptapeptide formed from the fragmentation of angiotensin I by endopeptidases or as a final product of the conversion of angiotensin II by the angiotensin II converting enzyme (ACE II) has primarily been identified as a molecule that exerts counter-regulatory actions of angiotensin-II in the homeostasis of the cardio-vascular system [2]. Most available evidence supports a counter-regulatory role for Ang-(1-7) by opposing many actions of Ang II on AT1 receptors, especially vasoconstriction and proliferation .In fact, previously, a series of studies showed an anti-angiogenic effect of this peptide in the mouse sponge model [88, 89]. More recently, studies have shown that Ang-(1-7) acting via Mas receptor exerts inhibitory effects on inflammation and on vascular and cellular growth mechanisms. Ang-(1-7) has also been shown to reduce key signaling pathways and molecules thought to be relevant for modifying processes associated with acute and chronic inflammation, including leukocyte influx, fibrogenesis and proliferation of cell types [63].However, simultaneous assessment of this effects on the development of the components of fibroproliferative diseases *in vivo* (angiogenesis, inflammation and fibrogenesis) has not been investigated. Accordingly, we applied a model of subcutaneous fibroproliferation induced by implantation of a synthetic matrix to test our hypothesis that the Ang-(1-7) would modulate this process *in vivo*. In this chronic inflammation model, the acellular and avascular synthetic matrix, implanted subcutaneously in the animals' dorsa, induces the migration, proliferation, and activation of various cell types responsible for the development of a fibrovascular tissue that underlies chronic pathological conditions [90, 91]. We report here that Ang-(1-7) was able to attenuate inflammatory response and increase angiogenesis and collagen deposition *in vivo*, which reveals a potential therapeutic function in which chronic inflammation and repair are changed.

Material and methods

The present study was approved by the Ethics Committee in Animal Use (CEUA) of Federal University of Uberlândia (UFU) (approval number 092/12).

Animals

Male Swiss mice 7–8 weeks old (30–35 g body weight) used in these experiments were provided by the animal facility of Pentapharm do Brasil Com. e Exp. (Uberlândia-MG, Brazil). The animals were housed individually and provided with chow pellets, water *ad libitum* and 12h light/dark cycles. Efforts were made to avoid all unnecessary distress to the animals. Housing, anesthesia and postoperative care concurred with the guidelines established by our local Institutional Animal Welfare Committee.

Preparation of sponge discs and implantation

Polyether-polyurethane sponge (Vitafoam Ltd., Manchester,UK) was used as the implanted material. The implants were discs, 5-mm thick x 8-mm diameter and were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 minutes before implantation. For that, the animals were anaesthetized by an intraperitoneal injection of ketamine and xylazine (60mg/kg and 8mg/Kg, respectively), the dorsal hair shaved and the skin wiped with 70% ethanol. The sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. Postoperatively, the animals were monitored for any signs of infection at the operative site, discomfort or distress; any animal showing such signs were immediately humanely killed.

Experimental procedure

Our protocol was made in three phases. For phase I (dose-response curve), animals were divided in 4 groups (n=10, each):Three treatment groups: ANG-(1-7)-10ng, 30ng, 55ng and 100ng dose and saline Control (CT group). ANG-(1-7) and vehicle saline were given by intraimplant injection (10uL, each).The doses of the compound and the treatment regimen were chosen based on pilot experiments and data from the literature [89]. Chosen doses were based on logarithmic calculations. Machado et al., 2001[89], to evaluate angiogenesis used Ang-(1-7)-20ng for 7 days of treatment. Our doses were chosen based on the logarithms of 10, 30, 55 and 100 in base 10 to have

a similar distance of values. Treatment started just after implant surgery and lasted for 9 days. For phase II (kinetics of inflammatory response) the selected dose of ANG-(1-7) at phase I was tested at different times after surgery. Animals were divided in 2 groups (n=10, each): ANG-(1-7)-30ngand saline control (CT) group. Treatment started just after implant surgery and lasted for 1, 4, 7, 9 and 14 days. For phase III (histological analysis), animals were divided in 2 groups (n=6, each): ANG-(1-7)-30ng and CT group. Protocol was made according to the same schedule of phase II and lasted for 1, 4, 7, 9 and 14 days. Treatment and sponge implants were well tolerated by the mice over the experimental period.

Hemoglobin extraction (indirect assessment of neovascularization)

The extent of the vascularization of the sponge implants was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin's method [92]. Animals were euthanized and the sponge implants carefully removed, dissected from adherent tissue, weighed. Each implant was homogenized (Ultra Stirrer) in 2 ml of Drabkin reagent (Labtest, Lagoa Santa Brazil), and centrifuged at 10,000 g for 40 min. The supernatants were filtered through a 0.22-mm millipore filter. Hb concentration of the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and was compared against a standard curve of Hb. The content of Hb in the implant was expressed as mg Hb per mg wet tissue (implant).

Tissue extraction and determination of myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG enzyme activities

The extent of neutrophil accumulation in the implants was measured by assaying myeloperoxidase enzyme (MPO) activity as previously described [92, 93]. After processing the supernatant of the implants for the Hb determination, a part of the corresponding pellet was weighed, homogenized in (2 ml) pH 4.7 buffer (0.1 M NaCl, 0.02 M NaH_2PO4, 0.015M Na-EDTA) and centrifuged at 15,300g for 15 min. The pellets were then resuspended in 0.05 M Na2HPO4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by the addition of 50 µl of H_2SO_4 (4 M). Results were expressed as change in OD per gram of wet tissue. The infiltration of mononuclear cells into the implants

was quantified by measuring the levels of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) which is present in high levels in activated macrophages [92, 93]. Part of the pellet that remained after the Hb measurement was kept for this assay. These pellets were weighed, homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100, and centrifuged (960 g; 10 min at 4°C). Samples of the resulting supernatant (100 µl) were incubated for 10 min with 100µl of P-nitrophenyl-N-acetyl-beta-D-glucosaminide prepared in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na2HPO4; pH 4.5) to yield a final concentration of 2.24 mM. The reaction was stopped by the addition of 100 µl of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. The readings were interpolated on a standard curve constructed with p-nitrophenol (0–500 nmol ml^{-1}). Data are reported as nmol of products formed per milligram of wet tissue.

Collagen measurement

Total soluble collagen was measured in whole tissue homogenates by the Sirius Red reagent based-assay [90, 91]. The implants were homogenized in 1 ml of PBS and 50 µl of sample were mixed with 50 µl of Sirius Red reagent. Samples were mixed by gentle inversion. The collagen-dye complex was precipitated by centrifugation at 6,000 g for 10 min. The supernatants were drained off and discarded and the pellet washed with 500 µl of ethanol (99% pure and methanol free). One milliliter of a 0.5 M NaOH solution was added to the remaining pellet of collagen-bound dye. After solubilization, samples were transferred to a 96-well plate and read at 540 nm. The calibration curve was setup on the basis of a gelatin standard. The results are expressed as microgram collagen per milligram of wet tissue.

Measurement of cytokines/chemokines production in the sponge implants

The supernatants from centrifugation of sponge homogenates (see Hb measurement method) were used to examine the levels of VEGF, TNF- a, TGF-β1, FGF, CXCL1/KC, CCL2/JE produced in sponge implants by enzyme-linked immunosorbent assay (ELISA). The assays were performed using Kits from R&D systems and according to the manufacturer's instructions. The threshold of sensitivity for each cytokine/chemokine was 7.5 pg/ml. The results were expressed as pico cytokine per milligram of wet tissue.

Histological analysis

At each time interval in phase III, implants from both groups (ANG-(1-7)-30ng and CT) were methacarn-fixed [94] paraffin-embedded tissue samples and sections (6-μm thick) were stained with Gomori´s Trichrome [95] for evaluation of inflammatory infiltrate, formation of connective and vascular, Toluidine blue [96] for mast cell quantification and Picrosirius Red [97-99] to assess collagen deposition by histological and morphometrical analyses. Morphometric analysis was carried out using a light microscope (Olympus Ltd., Hertfordshire, UK) with a 40x objective (Gomori´s Trichrome e Toluidine blue sections) and 10x objective (Picrosirius red sections)The microscope was equipped with an Oly-200 CCD camera linked to a PC and a capture and image analysis system (HL-Image 97, Western Vision Software, UT, USA). Ten random fields were selected, and the percentage of blood vessels, mast cells and collagen pixels were measured per field area.

Statistics

Statistical analysis was performed using Statistica software (99ed.,StatSoftInc.) and graphs were performed with GraphPad Prism program, version 5. All values were expressed as mean ± S.E.M. At phase I, the results of dose-response curve were analyzed by one-way analysis of variance (ANOVA). In cases where ANOVAs howeds ignificant differences ($p < 0.05$), the Dunnett´s *post hoc* test was performed. In phases II and III, results of kinetics of inflammatory response and histological analysis were analyzed by two-way ANOVA considering factors treatment, time and interaction of this factors. In cases where ANOVA showed significant differences ($p<0.05$), it was performed planned comparisons between groups of interest.

Results

Dose-response curve

After nine days of treatment, neovascularization of the implants, tissue repair, macrophage and neutrophil recruitment were increased, as detected by changes in the Hb content, collagen deposition, *N*-acetyl-β-D-glucosaminidase activity and myeloperoxidase activity. Hb content (figure 1-a) was different (increased by 48%) between control group and ANG-(1-7)-30ng ($p<0.05$). Collagen deposition (figure 1-b) was different (increased by 69%) between control group and ANG-(1-7)-30ng ($p<0.05$). At the same dose, treatment with ANG-(1-7)-30ng was able to decrease neutrophil

content (figure 1-c) by 45% as showed by myeloperoxidase enzyme activity, when compared to control group (p<0.05).NAG activity (figure 1-d) was different (decreased by 25%) between control group and ANG-(1-7)-30ng (p<0.05).

Kinetics of inflammatory response

The extent of vascularization of the sponge was assessed by measuring Hb content. This content (figure 2-a) was different between groups. The two-way ANOVA showed significant differences for time factor ($F_{4,56}$ = 55.05, p<0.05) and the interaction between treatment factor x time factor ($F_{4,56}$ = 4.48, p<0.05). Hb content was different between control group and ANG-(1-7)-30ng group on days 4 (decreased by 52%), 7 (decreased by 33%), 9 (increased by 42%) and 14 (increased by 38%) (p< 0.05). No significant differences were found on day 1.

VEGF level (figure 2-b) was also different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,16}$ = 6.97, p<0.05) and time factor ($F_{4,64}$ = 11.22, p<0.05). VEGF level was different between control group and ANG-(1-7)-30ng group on days 7 (increased by 26%) and 9 (increased by 29%) (p< 0.05). No significant differences were found on days 1, 4 and 14.

FGF level (figure 2-c) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,17}$ = 6.86, p< 0.05), time factor ($F_{4,68}$ = 49.09, p< 0.05) and the interaction between treatment factor x time factor ($F_{4,68}$ = 2.53, p< 0.05). FGF level was different between control group and ANG-(1-7)-30ng group on days 7 (increased by 32%) and 9 (increased by 24%) (p< 0.05). No significant differences were found on days 1, 4 and 14.

Blood vessels (figure 2-d) content was quantified by Gomori´s Trichrome staining and showed difference between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,8}$ = 6.45, p < 0.05), time factor ($F_{4,32}$ = 30.32, p< 0.05) and the interaction between treatment factor x time factor ($F_{4,32}$ = 3.35, p< 0.05). Blood vessel content was different between control group and ANG-(1-7)-30ng group on days 9 (increased by 46%) and 14 (increased by 38%) (p< 0.05). No significant differences were found on days 1, 4 and 7 for blood vessel content.

TNF-α level (figure 3-a) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,15}$ = 211.67, p< 0.05), time factor ($F_{4,60}$ = 253.24, p< 0.05) and the interaction between treatment factor x time factor ($F_{4,60}$ = 3.17, p< 0.05). TNF-α level was different between control group and ANG-(1-

7)-30ng group on days 1 (decreased by 25%), 4 (decreased by 36%), 7 (decreased by 66%), 9 (decreased by 67%) and 14 (decreased by 74%) ($p< 0.05$).

MPO activity (figure 3-b) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,16} = 6.67$, $p< 0.05$), time factor ($F_{4,64} = 37.82$, $p< 0.05$) and the interaction between treatment factor x time factor ($F_{4,64} = 5.76$, $p< 0.05$). Neutrophil content was different between control group and ANG-(1-7)-30ng group on days 1 (increased by 74%), 4 (decreased by 27%), 7 (decreased by 22%) and 9 (decreased by 28%) ($p< 0.05$). No significant differences were found on day 14, suggesting that treatment did not affect the accumulation of stimulated neutrophils in the sponge matrix.

CXCL1/KC level (figure 3-c) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,16} = 65.61$, $p < 0.05$), time factor ($F_{4,64} = 5.17$, $p< 0.05$) and the interaction between treatment factor x time factor ($F_{4,64} = 3.71$, $p< 0.05$). CXCL1/KC level was different between control group and ANG-(1-7)-30ng group on days 4 (decreased by 48%), 7 (decreased by 55%), 9 (decreased by 38%) and 14 (decreased by 25%) ($p< 0.05$). No significant differences were found at day 1.

The extent of active monocytes/macrophage accumulation in the sponge discs in the control and treated groups was measured by NAG activity. The level of NAG activity (figure 3-d) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,15} = 29.54$, $p< 0.05$) and time factor ($F_{4,60} = 2.25$, $p< 0.05$). NAG activity was different between control group and ANG-(1-7)-30ng group on days 4 (decreased by 58%), 7 (decreased by 51%) and 9 (decreased by 33%) ($p< 0.05$). No significant differences were found at day 1 and 14.

CCL2/JE level (figure 3-e) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,16} = 34.20$, $p< 0.05$) and time factor ($F_{4,64} = 10.43$, $p< 0.05$). CCL2/JE level was different between control group and ANG-(1-7)-30ng group only at day 7 (decreased by 32%), 9 (decreased by 22%) and 14 (decreased by 10%) ($p< 0.05$). No significant differences were found on days 1 and 4.

Mast cells quantification (figure 3-f) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,8} = 35.72$, $p< 0.05$) and time factor ($F_{4,32} = 26.03$, $p< 0.05$). Mast cells quantification was different between control group and ANG-(1-7)-30ng group on days 1 (increased by 87%), 4 (increased

by 59%), 7 (increased by 50%), 9 (increased by 54%) and 14 (increased by 32%) ($p<$ 0.05).

Total soluble collagen content (figure 4-a) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,14} = 41.96$, $p<$ 0.05), time factor ($F_{4,56} = 81.67$, $p< 0.05$) and the interaction between treatment factor x time factor ($F_{4,56} = 3.24$, $p< 0.05$). Total soluble collagen was different between control group and ANG-(1-7)-30ng group on days 7 (increased by 105%) and 9 (increased by 50%) ($p< 0.05$).No significant differences were found on days 1, 4and 14.

TGF-β1 level (figure 4-b) was different between groups. The two-way ANOVA showed significant differences for treatment factor ($F_{1,15} = 11.00$, $p< 0.05$), time factor ($F_{4,60} = 55.88$, $p< 0.05$) and the interaction between treatment factor x time factor ($F_{4,60} = 2.82$, $p< 0.05$). TGF-β1 level was different between control group and ANG-(1-7)-30ng group on days 7 (increased by 50%) and 9 (increased by 30%) ($p< 0.05$). No significant differences were found on days 1, 4 and 14.

Collagen content (figure 4-c) was measured by Picrosirius red-staining sections and showed difference between groups. The two-way ANOVA showed significant differences for time factor ($F_{4,20} = 94.39$, $p< 0.05$) and the interaction between treatment factor x time factor ($F_{4,20} = 3.40$, $p< 0.05$). Collagen content was different between control group and ANG-(1-7)-30ng group only at day 7 (increased by 37%) ($p< 0.05$). No significant differences were found on days 1, 4, 9 and 14.

Discussion

Angiogenesis it is a multistep process that can be defined as the development of new blood vessels from preexisting vessels [91, 100, 101] and involves numerous soluble and cell surface-bound mediators. In this study, we investigated the effects of Ang-(1-7) on the angiogenic and inflammatory components of the fibrovascular tissue induced by implantation of synthetic matrix in mice.

In this model, cell recruitment, inflammation, angiogenesis and extracellular matrix deposition are induced by the implantation technique that has been shown to be modulated by a number of potential therapeutic compounds. Although, the effects of Ang-(1-7) in angiogenesis has been previously reported for Machado *et al.* (2000) and Machado *et al.* (2001) [88, 89]. However, we are not aware of any systematic study the such effects in fibrovascular tissue. [101-103]. Our first set of results showed that daily

injections of Ang(1-7)dose-dependent (30ng) exerted pro-angiogenic (increase hemoglobin), anti-inflammatory (decrease MPO and NAG) and pro-fibrotic (total collagen soluble) effects. In addition, sponge implants can be easily manipulated and examined at defined time points and thus facilitate the kinetic tracing of different cell lineages [104]. In this context, a more detailed analysis revealed an increase in the hemoglobin content on 9 and 14-day. Vascularization was assessed by evaluating the hemoglobin content, and this method bears a good correlation with other quantitative techniques to evaluate angiogenesis in sponges [105]. Although on the 3rd and 7th days ANG-(1-7) showed the opposite effect, these data are in accordance with previous studies [88, 89]. The effects of ANG 1-7 maybe exert dual effects depending on the treatment regimen and cell population in the inflammatory response. For example, Machado *et al.* (2001) [89], on the other hand, reported that ANG-(1-7) inhibited angiogenesis, results that are in sharp contrast with the data reported. Although, Machado *et al.* (2002) [106] reported a vasodilatory effect of Ang-(1-7) and it may act as a vasodilator mediator in pre-existing (skin) and newly formed vasculatures (14-day-old sponge implants). We further assessed the VEGF and FGF levels intraimplant, that decreased significantly in points 7 and 9 of the kinetic. VEGF and FGF have been shown to be an essential mediator of neovascularization [82, 107]. The fact is that ANG-(1-7) was able reduce number of blood vessels in fibrovascular tissue when compared to control group. Interestingly, some effects of Ang-(1-7) in fibrovascular tissue were observed only at 7, 9 and 14 post-implantation. Therefore, it seems reasonable to speculate that may be due to the that data discussed that the newly formed fibrovascular tissue in the implant compartment has a population of Ang-(1–7) receptors that is still immature on days1 and 4post-implantation or the availability of functional receptors are limited. Another possibility is that in a multimediated process such as new blood vessel formation, the contribution of one inhibitor for the whole process are also limited and is likely to be counteracted by other regulatory substances [89].

In this study of kinetics of inflammatory response, a marked effect of the ANG-(1-7)-30ng in decreasing angiogenesis was assessed by the measurement of the Hb implants content on days 4 and 7. This results were in accordance with those of Machado *et al.* (2001) [89] who had 7-day-old sponge implants treated with daily injections of ANG-(1–7)-20ng and inhibited significantly the angiogenesis in the implants compared to CT group showing good and reliable correlation with other biochemical and functional measures of new vessel formation [88, 89]. For 9 and 14

days of treatment with ANG-(1-7)-30ng was in agreement with Machado *et al.* (2002) [106] who found a similar pattern to Hb although they have made a sistemic treatment. The Hb content of the sponge tissues is a good marker for angiogenesis [105, 108] and it was used as an index of the vascularization [89, 109] and decreased due to the VEGF release. VEGF has been shown to be an essential mediator of neovascularization, inducing dose-related growth of new blood vessels [105].This dose of ANG-(1-7)-30ng was enough to inhibit the formation of new vessels in this experimental model on days 4 and 7. The obtained result was in line with previously published work by Machado *et al.*(2001) [89] who used a similar daily dose of ANG-(1-7)-20ng for 7 days and found reduced levels of Hb which were implicated in angiogenesis in a mice sponge model. This is in line with several reports showing that various ANG-(1–7) effects *in vivo* and *in vitro* are produced via activation of an ANG-(1–7)-specific receptor [89, 110-112]. After 9 and 14 days of treatment the response was different and had a pro-angiogenic effect, which agrees to Machado *et al.*(2000) [88] and the idea of direct vasoconstriction or vasodilatation within the implant would alter blood, and thus Hb content of the neovasculature since ANG-(1–7) is known to be a vasodilator[89, 110, 113].

The sequential development of inflammation and cytokine production in the fibrovascular tissue induced by sponge implantation in mice treated with ANG-(1-7) differed substantially from untreated group. We measured six markers of inflammation (MPO, NAG activities, mast cell count, three cytokines TNF-α, CXCL-1 and CCL2)[82, 101]. In this study, the MPO activity, as a measure of neutrophil accumulation, was affected by ANG-(1-7)-30ng treatment on days 4, 7 and 9.Similarly, there was a decrease in the concentration of the chemokine CXCL-1 and TNF-α in the treated implants. Therefore, Guabiraba et al, (2013) [83] explain that reduced pro-inflammatory cytokine production (i.e. TNF-a, CXCL1/KC) may directly or indirectly contribute to the accumulation and activation of neutrophils within the implants, which together with the local production of VEGF may lead activation of angiogenesis process [83]. TNF-α had the same reponse to Ang-(1-7) action. Our results are in concordance to Silveira et al. (2010) [63] who studied Ang-(1-7) treatment with Mas receptor agonists in experimental models of arthritis and concluded that the decreased accumulation of neutrophils and joint damage was associated with decreased local production of proinflammatory cytokines, including TNF-α and and neutrophil-active chemokines, as similar to our data. In addition, the decreased of both neutrophil influx and cytokine production demonstrated that activation of the Mas receptor is sufficient

for stopping the positive interaction between cytokines and leukocytes at inducing joint inflammation and damage [63]. Similar to the neutrophils, NAG, representing macrophage accumulation, was decrease early, but not at day 14 after implantation. There was also a decrease in chemokine CCL2, which is the major chemokine involved in the monocyte recruitment to inflammatory site [83, 104]

Ang-(1-7) implants may be affected for this inflammatory cell population of inflammatory process induced by the sponge discs on days 1, 4, 7, 9 and 14.Although mast cells are involved especially in the maintenance of pro-angiogenic and inflammatory responses. However, it is likely that the increased accumulation of mast cell in implants would be linked to the positive feedback between the decreases in pro-angiogenic factors in implants treated with ANG-(1-7) and intrinsic role of this cell type in angiogenesis to deal with the persistent inflammatory stimulus [67, 96, 114]. Recent studies suggest that mast cells are involved in the production of proteins and pro-fibrotic factors [115]. In additional, numerous mast cells are observed in tissue fibrosis in various organs such as liver, lung and pancreas [116-118]. Therefore, mast cells in liver are reported to secrete various mediators in granules, including tryptase, that promote fibroblast growth and collagen synthesis [99, 114, 119]. In fact, our measurements of the ANG-(1-7) effects on the fibrogenic response indicates a increases of total soluble collagen and sections marked with Picrosirius red staining on days 7 and 9. Importantly, there is a similar increase in TGF-β level, which plays a central role in fibrosis by inducing differentiation of fibrocytes and increases in the expression of collagen deposition [119]. In contrast, other studies suggest a protective role of Ang-(1–7) against fibrosis. Recently, studies showed that Ang-(1–7) causes apoptosis of these cells, inhibits proliferation and diminishes the secretion of collagen, leading to a regression of the cardiac fibrosis[105, 108, 109].

It is clear that Ang-(1–7) can influence many organs and systems. Additional effects of the compound are related to dose chosen and model for the study[89, 120-122]. Our results provides further confirmatory evidence for the anti-inflammatory, pro-angiogenic and pro-fibrotic effects of Ang-(1-7) and suggest that inhibitory functions of ANG-(1-7) are associated with regulation of angiogenic, inflammatory and fibrogenic cytokines in the sponge implant model.

FIGURE LEGENDS

Figure 1 – Dose-response curve - Effect of different doses of Angiotensin-(1-7) treatment in sponge implants. In (A) the hemoglobin (HB) content was increased after 9 days of treatment at ANG-(1-7)-30ng dose. In (B) collagen (COL) content was increased after 9 days of treatment at ANG-(1-7)-30ng dose. In (C) myeloperoxidase enzyme (MPO) activity decreased after 9 days of treatment at ANG-(1-7)-30ng dose. In (D) N-acetylglucosaminidase (NAG) content was decreased after 9 days of treatment at ANG-(1-7)-30ng dose. Data are mean ±SEM. (*) $p < 0.05$ *vs.*CT. n = 8-10 mice for each group.

Figure 2 – Angiogenesis effects in different treatment times of Angiotensin-(1-7)-30ng treatment in sponge implants. In (A) the hemoglobin (HB) content was decreased after 4 and 7 days of treatment with ANG-(1-7)-30ngdose and increased after 9 and 14 days treatment. In (B) VEGF level was increased after 7 and 9 days of treatment with ANG-(1-7)-30ng dose. In (C) FGF level was increased after 7 and 9 days of treatment with ANG-(1-7)-30ngdose. In (D) blood vessels index was increased after 9 and 14 days of treatment with ANG-(1-7)-30ng dose. Data are mean ±SEM. (*) $p < 0.05$ *vs.* CT. n = 8-10 mice for each group.

Figure 3 – Inflammation effects of different treatment times of Angiotensin-(1-7)-30ng treatment in sponge implants. In (A) TNF-α was decreased after 1, 4, 7, 9 and 14 days of treatment with ANG-(1-7)-30ng dose. In (B) MPO activity was decreased after 1, 4, 7 and 9 days of treatment with ANG-(1-7)-30ng dose. In (C) CXCL1 was decreased after 4, 7, 9 and 14 days after treatment with ANG-(1-7)-30ng dose. In (D) NAG activity was decreased after4, 7, 9 and 14 days of treatment with ANG-(1-7)-30ng dose. In (E) CCL2 level was decreased after 7, 9 and 14 days after treatment with ANG-(1-7)-30ng dose. In (F) mast cell index was increased after 1, 4, 7, 9, and 14 days after treatment with ANG-(1-7)-30ng dose. Data are mean ±SEM. (*) $p < 0.05$ *vs.*CT. n = 8-10 mice for each group.

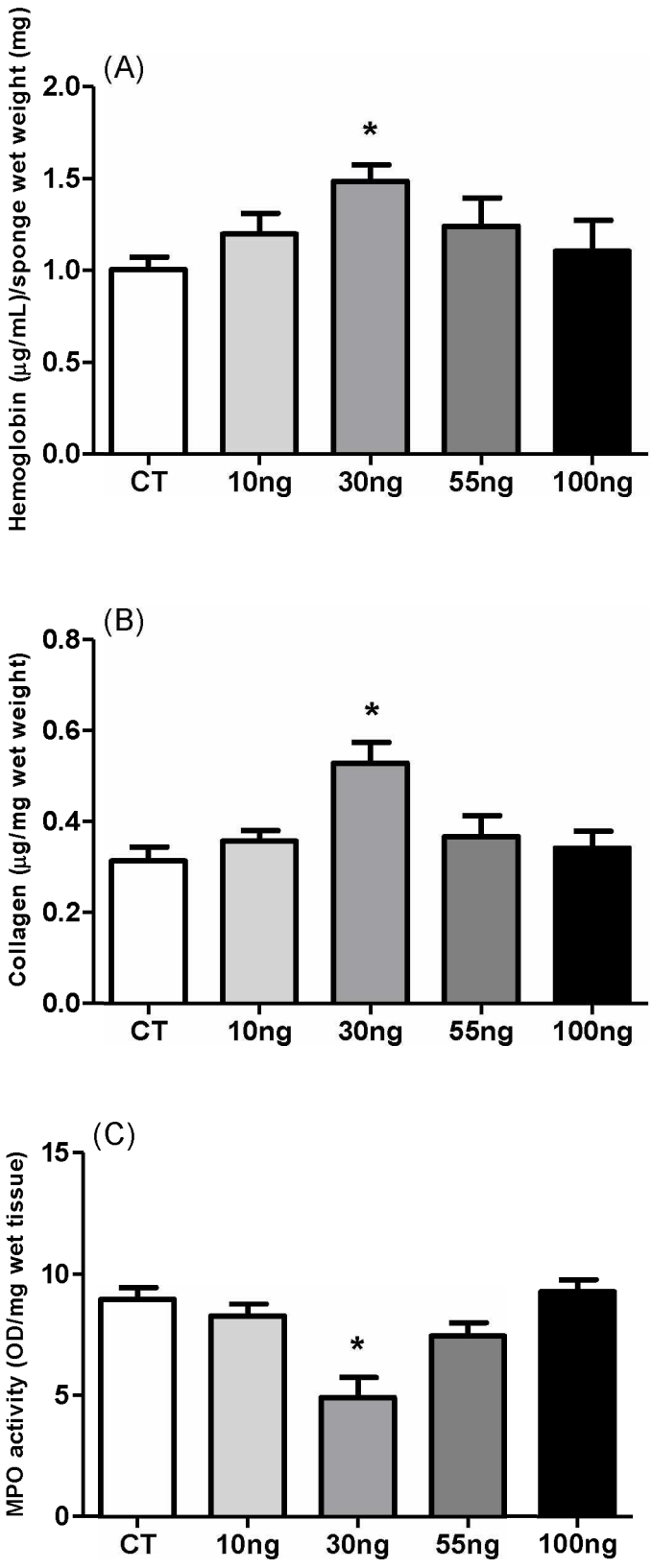
Figure 4 – Tissue repair aspects of different treatment times of Angiotensin-(1-7)-30ng treatment in sponge implants. In (A) Total soluble collagen activity was increased after 7 and 9 days of treatment with ANG-(1-7)-30ng dose. In (B) TGF-β1

level was increased after 7 and 9 days of treatment with ANG-(1-7)-30ng dose. In (C) collagen quantification sections were increased after 7, 9 and 14 days of treatment with ANG-(1-7)-30ng dose. Data are mean ±SEM. (*) $p < 0.05$ *vs.* CT. n = 8-10 mice for each group.

Figure 5 - Blood vessels quantification by Gomori´s Trichrome staining of different treatment times of Angiotensin-(1-7)-30ng treatment in sponge implants. Representative histological sections (6 µm) of sponge implants at days 1,4,7,9 and 14 post-implantation and quantification of blood vessels of sponge implants filled by conjunctive tissue. n = 5-6 mice for each group.

Figure 6- Collagen content measured by Picrosirius red-staining sections of different treatment times of Angiotensin-(1-7)-30ng treatment in sponge implants. Representative histological sections (6 µm) of sponge implants at days 1,4,7,9 and 14 post-implantation and morphometric analysis of the fibrovascular area (µm2). n = 5-6 mice for each group.

FIGURE 1


(A)
Hemoglobin (µg/mL)/sponge wet weight (mg)
2.0
1.5
1.0
0.5
0.0
*
CT
10ng
30ng
55ng
100ng
(B)
Collagen (µg/mg wet weight)
0.8
0.6
0.4
0.2
0.0
*
CT
10ng
30ng
55ng
100ng
(C)
MPO activity (OD/mg wet tissue)
15
10
5
0
*
CT
10ng
30ng
55ng
100ng

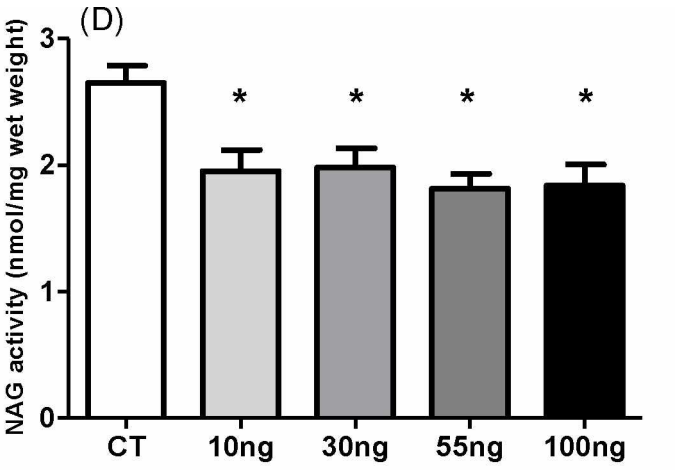
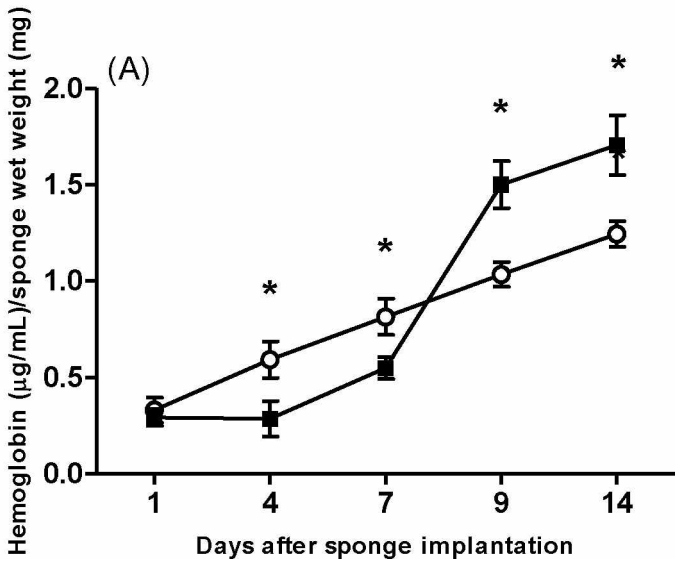
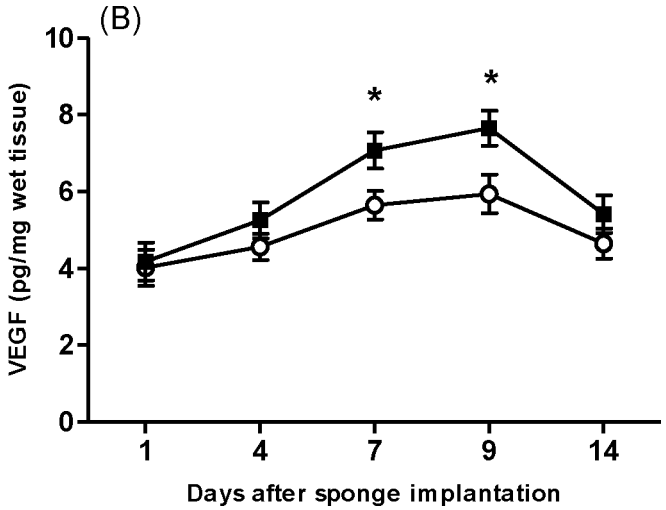
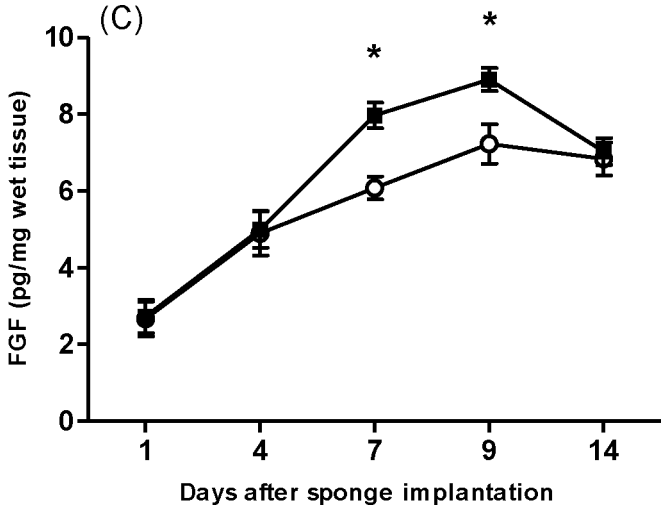

(D)
NAG activity (nmol/mg wet weight)
3
2
1
0
*
*
*
*
CT
10ng
30ng
55ng
100ng

FIGURE 2


(A)
Hemoglobin (µg/mL)/sponge wet weight (mg)
2.0
1.5
1.0
0.5
0.0
*
*
*
*
1
4
7
9
14
Days after sponge implantation


(B)
VEGF (pg/mg wet tissue)
10
8
6
4
2
0
*
*
1
4
7
9
14
Days after sponge implantation


(C)
FGF (pg/mg wet tissue)
10
8
6
4
2
0
*
*
1
4
7
9
14
Days after sponge implantation

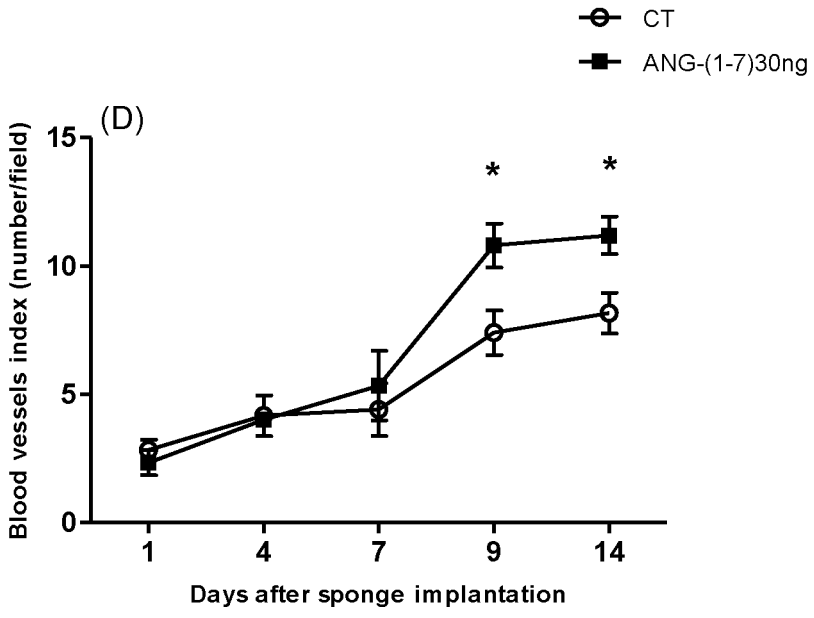
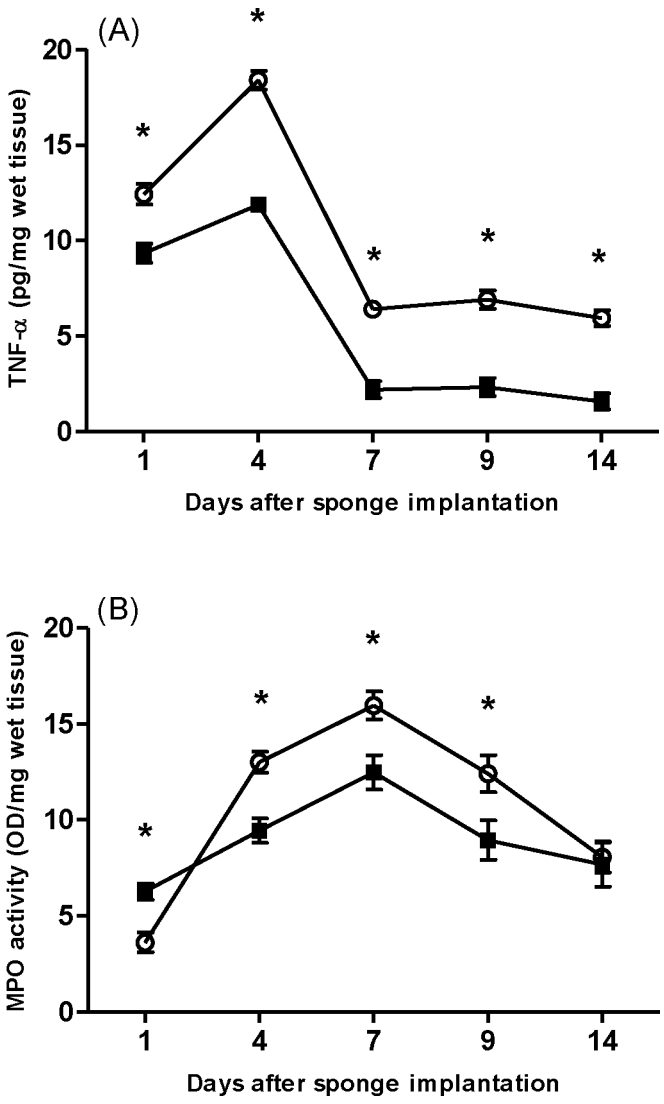
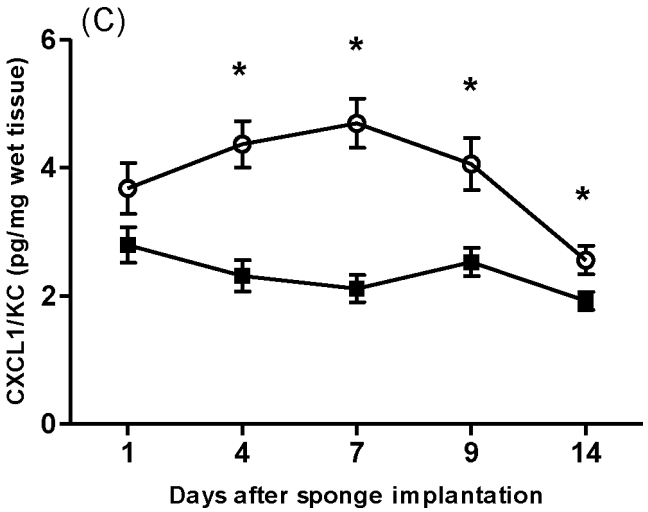
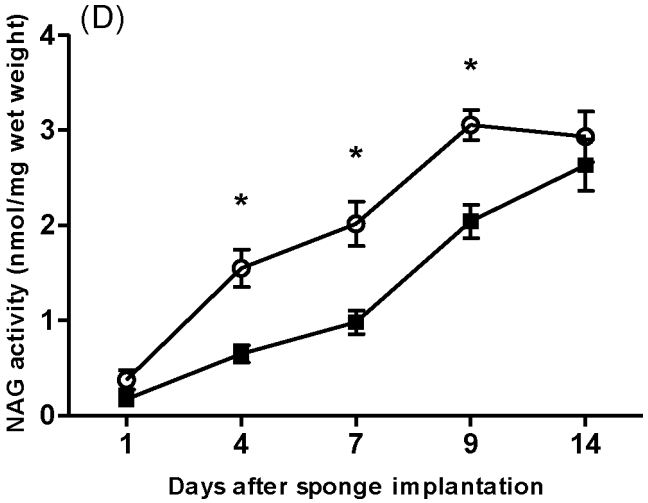
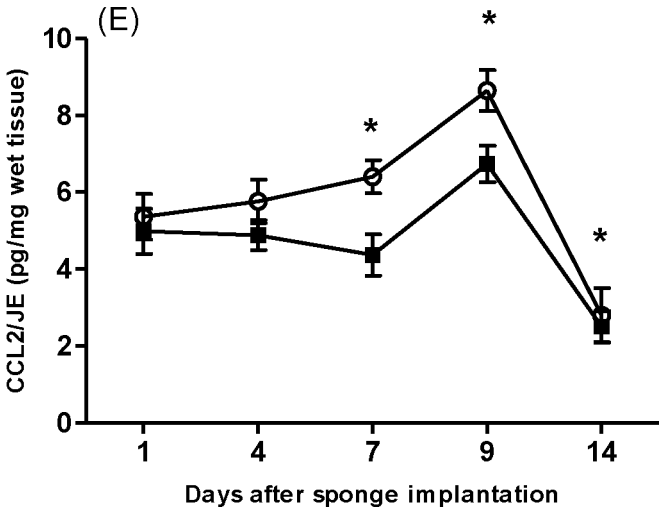

CT
ANG-(1-7)30ng
(D)
Blood vessels index (number/field)
15
10
5
0
*
*
1
4
7
9
14
Days after sponge implantation

FIGURE 3


(A)
TNF-α (pg/mg wet tissue)
20
15
10
5
0
1
4
7
9
14
Days after sponge implantation
(B)
MPO activity (OD/mg wet tissue)
20
15
10
5
0
1
4
7
9
14
Days after sponge implantation


(C)
CXCL1/KC (pg/mg wet tissue)
0
2
4
6
1
4
7
9
14
Days after sponge implantation
*
*
*
*


(D)
NAG activity (nmol/mg wet weight)
0
1
2
3
4
1
4
7
9
14
Days after sponge implantation
*
*
*


(E)
CCL2/JE (pg/mg wet tissue)
0
2
4
6
8
10
1
4
7
9
14
Days after sponge implantation
*
*
*

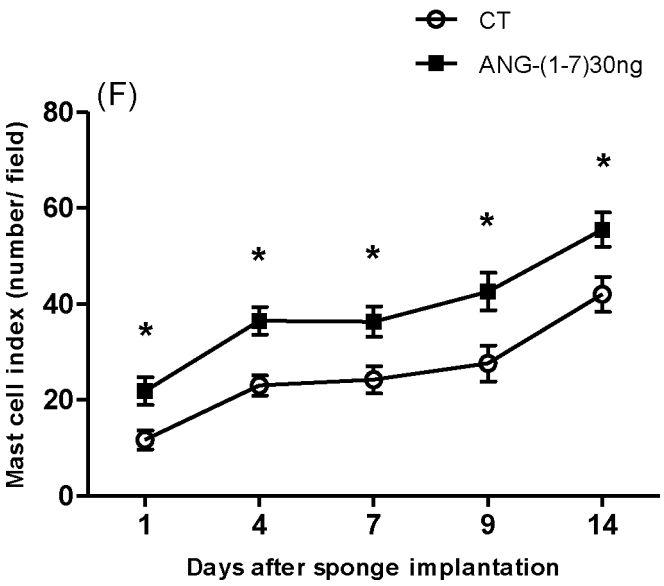
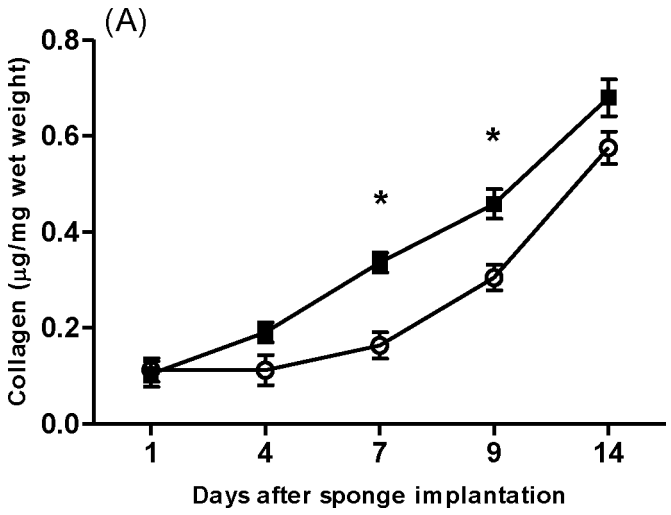
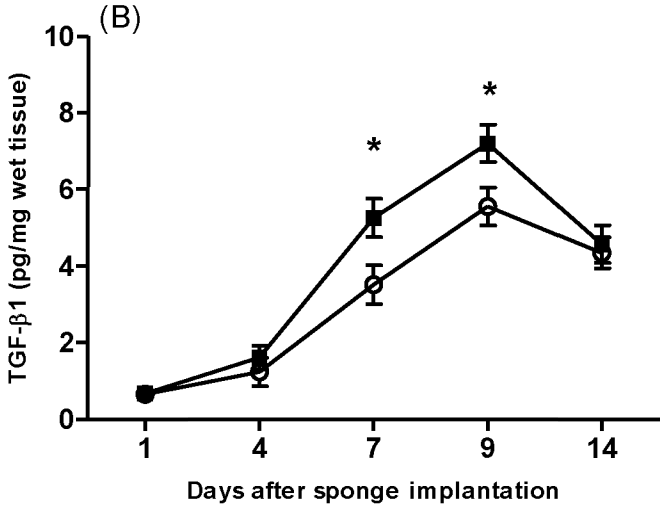

CT
ANG-(1-7)30ng
(F)
Mast cell index (number/ field)
80
60
40
20
0
1
4
7
9
14
Days after sponge implantation
*
*
*
*
*

FIGURE 4


(A)
Collagen (µg/mg wet weight)
0.8
0.6
0.4
0.2
0.0
1
4
7
9
14
Days after sponge implantation
*
*


(B)
TGF-β1 (pg/mg wet tissue)
10
8
6
4
2
0
*
*
1
4
7
9
14
Days after sponge implantation

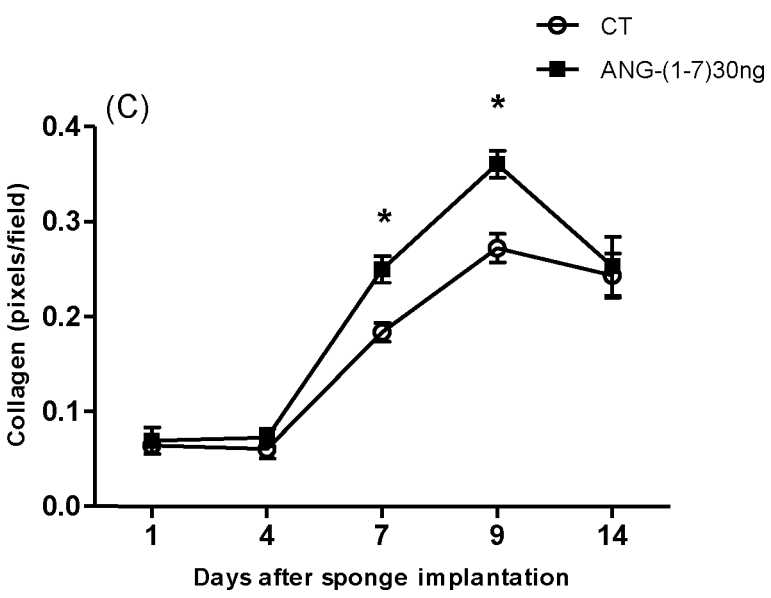

CT
ANG-(1-7)30ng
(C)
Collagen (pixels/field)
0.4
0.3
0.2
0.1
0.0
*
*
1
4
7
9
14
Days after sponge implantation

FIGURE 5

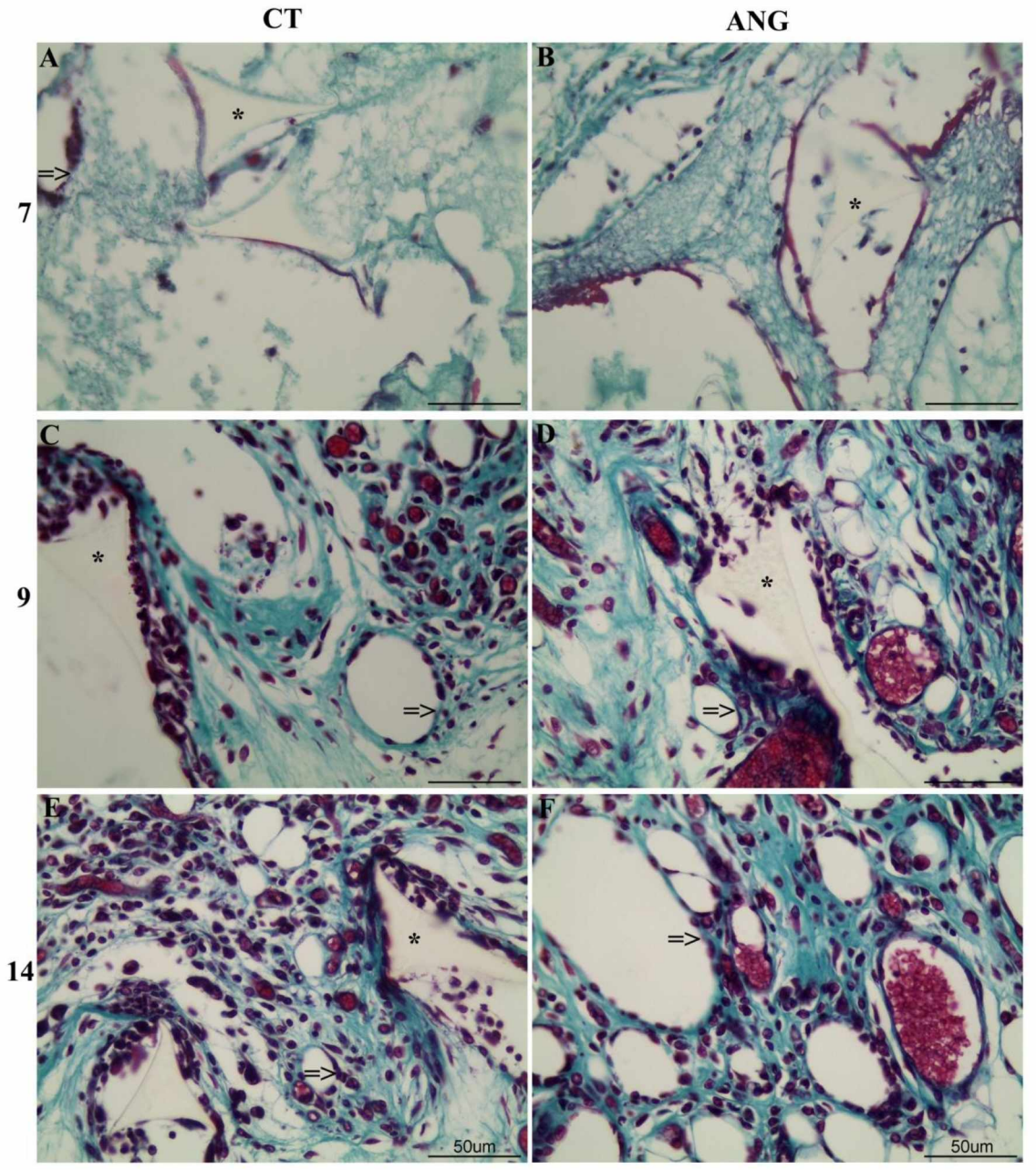
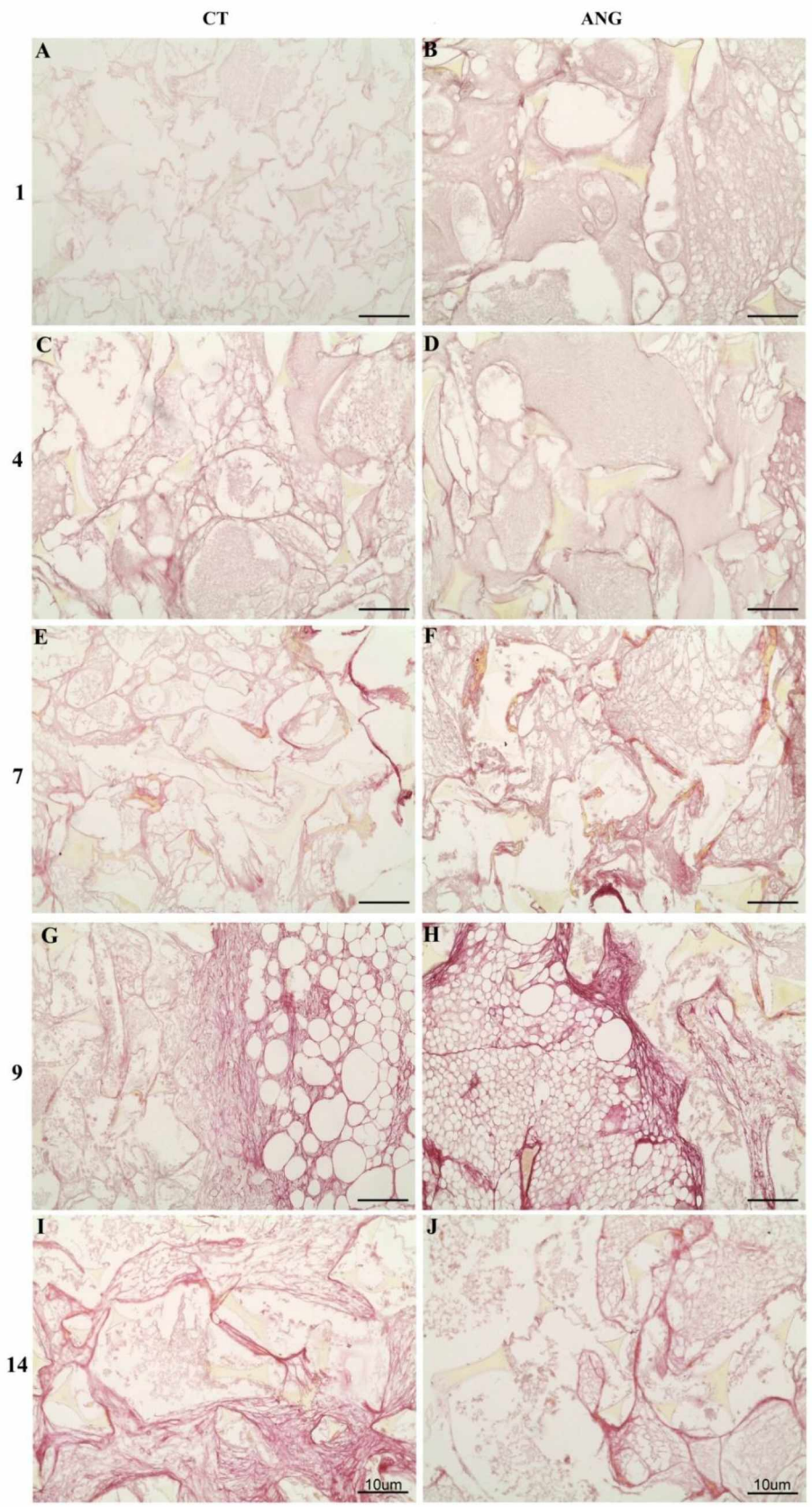


FIGURE 6



Conflict of interest statement

There are no conflicts of interest regarding to our manuscript.

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References

1. Meng, W., et al., *Autocrine and Paracrine Function of Angiotensin 1-7 in Tissue Repair During Hypertension.* Am J Hypertens, 2014.
2. Simoes e Silva, A.C., et al., *ACE2, angiotensin-(1-7) and Mas receptor axis in inflammation and fibrosis.* Br J Pharmacol, 2013. **169**(3): p. 477-92.
3. Machado, R.D., R.A. Santos, and S.P. Andrade, *Opposing actions of angiotensins on angiogenesis.* Life Sci, 2000. **66**(1): p. 67-76.
4. Machado, R.D., R.A. Santos, and S.P. Andrade, *Mechanisms of angiotensin-(1-7)-induced inhibition of angiogenesis.* Am J Physiol Regul Integr Comp Physiol, 2001. **280**(4): p. R994-R1000.
5. da Silveira, K.D., et al., *Anti-inflammatory effects of the activation of the angiotensin-(1-7) receptor, MAS, in experimental models of arthritis.* J Immunol, 2010. **185**(9): p. 5569-76.
6. Phillips, R.J., et al., *Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis.* J Clin Invest, 2004. **114**(3): p. 438-46.
7. Campos, P.P., Y.S. Bakhle, and S.P. Andrade, *Mechanisms of wound healing responses in lupus-prone New Zealand White mouse strain.* Wound Repair Regen, 2008. **16**(3): p. 416-24.
8. Araujo, F.A., et al., *Implant-induced intraperitoneal inflammatory angiogenesis is attenuated by fluvastatin.* Clin Exp Pharmacol Physiol, 2011. **38**(4): p. 262-8.
9. Barcelos, L.S., et al., *Production and in vivo effects of chemokines CXCL1-3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice.* Inflamm Res, 2004. **53**(10): p. 576-84.

10. Puchtler, H., et al., *Methacarn (methanol-Carnoy) fixation. Practical and theoretical considerations.* Histochemie, 1970. **21**(2): p. 97-116.
11. Leite, S.N., et al., *Experimental models of malnutrition and its effect on skin trophism.* An Bras Dermatol, 2011. **86**(4): p. 681-8.
12. Pereira, N.B., et al., *Apoptosis, mast cell degranulation and collagen breakdown in the pathogenesis of loxoscelism in subcutaneously implanted sponges.* Toxicon, 2014.
13. Puchtler, H., F.S. Waldrop, and L.S. Valentine, *Polarization microscopic studies of connective tissue stained with picro-sirius red FBA.* Beitr Pathol, 1973. **150**(2): p. 174-87.
14. Junqueira, L.C., G. Bignolas, and R.R. Brentani, *Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections.* Histochem J, 1979. **11**(4): p. 447-55.
15. Deconte, S.R., et al., *Alterations of antioxidant biomarkers and type I collagen deposition in the parotid gland of streptozotocin-induced diabetic rats.* Arch Oral Biol, 2011. **56**(8): p. 744-51.
16. Deryugina, E.I. and J.P. Quigley, *Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: contrasting, overlapping and compensatory functions.* Biochim Biophys Acta, 2010. **1803**(1): p. 103-20.
17. Moura, S.A., et al., *Local drug delivery system: inhibition of inflammatory angiogenesis in a murine sponge model by dexamethasone-loaded polyurethane implants.* J Pharm Sci, 2011. **100**(7): p. 2886-95.
18. Belo, A.V., et al., *Murine chemokine CXCL2/KC is a surrogate marker for angiogenic activity in the inflammatory granulation tissue.* Microcirculation, 2005. **12**(7): p. 597-606.
19. Ferreira, M.A., et al., *Tumor growth, angiogenesis and inflammation in mice lacking receptors for platelet activating factor (PAF).* Life Sci, 2007. **81**(3): p. 210-7.
20. Barcelos, L.S., et al., *Role of the chemokines CCL3/MIP-1 alpha and CCL5/RANTES in sponge-induced inflammatory angiogenesis in mice.* Microvasc Res, 2009. **78**(2): p. 148-54.
21. Saraswati, S. and S.S. Agarwal, *Strychnine inhibits inflammatory angiogenesis in mice via down regulation of VEGF, TNF-alpha and TGF-beta.* Microvasc Res, 2013. **87**: p. 7-13.
22. Machado, R.D., et al., *Vasodilator effect of angiotensin-(1-7) in mature and sponge-induced neovasculature.* Regul Pept, 2002. **107**(1-3): p. 105-13.

23. Mirabelli, P., et al., *Early effects of dexamethasone and anti-VEGF therapy in an inflammatory corneal neovascularization model.* Exp Eye Res, 2014. **125C**: p. 118-127.
24. Araujo, F.A., et al., *Atorvastatin inhibits inflammatory angiogenesis in mice through down regulation of VEGF, TNF-alpha and TGF-beta1.* Biomed Pharmacother, 2010. **64**(1): p. 29-34.
25. Araujo, F.A., et al., *3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (fluvastatin) decreases inflammatory angiogenesis in mice.* APMIS, 2012. **121**(5): p. 422-30.
26. Teixeira, A.S. and S.P. Andrade, *Glucose-induced inhibition of angiogenesis in the rat sponge granuloma is prevented by aminoguanidine.* Life Sci, 1999. **64**(8): p. 655-62.
27. Benter, I.F., D.I. Diz, and C.M. Ferrario, *Cardiovascular actions of angiotensin(1-7).* Peptides, 1993. **14**(4): p. 679-84.
28. Fontes, M.A., et al., *Evidence that angiotensin-(1-7) plays a role in the central control of blood pressure at the ventro-lateral medulla acting through specific receptors.* Brain Res, 1994. **665**(1): p. 175-80.
29. Santos, R.A., et al., *Characterization of a new angiotensin antagonist selective for angiotensin-(1-7): evidence that the actions of angiotensin-(1-7) are mediated by specific angiotensin receptors.* Brain Res Bull, 1994. **35**(4): p. 293-8.
30. Paula, R.D., et al., *Angiotensin-(1-7) potentiates the hypotensive effect of bradykinin in conscious rats.* Hypertension, 1995. **26**(6 Pt 2): p. 1154-9.
31. Guabiraba, R., et al., *Blockade of cannabinoid receptors reduces inflammation, leukocyte accumulation and neovascularization in a model of sponge-induced inflammatory angiogenesis.* Inflamm Res, 2013. **62**(8): p. 811-21.
32. Silver, R.B., et al., *Mast cells: a unique source of renin.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13607-12.
33. Marshall, J.S., *Mast-cell responses to pathogens.* Nat Rev Immunol, 2004. **4**(10): p. 787-99.
34. Jung, M., et al., *Mast cells produce novel shorter forms of perlecan that contain functional endorepellin: a role in angiogenesis and wound healing.* J Biol Chem, 2013. **288**(5): p. 3289-304.
35. Lu, D.Y., et al., *Mast cell leukemia: An extremely rare disease.* J Chin Med Assoc, 2014.
36. Arizmendi, N., et al., *Rac2 is involved in bleomycin-induced lung inflammation leading to pulmonary fibrosis.* Respir Res, 2014. **15**: p. 71.

37. Ammendola, M., et al., *Mast cells density positive to tryptase correlates with angiogenesis in pancreatic ductal adenocarcinoma patients having undergone surgery.* Gastroenterol Res Pract, 2014. **2014**: p. 951957.
38. Lu, J., et al., *Tryptase inhibitor APC 366 prevents hepatic fibrosis by inhibiting collagen synthesis induced by tryptase/protease-activated receptor 2 interactions in hepatic stellate cells.* Int Immunopharmacol, 2014. **20**(2): p. 352-7.
39. Marcus, Y., et al., *Angiotensin 1-7 as means to prevent the metabolic syndrome: lessons from the fructose-fed rat model.* Diabetes, 2013. **62**(4): p. 1121-30.
40. El-Hashim, A.Z., et al., *Angiotensin-(1-7) inhibits allergic inflammation, via the MAS1 receptor, through suppression of ERK1/2- and NF-kappaB-dependent pathways.* Br J Pharmacol, 2012. **166**(6): p. 1964-76.
41. Feltenberger, J.D., et al., *Oral formulation of angiotensin-(1-7) improves lipid metabolism and prevents high-fat diet-induced hepatic steatosis and inflammation in mice.* Hypertension, 2013. **62**(2): p. 324-30.

CAPÍTULO 3: MANUSCRITO 2

Title:

LOCAL TREATMENT WITH ANGIOTENSIN-1-7 IMPROVES SKIN WOUND HEALING IN MICE IN A MAS RECEPTOR-DEPENDENT MANNER

Authors:

Simone Ramos Deconte[1,2,4], Puebla Cassini Vieira[2,3],Leandro Ceotto Freitas Lima[3], Luiza Dias da Cunha Lima[3],Robson Augusto Souza Santos[3,4], Tatiana Carla Tomiosso[2],Fábio de Oliveira[1,2,4],Silvia Passos Andrade[3,4], Lucíola da Silva Barcelos*[3,4], Fernanda de Assis Araújo*[2,4].

Affiliation:

[1]Institute Genetics and Biochemistry (INGEB), Federal University of Uberlândia (UFU)

[2]Institute of Biomedical Sciences (ICBIM), Federal University of Uberlândia (UFU), Uberlândia - MG, Brazil.

[3]Department of Phisiology and Biophysics, Federal University of Minas Gerais (UFMG), Belo Horizonte - MG, Brazil.

[4]National Institute in Science and Technology in Nanobiopharmaceutics (NanoBiofar), BeloHorizonte-MG, Brazil.

*These authors contributed equally to this work

***Corresponding authors:**

Lucíola da Silva Barcelos

E-mail address: luciolasbarcelos@gmail.com

Current address:Universidade Federal de Minas Gerais (UFMG), Instituto de Ciências Biológicas (ICB), Bloco D4, sala 256. Av. Antônio Carlos 6627, Pampulha, CEP31270-901, Belo Horizonte-MG, Brazil. Tel: +55 31 3409-2955

Fernanda de Assis Araújo

E-mail address: folaraujo@gmail.com

Current address: Instituto de Ciências Biomédicas (ICBIM) – Área de Ciências Fisiológicas (ARFIS), Universidade Federal de Uberlândia (UFU).Av. Pará 1720 - Bloco 2A, piso superior – sala 120, CEP 38400-902, Uberlândia-MG, Brazil.Tel.: +55 34 3218-2200

Resumo

Angiotensina-(1-7) (Ang-(1-7)) é um componente bioativo do Sistema de Renina-Angiotensina conhecido por exercer propriedades antifibroticas e antiproliferativas, além de inibir a inflamação. A cicatrização de feridas é um processo de reparo de tecido altamente dinâmico que envolve uma sequência complexa de eventos celulares e bioquímicos, incluindo inflamação, proliferação, fibroplasia e cicatrização. A cicatrização inadequada pode levar a feridas crônicas, causando desconforto, morte física e/ou psicológica em casos extremos. Portanto, há um grande interesse no estudo de drogas que podem modular e melhorar o processo de cicatrização de feridas. Aqui, objetivamos avaliar os efeitos do tratamento local com Ang-(1-7) em um modelo de cicatrização de feridas na pele. Foram Utilizados camundongos C57BL/6 machos 7-8 semanas (20-25 g de peso corporal), quatro feridas circulares (25mm^2 cada) foram produzidas nas costas de camundongos. Os animais foram divididos em três grupos (n=6, cada): Ang-(1-7) 30ng, Ang-(1-7) 30ng + A779 e controle do veículo. O tratamento foi realizado diariamente começando com uma injeção intraperitoneal do antagonista do receptor MAS A779 (25 ug/animal) ou solução salina 20 min antes da aplicação local de Ang-(1-7) (30 ng/ferida) ou salina. Os animais foram eutanásicos nos dias 3, 7 e 14 para a coleta e avaliação da vascularização (fluxo sanguíneo da ferida, conteúdo de hemoglobina e níveis de citociinas), marcadores inflamatórios (atividades de Myeloperoxidase-MPO, N-acetil-β-D-glucosamindase-NAG, e níveis de quimiocina) e marcadores de fibroplasia (colágeno solúvel total e níveis de TGF-β1). *Resultados:* O teor de neutrófilos (atividade MPO) e os níveis de TNF-alfa e CXCL1 / KC foram diminuídos pelo tratamento Ang-(1-7) quando comparado ao controle do veículo e grupos Ang- (1-7)/A779 ($p < 0,05$). Em contraste, o conteúdo de macrófagos (atividade NAG) e os marcadores de angiogênese (hemoglobina, VEGF, FGF e fluxo sanguíneo) aumentaram no grupo Ang-(1-7) quando comparados ao controle do veículo e Ang-(1-7)/A779 Grupos ($p < 0,05$). Além disso, os marcadores de fibroplasia (níveis totais de colágeno solúvel e TGF-β1) também aumentaram no grupo Ang-(1-7), bem como a taxa de fechamento da ferida foi acelerada neste grupo quando comparada ao controle do veículo e Ang-(1-7)/A779 ($p < 0,05$). Em geral, observamos que o tratamento com Ang-(1-7) acelerou o encerramento da ferida, inibiu a inflamação e estimulou a formação de um novo tecido fibrovascular em feridas de pele excisional. Além disso, o bloqueio da ativação do receptor MAS inibiu completamente os efeitos de ANG- (1-7). Em

conclusão, nossos dados sugerem que o tratamento local com Ang-(1-7) melhora a cicatrização de feridas cutâneas de uma maneira dependente do receptor MAS.

Palavras-chave: Angiotensina- (1-7), cicatrização de feridas na pele, inflamação, angiogênese

Abstract

Angiotensin-(1-7) (Ang-(1-7)) is a bioactive component of the Renin-Angiotensin System that, by counteracting effects on angiotensin II, is known to exert antifibrotic and antiproliferative properties, as well as inhibiting inflammation. The wound healing is a highly dynamic tissue repair process that involves a complex sequence of cellular and biochemical events including inflammation, proliferation, fibroplasia, and scar formation. Inadequate healing can lead to chronic wounds, causing physical and/or psychological discomfort and death in extreme cases. Therefore, there is great interest in the study of drugs which can modulate and improve the wound healing process. Here, we aim to evaluate the effects of local treatment with Ang-(1-7) in a mouse model of skin wound healing. Male C57BL/6 mice 7–8 weeks (20–25 g body weight) were used, four circular excisional wounds (25mm2 each) were produced on the back of mice. Animals were divided into three groups (n=6, each): ANG-(1-7)30ng, ANG-(1-7)30ng+A779 and vehicle control. Treatment was performed daily beginning with an intraperitoneal injection of the MAS receptor antagonist A779 (25ug/animal) or saline 20 min before local application of Ang-(1-7)(30ng/wound) or saline. Animals were euthanized at days 3, 7 and 14 for wound collection and assessment of vascularization (wound blood flow, hemoglobin content, and cytokine levels), inflammatory markers (Myeloperoxidase-MPO,N-acetyl-β-D-glucosamindase-NAG activities, and chemokine levels) and fibroplasia markers (total soluble collagen and TGF-β1 levels).Neutrophil content (MPO activity) and levels of TNF-alpha and CXCL1/KC were decreased by Ang-(1-7) treatment when compared to vehicle control and ANG-(1-7)/A779 groups ($p<0.05$). In contrast, macrophage content (NAG activity) and angiogenesis markers (hemoglobin, VEGF, FGF, and blood flow)were increased in the ANG-(1-7) group when compared to vehicle control and ANG-(1-7)/A779 groups($p<0.05$). In addition, markers of fibroplasia (total soluble collagen and TGF-β1 levels) were also increased in the ANG-(1-7) group as well as the wound closure rate was accelerated in this group when compared to vehicle control and ANG-(1-7)/A779

groups (p<0.05).Overall, we observed that treatment with Ang-(1-7) accelerated wound closure, inhibited inflammation and stimulated the formation of a new fibrovascular tissue in excisional skin wounds. In addition, the blockade of MAS receptor activation fully inhibited ANG-(1-7) effects. In conclusion, our data suggests local treatment with Ang-(1-7) improves skin wound healing in a MAS receptor-dependent manner.

Keywords: Angiotensin-(1-7), skin wound healing, inflammation, angiogenesis, fibroplasia

Introduction

Angiotensin-(1-7) (Ang-(1-7)) is a metabolite of the Renin-angiotensin System (RAS) that for a long time was considered inactive[54]. This concept began to change from the studies showing that Ang-(1-7) exerted biological effects on *in vitro* release of vasopressin in cultures of posterior pituitary extract[55]. Since then, several studies have been demonstrating different effects and conceptual changes of fundamental importance related to Ang-(1-7) have arisen. Noteworthy, among them are the discovery of ACE2 (angiotensin-converting enzyme 2), as responsible for the formation of Ang-(1-7), and the identification of the receptor MAS which has high affinity to Ang-(1-7)[56, 57]. The current concept of RAS relies on two opposing main axes: the classic ACE-AngII-AT1 receptor and the counter-regulating ACE2-Ang-(1-7)-MAS receptor axis. Activation of MAS by Ang-(1-7) has antagonistic effects of that caused by the interaction of Ang II to the AT1 receptor and causes vasodilation, reduced fibrosis, reduced cell proliferation, reduced hypertrophy, among others [51, 58]. There are also crescent evidences that this peptide acts as a counteracting of angiotensin II in inflammatory conditions, by exerting anti-inflammatory actions [51, 59-61].

The wound healing is a highly dynamic tissue repair process that involves a complex sequence of cellular and biochemical events including hemostasis/inflammation, proliferation, fibroplasia and scar formation/extracellular matrix remodeling [123]. After an initial inflammatory phase characterized by infiltration of neutrophils and macrophages, follows the formation of a fibroproliferative tissue rich in fibroblast-derived collagen and newly formed blood vessels, and finally the fully epithelialization and dermis maturation phase that involves resolution of inflammation and remodeling of the extracellular matrix[124].When tissue repair occurs

inappropriately, i.e., the process fails to proceed through this series of events, a chronic response characterized by persistent inflammation and non-closure opened wounds may be installed. This is a direct problem for the individual, causing physical and/or psychological discomfort and delay in rehabilitation as well as can lead to limb amputation, septicemia and/or death in extreme cases[125].

The recruitment of inflammatory leukocytes to a wound, and is an evolutionary acquisition of mammals to prevent the invasion of microbes, is also a critical component during tissue repair due to the broad spectrum of released mediators that can help or even impair wound healing [36]. In addition, neovascularization is also critical to the healing process, because the growth of new blood vessels provides adequate supply of oxygen and for the healing tissue nutrients, to facilitate access of leukocytes and removal of waste material [36]. However, impaired and/or excessive growth of new blood vessels is associated with chronic wounds and development of fibrosis[40].Therefore, there is great interest in the study of drugs which can modulate and improve the wound healing process.

It is necessary to understand how we can manipulate the presence of leukocytes and the formation of new blood vessels at sites of injury in order to maintain the signals that are critical for targeting the repair process and, at the same time, block the signals that are not beneficial. This work aims to evaluate a new therapeutic strategy for the treatment of skin excisional wounds through the local application of Angiotensin-(1-7) in a mouse model.

Material and methods

Animals

Male C57BL/6 mice 7–8 weeks (20–25 g body weight) were used in these experiments, provided by the animal facility (Centro de bioterismo- CEBIO) of the Federal University of Minas Gerais-Brazil (CEBIO-UFMG). The animals were housed individually and provided with chow pellets, water *ad libitum* and 12h light/dark cycles. Efforts were made to avoid all unnecessary distress to the animals. Housing, anesthesia and postoperative care concurred with the guidelines established by our local Institutional Animal Welfare Committee.

Experimental procedure

Animals were anaesthetized by an intraperitoneal injection of ketamine and xylazine (60mg/kg and 8mg/Kg, respectively), the dorsal hair shaved and the skin wiped with 70% ethanol. Four circular excisional wounds in equidistant points, with an area of 25mm2, were produced on each mouse by using a surgical tool commonly used for procedures associated with biopsies. Animals were divided into three groups (n=6,each):ANG-(1-7)30ng, ANG-(1-7)30ng+A779 and Saline (vehicle) control. Postoperatively, the animals were monitored for any signs of infection at the operative site, discomfort or distress; any showing such signs were immediately humanely killed. The doses of the compound and the treatment regimen were chosen based on pilot experiments (data not shown) and data from the literature[63, 89]. Treatment and wounds were well tolerated by the mice over the experimental period. At the moment and every three days (until the 14th day) after surgery, the animals are anesthetized and the wound area was measured with the aid of a digital caliper to monitor the temporal profile of wound closure.

Treatment was performed daily beginning by an intraperitoneal injection of A779 (25ug/animal)or saline 20 min before local application of ANG-(1-7) or saline. For ANG-(1-7) injection animals were anesthetized by inhalation of Halotane (2-Bromo-2-chloro-1,1,1-trifluoroethane). Each wound received 4 points of injection of 2,5ul of ANG-(1-7) (30ng/10ul, each wound) using a Hamilton® syringe.

At different time-points (3, 7 and 14 days post-wounding and treatment) animals were euthanized by an intraperitoneal injection of ketamine and xylazine (60mg/kg and 8mg/Kg, respectively) and wounds were removed to assess vascularization (blood flow, hemoglobin content, cytokines levels), inflammatory markers (MPO, NAG activities and chemokines levels) and fibrogenesis markers (total collagen soluble and TGF-β1).

Wound blood flow evaluation

This was carried out in anesthetized mice (ketamine and xylazine (60mg/kg and 8mg/Kg, respectively)) immediatelyafter wounding and at 3, 7, 10 and 14 days after wounding. The blood flow was assessed using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, U.K). While capturing perfusion images, the ambient light level was kept at a minimum to impede any influence on the laser light and recorded signals. The animals were kept at a constant temperature of 37ºC for 5 minutes before and during measurements to control for temperature variations. The mean pixel value of each scanned image was calculated using MoorLDI V5.3 software

and the calculated mean flux was expressed as perfusion units (PU), which represent the average blood flow in each wound.

Hemoglobin extraction (indirect assessment of neovascularization)

The extent of the vascularization of wound closure level was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin's method [90]. Animals were euthanized and wounds were carefully removed, dissected from adherent tissue, weighed. Half of a wound of each animal was weighed and homogenized (Ultra Stirrer) in 2 ml of Drabkin reagent (Labtest, Lagoa Santa Brazil), and centrifuged at 10,000 g for 40 min. The supernatants were filtered through a 0.22-mm millipore filter. Hb concentration of the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and was compared against a standard curve of Hb. The content of Hb wound closure was expressed as mg Hb per mg wet tissue.

Tissue extraction and determination of myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAG)enzyme activities

The extent of neutrophil accumulation in wound closure was measured by assaying myeloperoxidase enzyme (MPO) activity as previously described [89, 100]. An entire wound of each animal was weighed, homogenized in (2 ml) pH 4.7 buffer (0.1 MNaCl, 0.02 MNaH_2PO4, 0.015 MNa-EDTA) and centrifuged at 15,300g for 15 min. The pellets were then resuspended in 0.05M Na2HPO4 buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (3.2mM) and H_2O_2 (0.3 mM). The reaction was terminated by the addition of 50 µl of H_2SO_4 (4 M). Results were expressed as change in OD per gram of wet tissue. The infiltration of mononuclear cells into wound was quantified by measuring the levels of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) which is present in high levels in activated macrophages[89, 92]. The other wound half part (of hemoglobin extraction) was weighed, homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100, and centrifuged (960 g; 10 min at 4°C). Samples of the resulting supernatant (100 µl) were incubated for 10 min with 100µl of P-nitrophenyl-N-acetyl-beta-D-glucosaminide prepared in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na2HPO4; pH 4.5) to yield a final

concentration of 2.24 mM. The reaction was stopped by the addition of 100 µl of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. The readings were interpolated on a standard curve constructed with p-nitrophenol (0–500 nmol ml^{-1}). Data are reported as nmol of products formed per milligram of wet tissue.

Total soluble collagen content

Total soluble collagen was measured using half part of a wound of each animal by the Sirius Red reagent based-assay[90, 92]. Wounds were weighed, homogenized in 1 ml of PBS and 50 µl of sample were mixed with 50 µl of Sirius Red reagent. Samples were mixed by gentle inversion. The collagen-dye complex was precipitated by centrifugation at 6000 g for 10 min. The supernatants were drained off and discarded and the pellet washed with 500 µl of ethanol (99% pure and methanol free). One milliliter of a 0.5 M NaOH solution was added to the remaining pellet of collagen-bound dye. After solubilization, samples were transferred to a 96-well plate and read at 540 nm. The calibration curve was setup on the basis of a gelatin standard. The results are expressed as microgram collagen per milligram wet tissue.

Evaluation of cytokines/ chemokines levels by ELISA

An entire wound of each animal was weighed (see Hb measurement) and used to examine the levels of VEGF, TNF-α, TGF-β1, FGF, CXCL1/KC, CCL2/MCP1/JE produced wounds by enzyme-linked immunosorbent assay (ELISA). The assays were performed using Kits from R&D systems and according to the manufacturer's instructions. The threshold of sensitivity for each cytokine/chemokine was 7.5 pg/ml. The results were expressed as pictogram of cytokine per milligram wet tissue.

Statistics

Statistical analysis was performed using Statistica software (99ed.,StatSoftIncand graphs were performed with GraphPad Prism program, version 5. All values were expressed as mean ± S.E.M. Kinetics of inflammatory response were analyzed by three-way ANOVA considering ANG-(1-7) factor, time factor and interaction of ANG-(1-7)/A779 and ANG-(1-7) factor. In cases where ANOVA showed significant differences ($p \leq 0.05$), it was performed the planned comparison test between groups of interest.

Results

Inflammation

The inflammatory components of the wound inflammation were determined by estimating the numbers of the leukocytes in the implant by assaying inflammatory enzyme activities and levels of pro-inflammatory cytokines. As shown in figure 1,TNF-α level was different between groups. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20}$ = 15.23, $p \leq 0.05$), time factor ($F_{3,60}$ = 352.93, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20}$ = 11.49, $p \leq 0.05$). On days 3 (decreased by 24%), 7 (decreased by 37%) and 14 (decreased by 44%) TNF-α level was different between saline control and ANG-(1-7) group ($p \leq 0.05$). When compared saline control x ANG-(1-7)/A779 treatment no significant differences were found on days 3, 7 and 14 ($p > 0.05$). ANG-(1-7)/A779 treatment was different to TNF-α level of ANG-(1-7) group on days 3 (increased by 26%), 7 (increased by 56%) and 14 (increased by 63%) when compared to ANG-(1-7) group ($p \leq 0.05$).

CCL2 (MCP1/JE)level was also changed between groups, as shown in figure 2. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20}$ = 9.08, $p \leq 0.05$), time factor ($F_{3,60}$ = 96.65, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20}$ = 7.39, $p \leq 0.05$). On days 3 (increased by 63%), 7 (increased by 47%) and 14 (increased by 54%) CCL2 level was different between saline control and ANG-(1-7) group ($p \leq 0.05$). When compared saline control x ANG-(1-7)/A779 treatment no significant differences were found on days 3, 7 and 14 ($p > 0.05$). ANG-(1-7)/A779 treatment was different to CCL2 level of ANG-(1-7) group on days 3 (decreased by 38%), 7 (decreased by 30%) and 14 (decreased by 28%) when compared to ANG-(1-7) group ($p \leq 0.05$).

Neutrophil content affected treatment groups, as shown in figure 3. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20}$ = 35.52, $p \leq 0.05$), time factor ($F_{3,60}$ = 219.50, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20}$ = 31.15, $p \leq 0.05$). On days 3 (decreased by 39%), 7 (decreased by 42%) and 14 (decreased by 59%) MPO content was different between saline control and ANG-(1-7) group ($p \leq 0.05$).When compared saline control x ANG-(1-7)/A779 treatment no significant differences were found on days 3, 7 and 14 ($p > 0.05$). ANG-(1-7)/A779 treatment was different to MPO content of ANG-(1-7) group on days 3 (increased by

58%), 7 (increased by 70%) and 14 (increased by 28%) when compared to ANG-(1-7) group ($p \leq 0.05$).

CXCL1 (KC) level was changed by treatments, as shown in figure 4. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20} = 20.55$, $p \leq 0.05$), time factor ($F_{3,60} = 259.35$, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20} = 12.12$, $p \leq 0.05$). On days 3 (decreased by 34%), 7 (decreased by 42%) and 14 (decreased by 46%) CXCL1 level was different between saline control and ANG-(1-7) group ($p \leq 0.05$).When compared saline control x ANG-(1-7)/A779 treatment no significant differences were found on days 3, 7 and 14 ($p > 0.05$). ANG-(1-7)/A779 treatment was different to CXCL1 level of ANG-(1-7) group on days 3 (increased by 43%), 7 (increased by 46%) and 14 (increased by 66%) when compared to ANG-(1-7) group ($p \leq 0.05$).

The extent of active monocytes/macrophage accumulation in wound was measured by NAG activity, as shown in figure 5. The level of NAG activity was also changed by treatments. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20} = 6.62$, $p \leq 0.05$), time factor ($F_{3,60} = 92.57$, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20} = 5.19$, $p \leq 0.05$). On days 3 (increased by 90%) and 14 (increased by 42%) NAG activity was different between saline control and ANG-(1-7) group ($p \leq 0.05$) and no significant differences were found on day 7 ($p > 0.05$).When compared saline control x ANG-(1-7)/A779 treatment no significant differences were found on days 3, 7 and 14 ($p > 0.05$). ANG-(1-7)/A779 treatment was different to NAG activity of ANG-(1-7) group only on day 14 (decreased by 28%) when compared to ANG-(1-7) group ($p \leq 0.05$) and no significant differences were found on days 3 and 7 ($p > 0.05$).

Angiogenesis

The extent of vascularization of wounds were assessed by measuring Hb content. As shown in figure 6, Hb content was different between groups. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20} = 12.82$, $p \leq 0.05$),time factor ($F_{3,60} = 304.42$, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor($F_{1,20} = 9.41$, $p \leq 0.05$). On days 7 (increased by 62%) and 14 (increased by 71%) Hb content was different between saline control and ANG-(1-7) group($p \leq 0.05$) and no significant differences were found on day 3 ($p > 0.05$). When compared saline control x ANG-(1-7)/A779 treatment no significant differences were found on days 3,7

and 14(p> 0.05).ANG-(1-7)/A779 treatment was different to Hb content of ANG-(1-7) group on days 7 (decreased by 34%) and 14 (decreased by 33%) ($p\leq0.05$) and no significant differences were found on day 3 (p> 0.05) when compared to ANG-(1-7) group.

As shown in figure 7, VEGF level was different between groups. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20}$ = 7.00, $p\leq0.05$), time factor ($F_{3,60}$ = 122.41, $p\leq0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20}$ = 8.90, $p\leq0.05$). On days 3 (increased by 83%) and 14 (increased by 53%) VEGF level was different between saline control and ANG-(1-7) group ($p\leq0.05$) and no significant differences were found on day 7 (p> 0.05).On days 3,7 and 14 no significant differences were found between saline control x ANG-(1-7)/A779 treatment related to vascular endothelial level. ANG-(1-7)/A779 treatment was different to VEGF level of ANG-(1-7) group on days 3 (decreased by 47%) and 14 (decreased by 44%) ($p\leq0.05$) and no significant differences were found on day 7when compared to ANG-(1-7) group (p> 0.05).

Figure 8 contains data obtained from FGF level, and it was different between groups. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20}$ = 5.30, $p\leq0.05$), time factor ($F_{3,60}$ = 262,63, $p\leq0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20}$ = 6.59, $p\leq0.05$).During the time FGF level was different between saline control and ANG-(1-7) group, as shown on days 3 (increased by 47%) 7 (increased by 24%) and 14 (increased by 25%) ($p\leq0.05$). On days 3, 7 and 14 no significant differences were found between saline control x ANG-(1-7)/A779.ANG-(1-7)/A779 treatment was different to FGF level of ANG-(1-7) group on days 3 (decreased by 32%), 7(decreased by 23%) and 14 (decreased by 22%) when compared to ANG-(1-7) group ($p\leq0.05$).

Blood flow measurement using a laser Doppler perfusion imagewas different between groups, as shown in figure 9. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,76}$ = 46.36, $p\leq0.05$), time factor ($F_{4,304}$ = 374.20, $p\leq0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,76}$ = 61.90, $p\leq0.05$).Blood flow level was different between saline control and ANG-(1-7) group, as shown on days 3 (increased by 32%), 7 (increased by 59%), 10 (increased by 71%) and 14 (increased by 204%) ($p\leq0.05$). On days 3, 7, 10 and 14 no significant differences were found between saline control x ANG-(1-7)/A779. ANG-(1-7)/A779 treatment was different to blood flow level of ANG-(1-7) group on days 3 (decreased by 30%), 7

(decreased by 44%), 10 (decreased by 48%) and 14 (decreased by 69%) when compared to ANG-(1-7) group ($p \leq 0.05$).

Fibrogenesis

Total soluble collagen content was different between groups, as shown in figure 10. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,20} = 9.03$, $p \leq 0.05$), time factor ($F_{3,60} = 474.12$, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20} = 10.06$, $p \leq 0.05$). Collagen content was different between saline control and ANG-(1-7) group, as shown on days 7 (increased by 43%) and 14 (increased by 39%) ($p \leq 0.05$) and no significant differences were found on day 3 ($p > 0.05$). On days 3, 7 and 14 no significant differences were found between saline control x ANG-(1-7)/A779. ANG-(1-7)/A779 treatment was different to collagen content of ANG-(1-7) group on days 7 (decreased by 28%)and 14 (decreased by 31%) when compared to ANG-(1-7) group ($p \leq 0.05$)and no significant differences were found on day 3 ($p > 0.05$).

Transforming growth factor beta isoform 1(TGF-β1) is a protein that controls proliferation, cellular differentiation, and other functions in most cells. As shown in figure 11, the three-way ANOVA showed significant differences for time factor ($F_{3,60} = 136.37$, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,20} = 5.13$, $p \leq 0.05$) and no significant differences were found for ANG-(1-7) factor TGF-β1levelwas different between saline control and ANG-(1-7) group only on day 14 (increased by 51%) ($p \leq 0.05$) and no significant differences were found on day 3 and 7($p > 0.05$). On days 3, 7 and 14 no significant differences were found between saline control x ANG-(1-7)/A779. ANG-(1-7)/A779 treatment was different to collagen TGF-β1 level of ANG-(1-7) group only on day 14 (decreased by 38%) when compared to ANG-(1-7) group ($p \leq 0.05$) and no significant differences were found on day 3 and 7($p > 0.05$).

Wounding closure level was different between groups, as shown in figure 12. The three-way ANOVA showed significant differences for ANG-(1-7) factor ($F_{1,76} = 43.54$, $p \leq 0.05$), time factor ($F_{4,304} = 984.08$, $p \leq 0.05$) and the interaction between ANG-(1-7) x A779 factor ($F_{1,76} = 4.94$, $p \leq 0.05$). Wound closure level was different between saline control and ANG-(1-7) group, as shown on days 7 (increased by 73%), 10 (increased by 29%) and 14 (increased by 33%) ($p \leq 0.05$) and no significant differences were found on day 3 ($p > 0.05$). Significant differences were found in ANG-(1-7)/A779

on days 10 (decreased by 12%) and 14 (decreased by 10%) when compared to saline control group ($p \leq 0.05$)and no significant differences on wound closure were found on day 3 and 7 ($p > 0.05$). ANG-(1-7)/A779 treatment was different to wound closure level on days 7 (decreased by 31%), 10 (decreased by 32%) and 14 (decreased by 31%) when compared to ANG-(1-7) group ($p \leq 0.05$) and no significant differences were found on day 3 ($p > 0.05$).

Figures

FIGURE 1

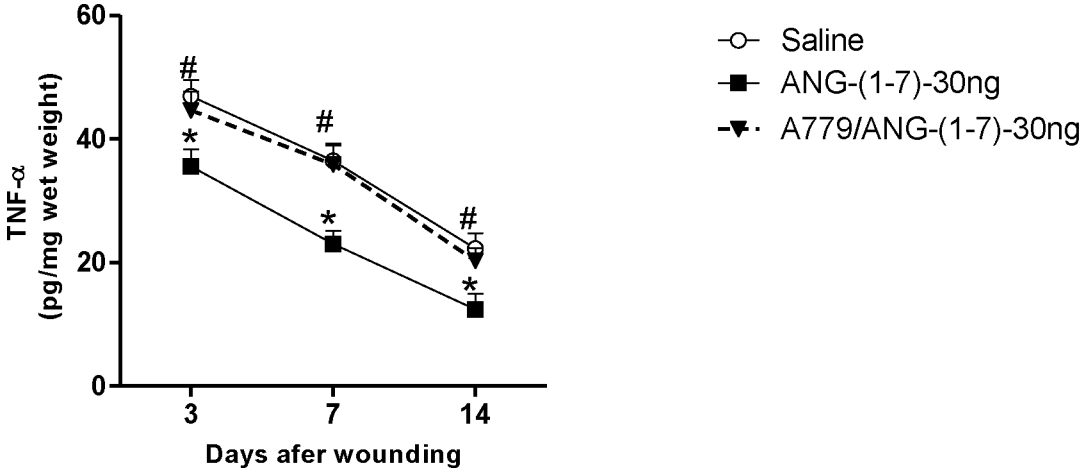


Figure 1 – TNF-α level on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. TNF-α level decreased after3, 7 and 14 days between saline control and ANG-(1-7) group ($p \leq 0.05$). TNF-α level increased after 3, 7 and 14 days after treatment with A779/ANG-(1-7) when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 2

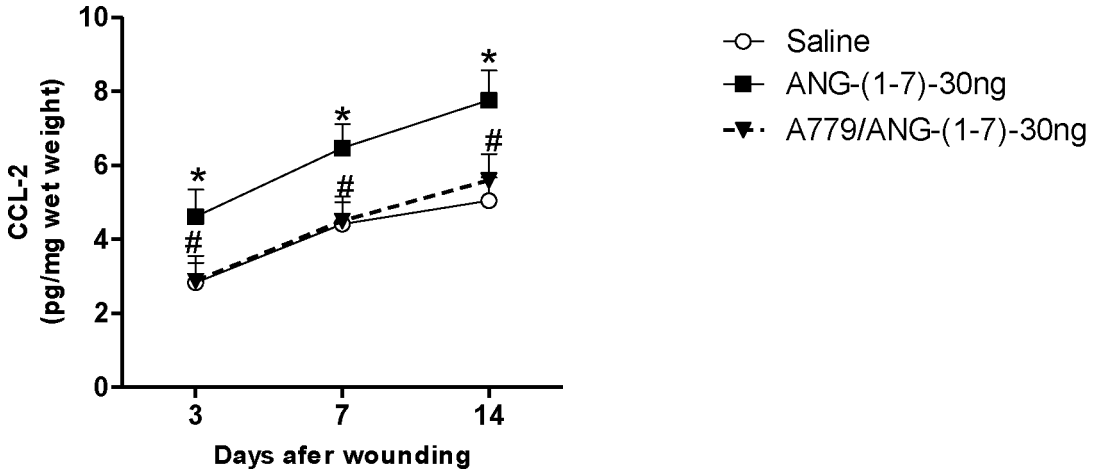


Figure 2 – CCL2 (MCP1/JE) level on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds.CCL2level was increased after 3, 7 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group($p \leq 0.05$). This activity was decreased after treatment A779/ANG-(1-7) on days 3, 7 and 14) when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 3

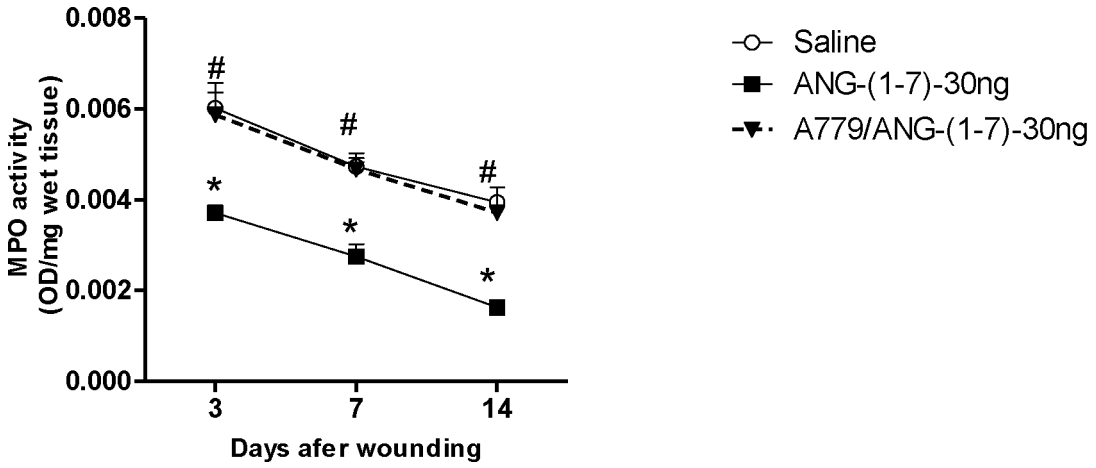


Figure 3 – Neutrophil content on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. MPO content was increased after 3, 7 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$). This activity was decreased after treatment A779/ANG-(1-7) on days 3, 7 and 14when compared to ANG-(1-7) group ($p \leq 0.05$).Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 4

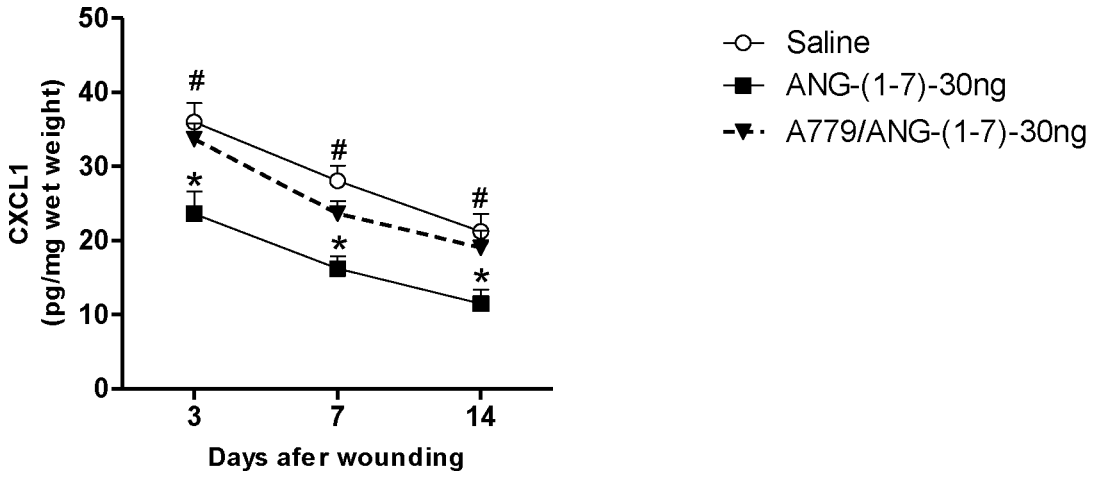


Figure 4 – CXCL1 (KC) level on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds.CXCL1level was decreased after 3, 7 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$). This activity was increased after treatment A779/ANG-(1-7) on days 3, 7 and 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 5

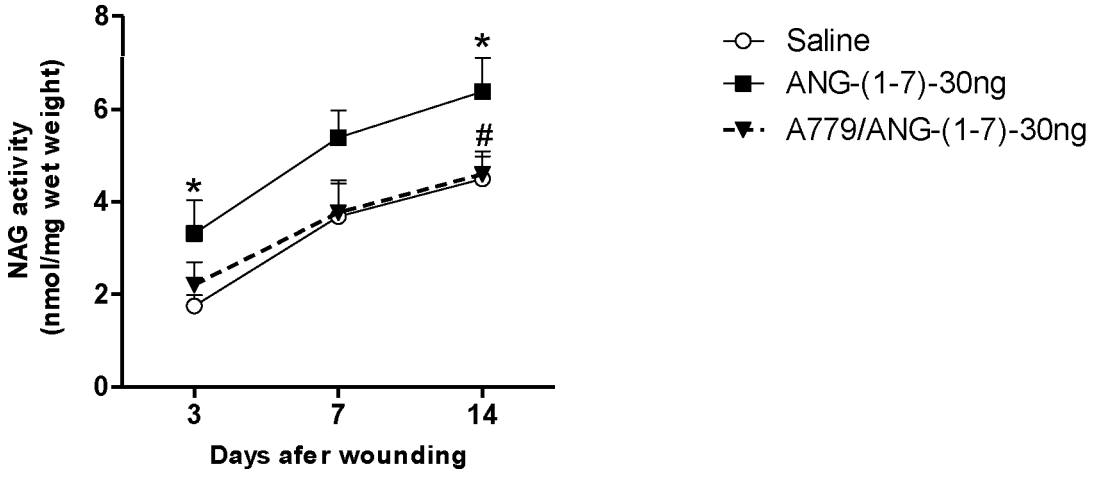


Figure 5 – Extent of active monocytes/macrophage accumulation was assessed on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. NAG activity was increased after 3 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$). This activity was decreased after treatment A779/ANG-(1-7) only on day 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 6

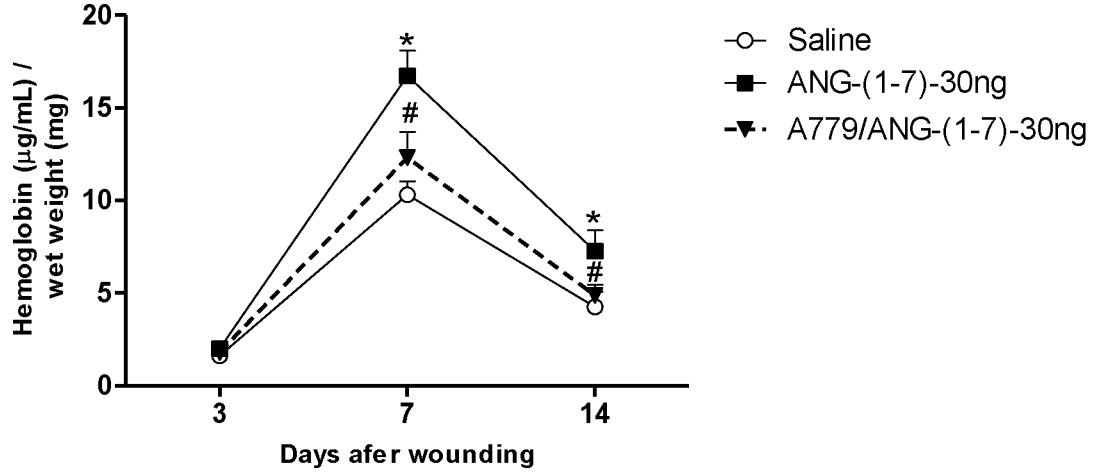


Figure 6 – Extent of vascularization was assessed on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. Hb content was increased after 7 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group

($p \leq 0.05$). This activity was decreased after treatment A779/ANG-(1-7) on days 7 and 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 7

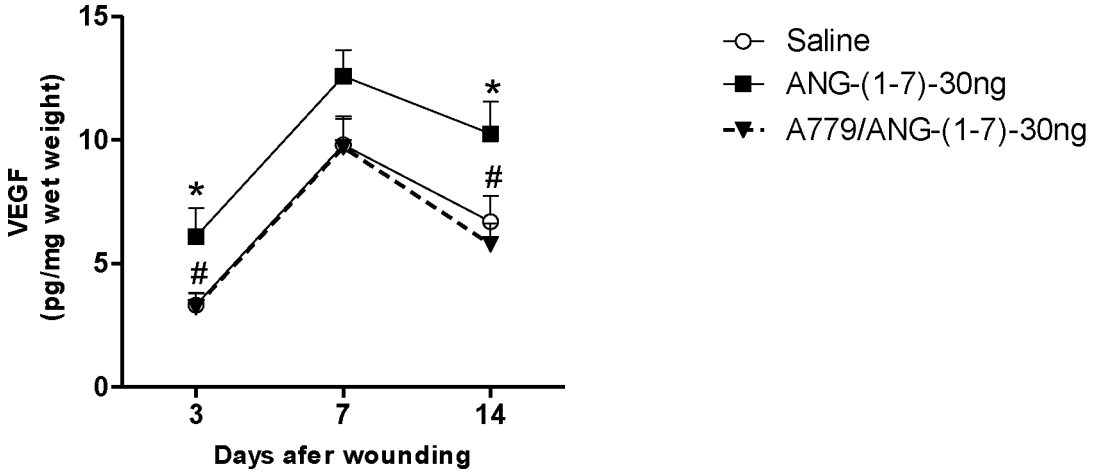


Figure 7 – VEGF level on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. VEGF level was increased after 3 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$). This activity was decreased after treatment A779/ANG-(1-7) on days 3 and 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 8

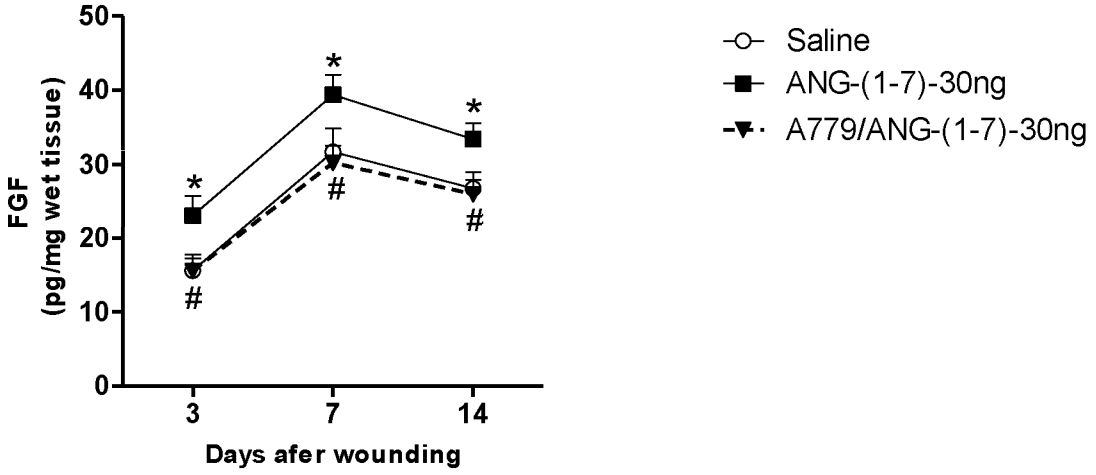
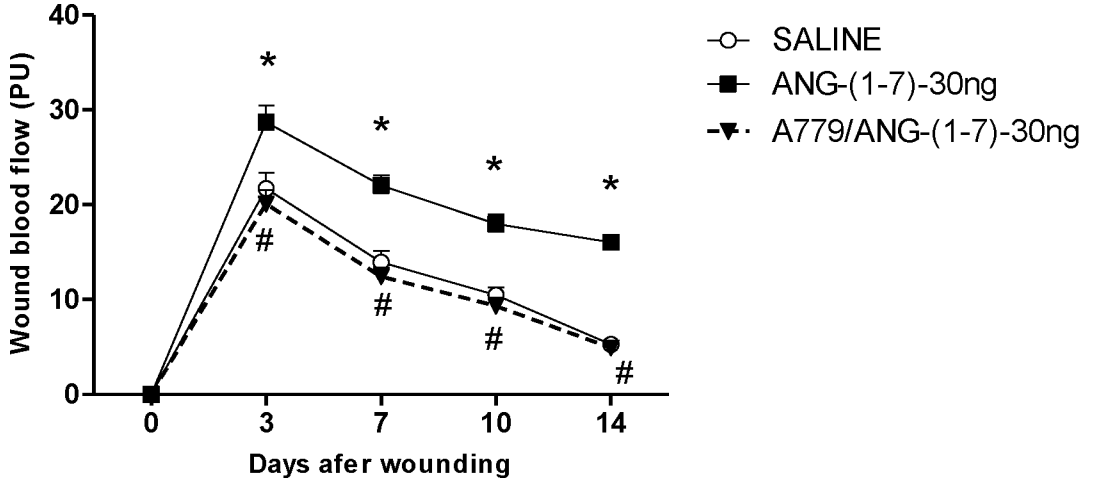


Figure 8 – FGF level on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. FGF level was increased after 3, 7 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$). This activity was decreased after treatment A779/ANG-(1-7) on days 3, 7 and 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 9



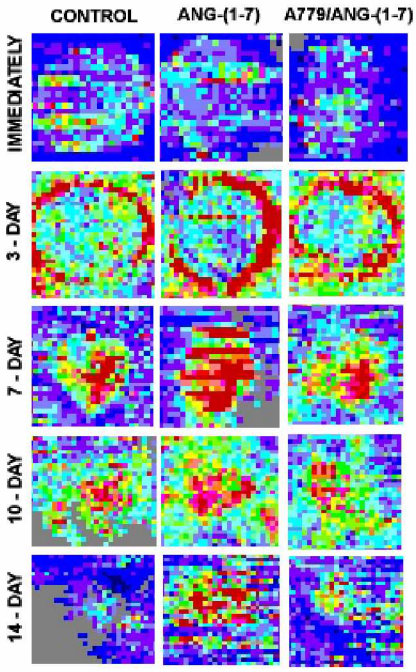


Figure 9 – Blood flow measurement using a laser Doppler perfusion image on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. Blood flow was increased after 3, 7, 10 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$). This level was decreased after treatment A779/ANG-(1-7) on days 3,7,10 and 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group

FIGURE 10

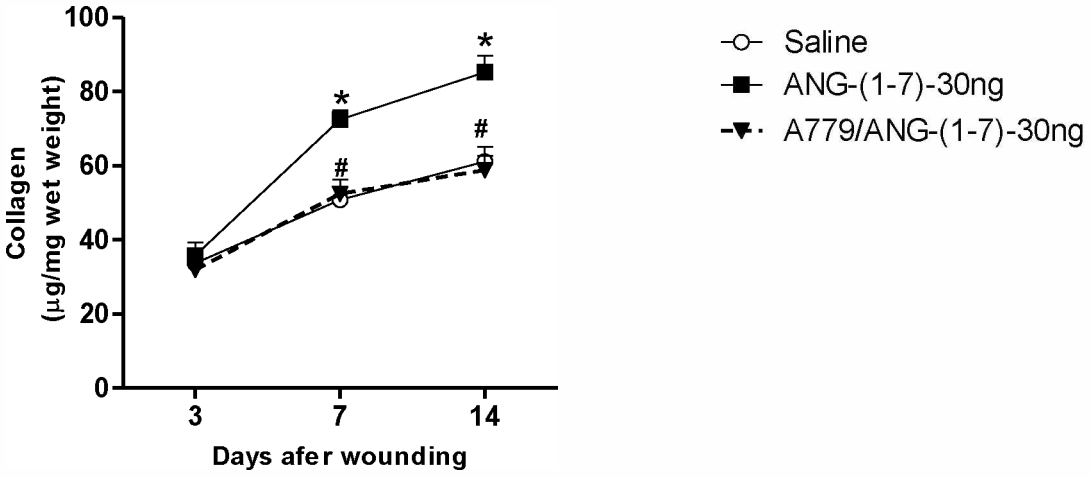


Figure 10 – Collagen soluble content on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. Total soluble collagen activity was increased after 7 and 14 days of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$).This activity was decreased after treatment A779/ANG-(1-7) on days 7 and 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 11

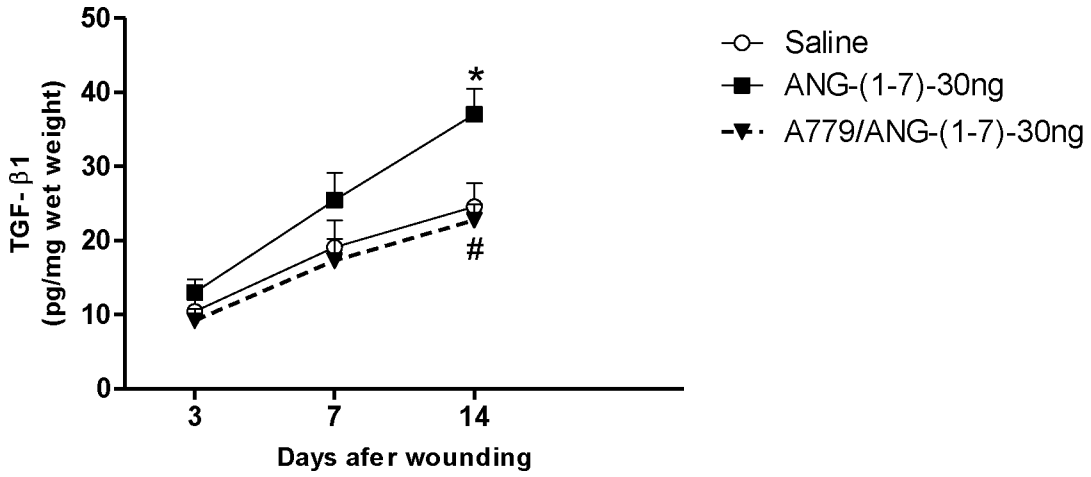


Figure 11 – TGF-β1 level on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. TGF-β1 level was increased only on day 14 of treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$).This activity was decreased after treatment A779/ANG-(1-7) only on day 14 when compared to ANG-(1-7) group ($p \leq 0.05$). Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

FIGURE 12

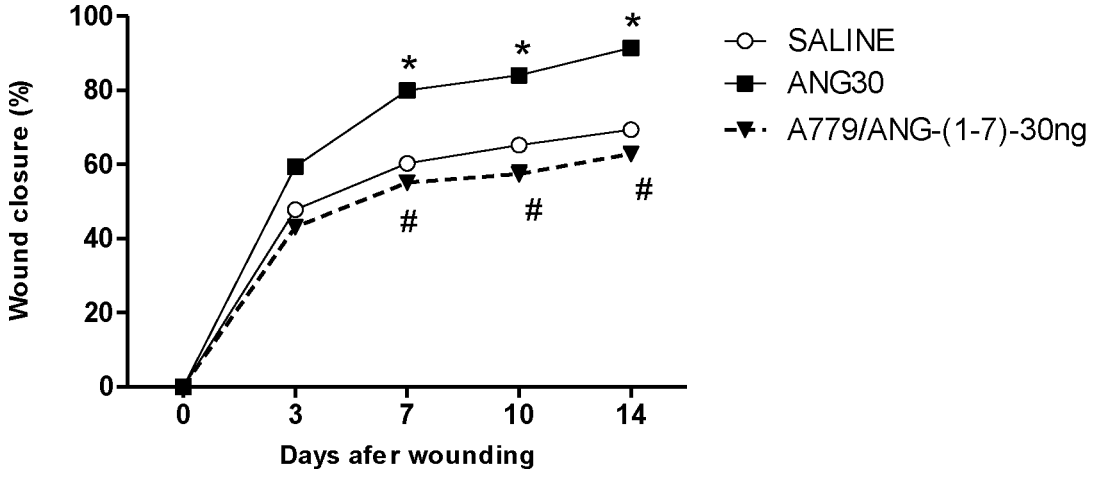


Figure 12 – Wounding closure level on different treatment times of Angiotensin-(1-7)-30ng treatment in wounds. Wound closure level was increased after 7, 10 and 14 days treatment with ANG-(1-7)-30ng dose when compared to saline control group ($p \leq 0.05$).This level decreased after treatment A779/ANG-(1-7) on days 10 and 14 when compared to saline control group ($p \leq 0.05$). ANG-(1-7)/A779 treatment decreased wound closure level on days 7, 10 and 14 when compared to ANG-(1-7) group ($p \leq 0.05$) Data are as mean ±SEM. (*) $p \leq 0.05$ *vs.* saline control. (#)$p \leq 0.05$ *vs.* ANG-(1-7). $n = 6$ mice for each group.

Discussion

Ang-(1-7) is a heptapeptide which can be formed directly from Ang II by angiotensin converting enzyme 2 (ACE2) or other peptidases [126].The actions of Ang-(1-7) are related to MAS activation, a seven-transmembrane domain G-protein-coupled receptor[56, 126], and has been associated to anti-inflammatory, anti-angiogenic and anti-fibrotic effects in different animal models [56] . However, the effects of Ang-(1-7) on skin wound healing, a process where inflammation, angiogenesis, and fibrogenesis are associated and critical, are not known. Therefore, in this study, we investigated the effects of Ang-(1-7) on the closure rate and the angiogenic, inflammatory and fibrogenic components of excisional skin wounds in mice.

Wound healing is a complex, biological process which concerns replacing damaged tissue by a living one [126, 127], and it is initiated by the hemostasis stage, which is connected with forming a temporary matrix, secreting cytokines and other growth factors, and interaction of the latter ones with extracellular matrix components,

which initiates the repairing process, preparing the wound bed to the next stage of the process, the inflammatory stage [126, 128, 129]. CXCL1/KC is a chemokine responsible for the recruitment and activation of neutrophils and the content of neutrophils may be directly related to its levels. In our study, the MPO activity (an indirect measurement of neutrophils content), CXCL1 and TNF-alpha levels were kept reduced during treatment with Ang-(1-7)when compared with the control group and the ANG-(1-7)+A779 group, suggesting Ang-(1-7) has anti-inflammatory effects on skin wounds and this effect is dependent on MAS receptor activation. In accordance, recently, Silveira *et al.* (2010) demonstrated anti-inflammatory effects of Ang-(1-7) in experimental models of inflammatory arthritis[63]. Those proinflammatory cytokines, TNF and CXCL1,are related to the recruitment of inflammatory cells, including polymorphonuclear cells and macrophages into the wound. Once in the wound, inflammatory cells become activated and produce a range of inflammatory cytokines that can stimulate the healing process, although it can perpetuate and chronifying inflammatory cell accumulation and activation if not controlled[130, 131]. Therefore, modulating inflammatory cell accumulation into skin wounds could improve tissue healing.

In addition, we observed that during treatment with Ang-(1-7) there was an increasing in CCL2/MCP-1 levels and NAG activity, suggesting the macrophage accumulation was increased by Ang-(1-7). The antagonist A779 abolished these effects, suggesting they are dependent on MAS receptor activation. Sinno & Prakash (2013) [127] reported that the extravasation of blood constituents provides the formation of the blood clot reinforcing the hemostatic plug. Platelets secrete several chemokines, among them CCL2, and these factors help to stabilizing the clot and also to attracting and activate macrophages and fibroblasts that are important to the healing process [127, 132].Although more efforts are needed to fully access the macrophage profile under Ang-(1-7) treatment, one could speculate that the increased accumulation of macrophages observed in this work is qualitatively related to a healing-prone macrophage phenotype.

Angiogenesis is a process of creating new blood vessels, in which restores blood circulation in the place of damage and prevents the development of ischemic necrosis simultaneously stimulating the tissue repair process [133, 134]. Many soluble mediators, such as bFGF, TGF-β, and VEGF secreted by epithelial cells, fibroblasts, endothelial cells, and macrophages demonstrate a strong proangiogenic activity [127,

134, 135].Interestingly, here we observed that Ang-(1-7) local treatment increased the levels bFGF, TGF-β1, and VEGF on wound tissue. In addition, and with a strong correlation with this data, we observed an increasing the blood flow and in the hemoglobin content of wounds, suggesting that angiogenesis is stimulated under Ang-(1-7) treatment. All these affects were also abolished in animals that received the antagonist A779, suggesting the effects of Ang-(1-7) are dependent on MAS activation.

Besides new blood vessels, granulation tissue is also constituted of collagen, elastin, proteoglycans, glycosaminoglycans, and non-collagenous proteins synthesized mainly by fibroblasts whose activity is regulated by PDGF and TGF-β [136]. The matrix of the early granulation tissue also contains great amount of hyaluronic acid and fibronectin that creates a "scaffold" facilitating the deposition and maturation of collagen [135]. Collagen deposition is, however, a vital component of the new tissue contraction and stability and its deposition and organization are progressively altered to generate a denser matrix [76]. In relation to matrix deposition, our data showed that treatment with Ang-(1-7) was able to increase total soluble collagen content and TGF-β1levels in wounds and that these effects seems to be crucially dependent on MAS activation, since they were inhibited when mice were treated with the antagonist A779.

To our knowledge, this is the first demonstration that Ang-(1-7) improves the skin wound healing process. Overall, our results shows that local treatment with Ang-(1-7) accelerated wound closure by inhibiting neutrophil accumulation and stimulating the formation of a new fibrovascular tissue as assessed by a combination of biochemical and functional approaches. Interestingly, macrophage content was increased under treatment with Ang-(1-7). Blockade of MAS activation by using the antagonist A779 fully inhibited Ang-(1-7) effects. In conclusion, our data suggests local treatment with Ang-(1-7) improves skin wound healing in a MAS receptor-dependent manner.

Conflict of interest statement

There are no conflicts of interest with regard to our manuscript.

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References

1. *Okin, D. and R. Medzhitov, Evolution of inflammatory diseases. Curr Biol, 2012.* ***22****(17): p. R733-40.*
2. *Libby, P., Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutr Rev, 2007.* ***65****(12 Pt 2): p. S140-6.*
3. *Barton, G.M., A calculated response: control of inflammation by the innate immune system. J Clin Invest, 2008.* ***118****(2): p. 413-20.*
4. *Pober, J.S. and W.C. Sessa, Evolving functions of endothelial cells in inflammation. Nat Rev Immunol, 2007.* ***7****(10): p. 803-15.*
5. *Burg, N.D. and M.H. Pillinger, The neutrophil: function and regulation in innate and humoral immunity. Clin Immunol, 2001.* ***99****(1): p. 7-17.*
6. *Charo, I.F. and R.M. Ransohoff, The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med, 2006.* ***354****(6): p. 610-21.*
7. *Charo, I.F. and M.B. Taubman, Chemokines in the pathogenesis of vascular disease. Circ Res, 2004.* ***95****(9): p. 858-66.*
8. *de Visser, K.E., A. Eichten, and L.M. Coussens, Paradoxical roles of the immune system during cancer development. Nat Rev Cancer, 2006.* ***6****(1): p. 24-37.*
9. *Filho, G.B.B., Patologia. 7a. ed, ed. 7a. 2006, Rio de Janeiro: Guanabara Koogan.*
10. *Moore, M.M., et al., Inflammation and cancer: causes and consequences. Clin Pharmacol Ther, 2010.* ***87****(4): p. 504-8.*
11. *Hasko, G. and P. Pacher, Regulation of macrophage function by adenosine. Arterioscler Thromb Vasc Biol, 2012.* ***32****(4): p. 865-9.*
12. *Kolaczkowska, E. and P. Kubes, Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol, 2013.* ***13****(3): p. 159-75.*
13. *Ortega-Gomez, A., M. Perretti, and O. Soehnlein, Resolution of inflammation: an integrated view. EMBO Mol Med, 2013.* ***5****(5): p. 661-74.*

14. *Schultz, G.S. and A. Wysocki, Interactions between extracellular matrix and growth factors in wound healing. Wound Repair Regen, 2009.* ***17****(2): p. 153-62.*
15. *Medzhitov, R., Inflammation 2010: new adventures of an old flame. Cell.* ***140****(6): p. 771-6.*
16. *Szekanecz, Z. and A.E. Koch, Vascular endothelium and immune responses: implications for inflammation and angiogenesis. Rheum Dis Clin North Am, 2004.* ***30****(1): p. 97-114.*
17. *Ferguson, M.E., et al., Results of intravascular stent placement for fibrosing mediastinitis. Congenit Heart Dis, 2010.* ***5****(2): p. 124-33.*
18. *Wynn, T.A., A. Chawla, and J.W. Pollard, Macrophage biology in development, homeostasis and disease. Nature, 2013.* ***496****(7446): p. 445-55.*
19. *Hotamisligil, G.S., Inflammation and metabolic disorders. Nature, 2006.* ***444****(7121): p. 860-7.*
20. *Tracey, K.J., The inflammatory reflex. Nature, 2002.* ***420****(6917): p. 853-9.*
21. *Mrowietz, U. and W.H. Boehncke, Leukocyte adhesion: a suitable target for anti-inflammatory drugs. Curr Pharm Des, 2006.* ***12****(22): p. 2825-31.*
22. *van Zuijlen, P.P., et al., Scar assessment tools: implications for current research. Plast Reconstr Surg, 2002.* ***109****(3): p. 1108-22.*
23. *Balbino, C.A.P., L.M.; Curi, R., Mecanismos envolvidos na cicatrização: uma revisão. Rev Bras Ciências Farmacêuticas, 2005.* ***41****(1): p. 27-51.*
24. *Lazarus, G.S., et al., Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol, 1994.* ***130****(4): p. 489-93.*
25. *Diegelmann, R.F. and M.C. Evans, Wound healing: an overview of acute, fibrotic and delayed healing. Front Biosci, 2004.* ***9****: p. 283-9.*
26. *Roberts, A.B. and M.B. Sporn, Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors, 1993.* ***8****(1): p. 1-9.*
27. *Hall, M.C., et al., The comparative role of activator protein 1 and Smad factors in the regulation of Timp-1 and MMP-1 gene expression by transforming growth factor-beta 1. J Biol Chem, 2003.* ***278****(12): p. 10304-13.*
28. *Vorstenbosch, J., et al., Transgenic mice overexpressing CD109 in the epidermis display decreased inflammation and granulation tissue and improved collagen architecture during wound healing. Wound Repair Regen, 2013.* ***21****(2): p. 235-46.*
29. *Hinz, B., Formation and function of the myofibroblast during tissue repair. J Invest Dermatol, 2007.* ***127****(3): p. 526-37.*

30. *Hendricks, T., et al., Inhibition of basal and TGF beta-induced fibroblast collagen synthesis by antineoplastic agents. Implications for wound healing. Br J Cancer, 1993.* ***67****(3): p. 545-50.*
31. *Duffield, J.S., The inflammatory macrophage: a story of Jekyll and Hyde. Clin Sci (Lond), 2003.* ***104****(1): p. 27-38.*
32. *Hunt, T.K., et al., Studies on inflammation and wound healing: angiogenesis and collagen synthesis stimulated in vivo by resident and activated wound macrophages. Surgery, 1984.* ***96****(1): p. 48-54.*
33. *Tonnesen, M.G., X. Feng, and R.A. Clark, Angiogenesis in wound healing. J Investig Dermatol Symp Proc, 2000.* ***5****(1): p. 40-6.*
34. *Nissen, N.N., et al., Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am J Pathol, 1998.* ***152****(6): p. 1445-52.*
35. *Shenoy, V., et al., The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. Am J Respir Crit Care Med, 1997.* ***182****(8): p. 1065-72.*
36. *Eming, S.A., et al., Regulation of angiogenesis: wound healing as a model. Prog Histochem Cytochem, 2007.* ***42****(3): p. 115-70.*
37. *Stramer, B.M., R. Mori, and P. Martin, The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. J Invest Dermatol, 2007.* ***127****(5): p. 1009-17.*
38. *Dovi, J.V., L.K. He, and L.A. DiPietro, Accelerated wound closure in neutrophil-depleted mice. J Leukoc Biol, 2003.* ***73****(4): p. 448-55.*
39. *Pierce, G.F., Inflammation in nonhealing diabetic wounds: the space-time continuum does matter. Am J Pathol, 2001.* ***159****(2): p. 399-403.*
40. *Costa, C., J. Incio, and R. Soares, Angiogenesis and chronic inflammation: cause or consequence? Angiogenesis, 2007.* ***10****(3): p. 149-66.*
41. *Gurtner, G.C., et al., Wound repair and regeneration. Nature, 2008.* ***453****(7193): p. 314-21.*
42. *Shaw, T.J. and P. Martin, Wound repair at a glance. J Cell Sci, 2009.* ***122****(Pt 18): p. 3209-13.*
43. *Hatanaka, E.C., R., Ácidos graxos e cicatrização: uma revisão. . Rev Bras Farmacologia, 2007.* ***88****(2): p. 53-58.*
44. *Shimizu, N., et al., Development of a functional wound dressing composed of hyaluronic acid spongy sheet containing bioactive components: evaluation of wound healing potential in animal tests. J Biomater Sci Polym Ed, 2014: p. 1-14.*
45. *Howdieshell, T.R., et al., Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation. J Surg Res, 2001.* ***96****(2): p. 173-82.*

46. *Montesano, R. and L. Orci, Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing. Proc Natl Acad Sci U S A, 1988.* ***85****(13): p. 4894-7.*
47. *Beanes, S.R., et al., Skin repair and scar formation: the central role of TGF-beta. Expert Rev Mol Med, 2003.* ***5****(8): p. 1-22.*
48. *Clark, R.A., Biology of dermal wound repair. Dermatol Clin, 1993.* ***11****(4): p. 647-66.*
49. *Johnston, D.E., Wound healing in skin. Vet Clin North Am Small Anim Pract, 1990.* ***20****(1): p. 1-25.*
50. *Ackerly, J.A., A.F. Moore, and M.J. Peach, Demonstration of different contractile mechanisms for angiotensin II and des-Asp1-angiotensin II in rabbit aortic strips. Proc Natl Acad Sci U S A, 1977.* ***74****(12): p. 5725-8.*
51. *Santos, R.A., A.J. Ferreira, and E.S.A.C. Simoes, Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis. Exp Physiol, 2008.* ***93****(5): p. 519-27.*
52. *Carey, R.M., Angiotensin type-2 receptors and cardiovascular function: are angiotensin type-2 receptors protective? Curr Opin Cardiol, 2005.* ***20****(4): p. 264-9.*
53. *Ferrario, C.M., et al., Counterregulatory actions of angiotensin-(1-7). Hypertension, 1997.* ***30****(3 Pt 2): p. 535-41.*
54. *Greene, L.J., et al., Brain endo-oligopeptidase B: a post-proline cleaving enzyme that inactivates angiotensin I and II. Hypertension, 1982.* ***4****(2): p. 178-84.*
55. *Schiavone, M.T., et al., Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1-7) heptapeptide. Proc Natl Acad Sci U S A, 1988.* ***85****(11): p. 4095-8.*
56. *Santos, R.A., et al., Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci U S A, 2003.* ***100****(14): p. 8258-63.*
57. *Donoghue, M., et al., A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res, 2000.* ***87****(5): p. E1-9.*
58. *Tallant, E.A., D.I. Diz, and C.M. Ferrario, State-of-the-Art lecture. Antiproliferative actions of angiotensin-(1-7) in vascular smooth muscle. Hypertension, 1999.* ***34****(4 Pt 2): p. 950-7.*
59. *Pan, C.H., C.H. Wen, and C.S. Lin, Interplay of angiotensin II and angiotensin(1-7) in the regulation of matrix metalloproteinases of human cardiocytes. Exp Physiol, 2008.* ***93****(5): p. 599-612.*
60. *Esteban, V., et al., Angiotensin-(1-7) and the g protein-coupled receptor MAS are key players in renal inflammation. PLoS One, 2009.* ***4****(4): p. e5406.*

61. *Cook, K.L., et al., Angiotensin-(1-7) reduces fibrosis in orthotopic breast tumors. Cancer Res, 2010.* ***70****(21): p. 8319-28.*
62. *Shenoy, V., et al., The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. Am J Respir Crit Care Med, 2010.* ***182****(8): p. 1065-72.*
63. *da Silveira, K.D., et al., Anti-inflammatory effects of the activation of the angiotensin-(1-7) receptor, MAS, in experimental models of arthritis. J Immunol, 2010.* ***185****(9): p. 5569-76.*
64. *Nabah, Y.N., et al., Angiotensin II induces neutrophil accumulation in vivo through generation and release of CXC chemokines. Circulation, 2004.* ***110****(23): p. 3581-6.*
65. *Abu Nabah, Y.N., et al., CXCR2 blockade impairs angiotensin II-induced CC chemokine synthesis and mononuclear leukocyte infiltration. Arterioscler Thromb Vasc Biol, 2007.* ***27****(11): p. 2370-6.*
66. *Hagiwara, S., et al., Effects of an angiotensin-converting enzyme inhibitor on the inflammatory response in in vivo and in vitro models. Crit Care Med, 2009.* ***37****(2): p. 626-33.*
67. *Silver, R.B., et al., Mast cells: a unique source of renin. Proc Natl Acad Sci U S A, 2004.* ***101****(37): p. 13607-12.*
68. *Nahmod, K.A., et al., Control of dendritic cell differentiation by angiotensin II. FASEB J, 2003.* ***17****(3): p. 491-3.*
69. *Theuer, J., et al., Angiotensin II induced inflammation in the kidney and in the heart of double transgenic rats. BMC Cardiovasc Disord, 2002.* ***2****: p. 3.*
70. *Chiba, T. and K. Umegaki, Pivotal roles of monocytes/macrophages in stroke. Mediators Inflamm, 2013.* ***2013****: p. 759103.*
71. *Wassmann, S., et al., Interleukin-6 induces oxidative stress and endothelial dysfunction by overexpression of the angiotensin II type 1 receptor. Circ Res, 2004.* ***94****(4): p. 534-41.*
72. *Alvarez, A., et al., Direct evidence of leukocyte adhesion in arterioles by angiotensin II. Blood, 2004.* ***104****(2): p. 402-8.*
73. *Jacobi, J., et al., Exogenous superoxide mediates pro-oxidative, proinflammatory, and procoagulatory changes in primary endothelial cell cultures. Free Radic Biol Med, 2005.* ***39****(9): p. 1238-48.*
74. *Guo, Y.J., et al., ACE2 overexpression inhibits angiotensin II-induced monocyte chemoattractant protein-1 expression in macrophages. Arch Med Res, 2008.* ***39****(2): p. 149-54.*
75. *Weber, K.T., Fibrosis, a common pathway to organ failure: angiotensin II and tissue repair. Semin Nephrol, 1997.* ***17****(5): p. 467-91.*

76. *Li, J., Y.P. Zhang, and R.S. Kirsner, Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. Microsc Res Tech, 2003.* ***60****(1): p. 107-14.*
77. *Oudit, G.Y., et al., Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice. Cardiovasc Res, 2007.* ***75****(1): p. 29-39.*
78. *Grindlay, J.H. and J.M. Waugh, Plastic sponge which acts as a framework for living tissue; experimental studies and preliminary report of use to reinforce abdominal aneurysms. AMA Arch Surg, 1951.* ***63****(3): p. 288-97.*
79. *Andrade, S.P., T.P. Fan, and G.P. Lewis, Quantitative in-vivo studies on angiogenesis in a rat sponge model. Br J Exp Pathol, 1987.* ***68****(6): p. 755-66.*
80. *Andrade, S.P., et al., Effects of platelet activating factor (PAF) and other vasoconstrictors on a model of angiogenesis in the mouse. Int J Exp Pathol, 1992.* ***73****(4): p. 503-13.*
81. *Araújo, F.A., Avaliação dos efeitos de estatinas na angiogênese inflamatória em camundongos, in Department of Physiology and Biophysics. 2009, Universidade Federal de Minas Gerais: Belo Horizonte. p. 153.*
82. *Araujo, F.A., et al., Atorvastatin inhibits inflammatory angiogenesis in mice through down regulation of VEGF, TNF-alpha and TGF-beta1. Biomed Pharmacother, 2010.* ***64****(1): p. 29-34.*
83. *Guabiraba, R., et al., Blockade of cannabinoid receptors reduces inflammation, leukocyte accumulation and neovascularization in a model of sponge-induced inflammatory angiogenesis. Inflamm Res, 2013.* ***62****(8): p. 811-21.*
84. *Mendes, J.B., et al., Host response to sponge implants differs between subcutaneous and intraperitoneal sites in mice. J Biomed Mater Res B Appl Biomater, 2007.* ***83****(2): p. 408-15.*
85. *Andrade, S.P., et al., Sponge-induced angiogenesis in mice and the pharmacological reactivity of the neovasculature quantitated by a fluorimetric method. Microvasc Res, 1997.* ***54****(3): p. 253-61.*
86. *Meng, W., et al., Autocrine and Paracrine Function of Angiotensin 1-7 in Tissue Repair During Hypertension. Am J Hypertens, 2014.*
87. *Simoes e Silva, A.C., et al., ACE2, angiotensin-(1-7) and Mas receptor axis in inflammation and fibrosis. Br J Pharmacol, 2013.* ***169****(3): p. 477-92.*
88. *Machado, R.D., R.A. Santos, and S.P. Andrade, Opposing actions of angiotensins on angiogenesis. Life Sci, 2000.* ***66****(1): p. 67-76.*
89. *Machado, R.D., R.A. Santos, and S.P. Andrade, Mechanisms of angiotensin-(1-7)-induced inhibition of angiogenesis. Am J Physiol Regul Integr Comp Physiol, 2001.* ***280****(4): p. R994-R1000.*

90. *Phillips, R.J., et al., Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest, 2004.* ***114****(3): p. 438-46.*
91. *Campos, P.P., Y.S. Bakhle, and S.P. Andrade, Mechanisms of wound healing responses in lupus-prone New Zealand White mouse strain. Wound Repair Regen, 2008.* ***16****(3): p. 416-24.*
92. *Araujo, F.A., et al., Implant-induced intraperitoneal inflammatory angiogenesis is attenuated by fluvastatin. Clin Exp Pharmacol Physiol, 2011.* ***38****(4): p. 262-8.*
93. *Barcelos, L.S., et al., Production and in vivo effects of chemokines CXCL1-3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice. Inflamm Res, 2004.* ***53****(10): p. 576-84.*
94. *Puchtler, H., et al., Methacarn (methanol-Carnoy) fixation. Practical and theoretical considerations. Histochemie, 1970.* ***21****(2): p. 97-116.*
95. *Leite, S.N., et al., Experimental models of malnutrition and its effect on skin trophism. An Bras Dermatol, 2011.* ***86****(4): p. 681-8.*
96. *Pereira, N.B., et al., Apoptosis, mast cell degranulation and collagen breakdown in the pathogenesis of loxoscelism in subcutaneously implanted sponges. Toxicon, 2014.*
97. *Puchtler, H., F.S. Waldrop, and L.S. Valentine, Polarization microscopic studies of connective tissue stained with picro-sirius red FBA. Beitr Pathol, 1973.* ***150****(2): p. 174-87.*
98. *Junqueira, L.C., G. Bignolas, and R.R. Brentani, Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J, 1979.* ***11****(4): p. 447-55.*
99. *Deconte, S.R., et al., Alterations of antioxidant biomarkers and type I collagen deposition in the parotid gland of streptozotocin-induced diabetic rats. Arch Oral Biol, 2011.* ***56****(8): p. 744-51.*
100. *Deryugina, E.I. and J.P. Quigley, Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: contrasting, overlapping and compensatory functions. Biochim Biophys Acta, 2010.* ***1803****(1): p. 103-20.*
101. *Moura, S.A., et al., Local drug delivery system: inhibition of inflammatory angiogenesis in a murine sponge model by dexamethasone-loaded polyurethane implants. J Pharm Sci, 2011.* ***100****(7): p. 2886-95.*
102. *Belo, A.V., et al., Murine chemokine CXCL2/KC is a surrogate marker for angiogenic activity in the inflammatory granulation tissue. Microcirculation, 2005.* ***12****(7): p. 597-606.*
103. *Ferreira, M.A., et al., Tumor growth, angiogenesis and inflammation in mice lacking receptors for platelet activating factor (PAF). Life Sci, 2007.* ***81****(3): p. 210-7.*

104. *Barcelos, L.S., et al., Role of the chemokines CCL3/MIP-1 alpha and CCL5/RANTES in sponge-induced inflammatory angiogenesis in mice. Microvasc Res, 2009.* ***78****(2): p. 148-54.*
105. *Saraswati, S. and S.S. Agarwal, Strychnine inhibits inflammatory angiogenesis in mice via down regulation of VEGF, TNF-alpha and TGF-beta. Microvasc Res, 2013.* ***87****: p. 7-13.*
106. *Machado, R.D., et al., Vasodilator effect of angiotensin-(1-7) in mature and sponge-induced neovasculature. Regul Pept, 2002.* ***107****(1-3): p. 105-13.*
107. *Mirabelli, P., et al., Early effects of dexamethasone and anti-VEGF therapy in an inflammatory corneal neovascularization model. Exp Eye Res, 2014.* ***125C****: p. 118-127.*
108. *Araujo, F.A., et al., 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitor (fluvastatin) decreases inflammatory angiogenesis in mice. APMIS, 2012.* ***121****(5): p. 422-30.*
109. *Teixeira, A.S. and S.P. Andrade, Glucose-induced inhibition of angiogenesis in the rat sponge granuloma is prevented by aminoguanidine. Life Sci, 1999.* ***64****(8): p. 655-62.*
110. *Benter, I.F., D.I. Diz, and C.M. Ferrario, Cardiovascular actions of angiotensin(1-7). Peptides, 1993.* ***14****(4): p. 679-84.*
111. *Fontes, M.A., et al., Evidence that angiotensin-(1-7) plays a role in the central control of blood pressure at the ventro-lateral medulla acting through specific receptors. Brain Res, 1994.* ***665****(1): p. 175-80.*
112. *Santos, R.A., et al., Characterization of a new angiotensin antagonist selective for angiotensin-(1-7): evidence that the actions of angiotensin-(1-7) are mediated by specific angiotensin receptors. Brain Res Bull, 1994.* ***35****(4): p. 293-8.*
113. *Paula, R.D., et al., Angiotensin-(1-7) potentiates the hypotensive effect of bradykinin in conscious rats. Hypertension, 1995.* ***26****(6 Pt 2): p. 1154-9.*
114. *Marshall, J.S., Mast-cell responses to pathogens. Nat Rev Immunol, 2004.* ***4****(10): p. 787-99.*
115. *Jung, M., et al., Mast cells produce novel shorter forms of perlecan that contain functional endorepellin: a role in angiogenesis and wound healing. J Biol Chem, 2013.* ***288****(5): p. 3289-304.*
116. *Lu, D.Y., et al., Mast cell leukemia: An extremely rare disease. J Chin Med Assoc, 2014.*
117. *Arizmendi, N., et al., Rac2 is involved in bleomycin-induced lung inflammation leading to pulmonary fibrosis. Respir Res, 2014.* ***15****: p. 71.*
118. *Ammendola, M., et al., Mast cells density positive to tryptase correlates with angiogenesis in pancreatic ductal adenocarcinoma*

patients having undergone surgery. Gastroenterol Res Pract, 2014. ***2014****: p. 951957.*

119. *Lu, J., et al., Tryptase inhibitor APC 366 prevents hepatic fibrosis by inhibiting collagen synthesis induced by tryptase/protease-activated receptor 2 interactions in hepatic stellate cells. Int Immunopharmacol, 2014.* ***20****(2): p. 352-7.*
120. *Marcus, Y., et al., Angiotensin 1-7 as means to prevent the metabolic syndrome: lessons from the fructose-fed rat model. Diabetes, 2013.* ***62****(4): p. 1121-30.*
121. *El-Hashim, A.Z., et al., Angiotensin-(1-7) inhibits allergic inflammation, via the MAS1 receptor, through suppression of ERK1/2- and NF-kappaB-dependent pathways. Br J Pharmacol, 2012.* ***166****(6): p. 1964-76.*
122. *Feltenberger, J.D., et al., Oral formulation of angiotensin-(1-7) improves lipid metabolism and prevents high-fat diet-induced hepatic steatosis and inflammation in mice. Hypertension, 2013.* ***62****(2): p. 324-30.*
123. *Wilgus, T.A., Growth Factor-Extracellular Matrix Interactions Regulate Wound Repair. Adv Wound Care (New Rochelle), 2012.* ***1****(6): p. 249-254.*
124. *Martin, P., Wound healing--aiming for perfect skin regeneration. Science, 1997.* ***276****(5309): p. 75-81.*
125. *Braund, R., S. Hook, and N.J. Medlicott, The role of topical growth factors in chronic wounds. Curr Drug Deliv, 2007.* ***4****(3): p. 195-204.*
126. *Etelvino, G.M., A.A. Peluso, and R.A. Santos, New components of the renin-angiotensin system: alamandine and the MAS-related G protein-coupled receptor D. Curr Hypertens Rep, 2014.* ***16****(6): p. 433.*
127. *Sinno, H. and S. Prakash, Complements and the wound healing cascade: an updated review. Plast Surg Int, 2013.* ***2013****: p. 146764.*
128. *Shah, J.M., et al., Cellular events and biomarkers of wound healing. Indian J Plast Surg, 2012.* ***45****(2): p. 220-8.*
129. *Wu, Y.S. and S.N. Chen, Apoptotic cell: linkage of inflammation and wound healing. Front Pharmacol, 2014.* ***5****: p. 1.*
130. *Zgheib, C., J. Xu, and K.W. Liechty, Targeting Inflammatory Cytokines and Extracellular Matrix Composition to Promote Wound Regeneration. Adv Wound Care (New Rochelle), 2014.* ***3****(4): p. 344-355.*
131. *Roth, M., et al., Intracellular interleukin 6 mediates platelet-derived growth factor-induced proliferation of nontransformed cells. Proc Natl Acad Sci U S A, 1995.* ***92****(5): p. 1312-6.*

132. *Heldin, C.H. and B. Westermark, Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev, 1999.* ***79****(4): p. 1283-316.*
133. *Dulmovits, B.M. and I.M. Herman, Microvascular remodeling and wound healing: a role for pericytes. Int J Biochem Cell Biol, 2012.* ***44****(11): p. 1800-12.*
134. *Olczyk, P., L. Mencner, and K. Komosinska-Vassev, The role of the extracellular matrix components in cutaneous wound healing. Biomed Res Int, 2014.* ***2014****: p. 747584.*
135. *Busti, A.J., et al., Effects of perioperative antiinflammatory and immunomodulating therapy on surgical wound healing. Pharmacotherapy, 2005.* ***25****(11): p. 1566-91.*
136. *Wipff, P.J., et al., Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. J Cell Biol, 2007.* ***179****(6): p. 1311-23.*