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FRANCA STEDILE ANGELI

TERAPIA COMBINADA COM ERITROPOETINA E FATOR ESTIMULANTE DA COLÔNIA DE GRANULÓCITOS EM UM MODELO DE INFARTO AGUDO DO MIOCÁRDIO

Porto Alegre
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Tese de Doutorado Submetida ao Programa de Pós-Graduação em Medicina e Ciências da Saúde da Faculdade de Medicina da Pontifícia Universidade Católica do Rio Grande de Sul-PUCRS, como requisito à obtenção do título de Doutor em Medicina e Ciências da Saúde.

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Orientador: Prof. Dr. Luiz Carlos Bodanese

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Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Medicina e Ciências da saúde da Pontifícia Universidade Católica do Rio Grande do Sul.

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Dedicatória

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RESUMO

Introdução: Recentemente, a eritropoetina (EPO) e o fator estimulante de colônia de granulócitos (GCSF) surgiram como potenciais terapias no tratamento do infarto agudo do miocárdio (IAM). Contudo, os efeitos da terapia combinada ainda estão por ser investigados.

Objetivo: Investigar a eficácia e segurança da terapia combinada com EPO e GCSF pós-IAM em um modelo porcino.

Métodos: IAM foi induzido em porcos domésticos através da oclusão por 90 minutos da coronária descendente anterior esquerda. Dezesseis animais foram tratados com análogo de longa ação da EPO a GCSF ou solução salina (grupo controle). Função cardíaca foi avaliada via ecocardiografia e medidas de pressão-volume no inicio do estudo, uma e seis semanas após o IM. Histologia foi realizada seis semanas após o IAM.

Resultados: Seis semanas após o IAM, a terapia combinada com EPO e GCSF demonstrou estabilizar a fração de ejeção ventricular esquerda (41±1% vs. 33±1%, $p<0.01$) e melhorar a função diastólica quando comparada com o grupo controle. Avaliação histopatológica revelou aumento de áreas de miocárdio viável e de densidade vascular no grupo tratado com EPO e GCSF quando comparada com o grupo controle. Apesar dos resultados encorajadores, em uma avaliação histórica comparando a terapia combinada com a monoterapia com EPO ou GCSF, a terapia combinada não demonstrou ter beneficio adicional na preservação da fração de ejeção ou volumes ventriculares ao longo do período em estudo.

Conclusão: Os presentes achados sugerem que a terapia combinada com EPO e GCSF promove a estabilização da função cardíaca após o IAM. Contudo, a terapia combinada não aprece ser superior a monoterapia com EPO ou GCSF.

Palavras-chave: Eritropoetina, Fator estimulante da colônia de granulócitos, Infarto do miocárdio, Citoquina.

ABSTRACT

Background: Erythropoietin (EPO) and granulocyte colony stimulating factor (GCSF) have generated interest as novel therapies after myocardial infarction (MI), but the effect of combination therapy has not been studied in the large animal model.

Objetives: We investigated the impact of prolonged combination therapy with EPO and GCSF on cardiac function, infarct size, and vascular density after MI in a porcine model.

Methods: MI was induced in pigs by a 90 min balloon occlusion of the left anterior descending coronary artery. 16 animals were treated with EPO+GCSF, or saline (control group). Cardiac function was assessed by echocardiography and pressure-volume measurements at baseline, 1 and 6 weeks post-MI. Histopathology was performed 6 weeks post-MI.

Results: At week 6, EPO+GCSF therapy stabilized left ventricular ejection fraction, (41±1% vs. 33±1%, $p<0.01$) and improved diastolic function compared to the control group. Histopathology revealed increased areas of viable myocardium and vascular density in the EPO+GCSF therapy, compared to the control. Despite these encouraging results, in a historical analysis comparing combination therapy with monotherapy with EPO or GCSF, there were no significant additive benefits in the LVEF and volumes overtime using the combination therapy.

Conclusion: Our findings indicate that EPO+GCSF combination therapy promotes stabilization of cardiac function after acute MI. However, combination therapy does not seem to be superior to monotherapy with either EPO or GCSF.

Key-words: Erythropoietin, Granulocyte colony stimulating factor, Myocardial infarction, Cardiac remodeling, Cytokine.

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LISTA DE ABREVIATURAS

EPO	Eritropoetina
Bcl-2	B-cell Lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
dP/dt_{max}	Máxima razão de pressão do ventrículo esquerdo desenvolvida durante sístole
dP/dt_{min}	Declínio durante relaxamento isovolumétrico
GCSF	Fator Estimulante da colônia de granulócitos
IAM	Infarto agudo do miocárdio
IM	Infarto do miocárdio
IV	Intravenoso
JAK/STAT	Quinase Janus/sinalizador de transdução e ativação da transcrição
SC	Subcutâneo
VE	Ventrículo esquerdo

SUMARIO

ANEXOS

1 INTRODUÇÃO

O infarto do miocárdio (IM) é a principal causa de morbimortalidade nos países ocidentais[1]. Apesar dos avanços no manejo do IM, o número de pacientes com insuficiência cardíaca continua a crescer e permanece associado a risco elevado de morte[1]. Conseqüentemente, novos métodos terapêuticos voltados ao reparo do miocárdio danificado tem sido alvo de intensa pesquisa nos últimos anos[2-5]. Recentemente, várias moléculas, incluindo a eritropoetina (EPO) e o fator de crescimento das colônias de granulócitos (GCSF), demonstraram ter potencial efeito benéfico no remodelamento ventricular após o IM, emergindo como potenciais candidatos no tratamento dos pacientes pós MI[3, 6-8]. Contudo, varias questões relacionadas ao real efeito terapêutico destes agentes e aos mecanismos a eles associados, permanecem não respondidas.

1.1 INSUFICIÊNCIA CARDÍACA: EPIDEMIOLOGIA E AVANÇOS TERAPÊUTICOS

O aumento da prevalência de insuficiência cardíaca congestiva e uma realidade no mundo ocidental. Atualmente, só nos Estados Unidos, mais de cinco milhões de pessoas vivem com insuficiência cardíaca, e mais de 550.000 novos casos são diagnósticos a cada ano[1]. Pacientes com insuficiência cardíaca secundária a cardiomiopatia isquêmica tem no infarto agudo do miocárdio (IAM) e subsequente remodelamento uma das principais causas de deterioração da sua função ventricular[9, 10].

O IAM afeta mais de sete milhões de pessoas ano só nos Estados Unidos. Apesar do IAM ser a maior causa isolada de mortalidade neste pais, a maioria dos pacientes sobrevive ao evento, sendo alvo de significante morbidade. Aproximadamente 22% dos homens e 46 % das mulheres vítimas de IAM sofrem de insuficiência cardíaca seis anos após o evento agudo[10].

No tratamento do IAM, a melhor estratégia de preservação miocárdica é a reperfusão emergencial da coronária ocluída através do uso de fibrinolíticos ou,

preferencialmente, através de intervenção coronariana envolvendo angioplastia e implante de endopróteses. Nos últimos anos, a eficácia da terapia de reperfusão foi beneficiada pelo concomitante avanço nas técnicas percutâneas de reperfusão, das terapias medicamentosas adjuntas, e do reconhecimento da importância da limitação do intervalo de tempo entre o evento e a reperfusão[9]. Apesar destes avanços, muitos pacientes terminam sofrendo as consequências do infarto, quer pelo atraso na reperfusão ou pela presença de anatomia coronariana não susceptível as técnicas existentes de reperfusão.

Dentro deste cenário, novos agentes terapêuticos voltados a implementar a capacidade regenerativa miocárdica vêm sendo amplamente estudados. Dentre estes, a terapia celular figura como uma das áreas de pesquisa de maior impacto na cardiologia. Nos últimos anos, o uso de células tronco da medula óssea no reparo do miocárdio infartado foi alvo de vários estudos clínicos. Apesar de os resultados destes estudos terem sido variáveis, os mesmos foram responsáveis por significativo avanço na área da terapia regenerativa[11-14]. Dentre estes novos conceitos, o uso de agentes com a capacidade de modular respostas endógenas, ampliando o número de células precursoras disponíveis e amplificando vias anti-apoptóticas gerou interesse na comunidade científica[8, 15].

1.2 ERITROPOETINA

A EPO é um hormônio hematopoiético produzido preferencialmente pelos rins em resposta a hipóxia[16]. Apesar de não ser classicamente considerada uma citoquina, a eritropoetina tem estrutura e mecanismos de sinalização similares às citoquinas do tipo I[17]. A EPO demonstrou ter ações anti-apoptóticas amplas, mediadas pelas proteínas reguladoras da apoptose *B-cell Lymphoma 2* (Bcl-2) e *B-cell lymphoma-extra large* (Bcl-XL) e conectadas as vias da quinase Janus/sinalizador de transdução e ativação da transcrição (JAK/STAT)[18, 19]. Em paralelo, demonstrou ter efeitos pró-angiogênicos[20, 21] e anti-inflamatórios[19, 22], aumentar a síntese e biodisponibilidade do oxido nítrico[23], aumentar a reserva de fluxo coronariano[8] e, finalmente, mobilizar células da medula óssea e

precursores pluripotentes cardíacos[24-26]. Em modelos murinos demonstrou melhorar a contractilidade miocárdica[27], reduzir o dano celular e apoptose[22] e aumentar neovascularização, levando a redução do tamanho do infarto e melhorando a função cardíaca após o IAM[28-30].

Em um modelo de IM em roedores, a terapia prolongada (administração imediata após o IM seguida de três doses em intervalos semanais) com o análogo de longa ação da EPO (darbopoetina alfa) esteve associada à melhora da função cardíaca quando comparada com o tratamento em dose única[29]. Interessantemente, em um modelo de IM em porcos, a darbopoetina em dose única demonstrou reduzir a fibrose intersticial e aumentar a área capilar, levando a melhora da função cardíaca regional sem, contudo, ter efeito positivo na função cardíaca global[31]. Finalmente, a darbopoetina, quando testada clinicamente, demonstrou ser segura quando administrada em dose única antes da reperfusão de pacientes na fase aguda do IM[32].

Tendo em vista o potencial benefício clínico associado à terapia prolongada com EPO, nos conduzimos um estudo em porcos avaliando a segurança e eficácia da terapia prolongada (quatro semanas) com EPO (darbopoetina)[33], usando doses clinicamente relevantes desta droga [30, 32, 34]. Neste estudo, a terapia prolongada com EPO após IM demonstrou ser segura e estar associada à prevenção da deterioração da função sistólica. Adicionalmente, a terapia esteve associada ao aumento das áreas de miocárdio viável, ao aumento da densidade vascular, e a indução da mobilização de células da medula óssea[33]. Tais resultados sugerem que a melhora observada esteja relacionada ao aumento da densidade vascular e da razão capilar-para-miócito, ambos indicativos de aumento da vascularização.

1.3 FATOR DE CRESCIMENTO DA COLÔNIA DE GRANULÓCITOS

O GCSF é uma citoquina hematopoiética que promove a proliferação, sobrevida e diferenciação de células hematopoiéticas, estando envolvido na mobilização de células da medula óssea para a periferia sanguínea[35, 36]. Em

paralelo, o GCSF ativa várias vias sinalizadoras anti-apoptóticas, como a via JAK/STAT nos cardiomiócitos[6]. Dados pré-clínicos confirmaram tais benefícios, demonstrando a prevenção de apoptose e a indução de angiogênese em modelos de IAM[37, 38].

Estudos clínicos em pacientes com IAM confirmaram a segurança do uso de GCSF, mas apresentaram resultados desapontadores em termos de eficácia, levantando questionamentos em relação à posologia testada e da utilidade do seu uso como monoterapia[15, 39-44]. Contudo, na avaliação histórica dos subgrupos tratados, o GCSF demonstrou ter efeito benéfico em pacientes com fração de ejeção menor do que 50%, enquanto pacientes com infartos menores e fração de ejeção preservada não parecem se beneficiar desta terapia[40]. Interessantemente, a maior parte dos pacientes tratados com GCSF em estudos clínicos fazem parte do último grupo[40, 45, 46].

O tempo ideal para administração do GCSF após o infarto também é objeto de controvérsia. Dados oriundos do maior estudo randomizado, multicêntrico, utilizando células da medula óssea (REPAIR-AMI) sugerem que os pacientes beneficiados seriam aqueles com áreas significativas de infarto (fração de ejeção < 49%), e pacientes que receberam terapia após o quinto dia após o IM[47]. Assim, o momento de mobilização das células da medula óssea pelo GCSF parece ter um papel crucial na recuperação da função cardíaca após IM. Concomitantemente, dados oriundos de estudos prévios sugerem que a administração de GCSF iniciada nas primeiras 37 horas após inicio do IM estaria associada a melhora da função cardíaca[40]. Tal efeito parece estar em parte associado às propriedades anti-apoptóticas do GCSF[6].

Considerando estes achados, nos conduzimos um estudo em porcos com fração de ejeção menor ou igual a 49% avaliando a segurança e eficácia do GCSF administrado em doses clinicamente relevantes imediatamente após reperfusão, seguido de doses diárias entre o quinto e o nono dia após a criação do MI[48]. Neste estudo, a terapia com GCSF demonstrou ser segura e estar associada à preservação da função sistólica quando comparada com o grupo

controle. Em paralelo, a terapia GCSF esteve associada a aumento da vascularização das áreas de infarto e mobilização de células da medula óssea[48].

1.4 POTENCIAL BENEFICIO DA TERAPIA COMBINADA

As citoquinas, e moléculas correlatas, caracteristicamente têm a capacidade de exercer diferentes funções e, muitas vezes, de sobreposição das mesmas[17, 49, 50]. Acredita-se que esta redundância esteja associada à capacidade de amplificação das respostas a diferentes estímulos desta classe[49]. Reforçando esta hipótese, a terapia combinada com EPO e GCSF demonstrou ser útil no tratamento de pacientes com anemia refratária associada à síndrome displásica pela inibição sinergética da apoptose das células progenitoras hematopoiética e amplificação da mobilização das células progenitoras[51, 52]. Paralelamente, em um modelo de IM em rodentes, nós demonstramos que a combinação de EPO e GCSF resulta em aumento significativo da mobilização de células progenitoras da medula óssea quando comparada com monoterapia com EPO ou GCSF[53].

Assim, considerando o potencial beneficio do uso adjunto da EPO e do GCSF após o IM, e plausível que se teste a segurança e eficácia da combinação terapêutica de EPO com GCSF em um modelo porcino de IAM. Este trabalho complementa nossa linha de pesquisa testando o efeito de diferentes agentes na função cardíaca após IM ao examinar o potencial beneficio da terapia combinada com EPO e GCSF[33, 48].

2 OBJETIVOS

2.1 OBJETIVO GERAL

Testar a segurança e eficácia da combinação terapêutica de EPO com GCSF em um modelo porcino de IAM.

2.2 OBJETIVOS ESPECÍFICOS

Objetivo Específico 1 – Segurança do uso *In Vivo* da terapia combinada com EPO e GCSF no pós-IAM

Neste objetivo investigaremos a segurança do uso da terapia combinada com EPO e GCSF pós-IAM comparada com o grupo controle.

1a) Nos administraremos EPO e GCSF em um modelo de IAM, previamente caracterizado em nosso laboratório. Segurança será avaliada laboratorialmente (marcadores inflamatórios, de função renal e cardíaca, hemograma completo com diferencial, marcadores de necrose miocárdica) e clinicamente (desenvolvimento de novos eventos isquêmicos, potência coronariana, e hipertensão) por um período de seis semanas.

1b) Nos também examinaremos a segurança do uso da terapia com EPO e GCSF através de detalhada avaliação histopatológica. Especificamente serão avaliadas: presença de massas, ou formações tumorais; quantificação e caracterização das áreas de fibrose, peri-infarto e miocárdio contralateral; inflamação; e vascularização.

Objetivo Específico 2 – Eficácia do uso *In Vivo* da terapia combinada com EPO e GCSF no pós-IAM

Nós acessaremos a eficácia do uso combinado de EPO e GCSF pós-IAM comparada com o grupo controle através da avaliação dos seguintes parâmetros.comparada com

2a) Examinar as mudanças na recuperação funcional cardíaca pós-IAM através de medidas ecocardiográficas e de pressão e volume.

2b) Investigar os possíveis mecanismos associados a estas mudanças através de detalhada avaliação histológica.

Objetivo Específico 3 – Eficácia do uso *In Vivo* da terapia combinada com EPO e GCSF *versus* monoterapia com EPO ou GCSF no pós-IAM

Nós realizaremos uma avaliação histórica, nos acessaremos a eficácia do uso combinado de EPO e GCSF pós-IAM quando comparada com monoterapia com EPO ou GCSF.

3a) Examinar as mudanças na recuperação funcional cardíaca pós-IAM através de medidas ecocardiográficas e de pressão e volume.

3 HIPÓTESE

A terapia combinada com EPO+GCSF é segura e está associada a melhora da função ventricular pós-IAM.

4 MÉTODOS

4.1 DELINEAMENTO

Estudo experimental, controlado, realizado nas dependências da Universidade da Califórnia, San Francisco, no período de julho de 2007 a dezembro de 2010.

4.2 POPULAÇÃO E AMOSTRA

Dezoito porcos domésticos (Yorkshire–Landrace), com 3 a 4 meses de idade, foram adquiridos e mantidos nas dependências do *Large Animal Resource Center* da Universidade da Califórnia, San Francisco, conforme protocolo aprovado pelo comitê de uso e cuidado com animais desta instituição (ANEXO 2 e 3). Este estudo obedeceu a regulamentação desta instituição e do National Institutes of Health americano (*Guide for the Care and Use of Laboratory Animals).* Os animais foram monitorizados quanto ao seu comportamento, excreta, e atitude (estado de alerta, apetite, responsividade) ao longo de todo estudo.

O tamanho da amostra foi determinada tendo por base estudos prévios utilizando o mesmo modelo animal. A análise do poder da amostra indicou que cada grupo deveria ter pelos anos oito animais para poder determinar uma diferença de 10% entre os tratamentos, respeitando um poder de 90%, e um erro de 5% ($P<0.05$). Tendo em vista que o estudo experimental está associado

com uma mortalidade estimada em torno de 10%, o número necessário para cada grupo foi inicialmente definido como sendo de 9 animais.

4.3 PROTOCOLO DE ESTUDO

4.3.1 Protocolo de tratamento

Os animais receberam um dos seguintes tratamentos: 1) Análogo de longa ação da EPO/Darbopoetina (Aranesp, Amgen, Thousand Oaks, CA) foi administrado em bolo IV no momento da reperfusão (0.9 ug/Kg), e semanalmente na forma de injeções subcutâneas por quatro semanas (0.4 ug/Kg), e GCSF (Neupogen, Amgen, Thousand Oaks, CA) foi administrado na forma de bolo IV no momento da reperfusão (10 ug/kg) e após diariamente do dia 5 ao 9 pós-IAM (5 ug/kg); 2) Grupo controle (solução salina no volume e posologia acima descritos). A posologia de ambas as drogas foi definida com base em dados de segurança e eficácia disponíveis[30, 32, 40].

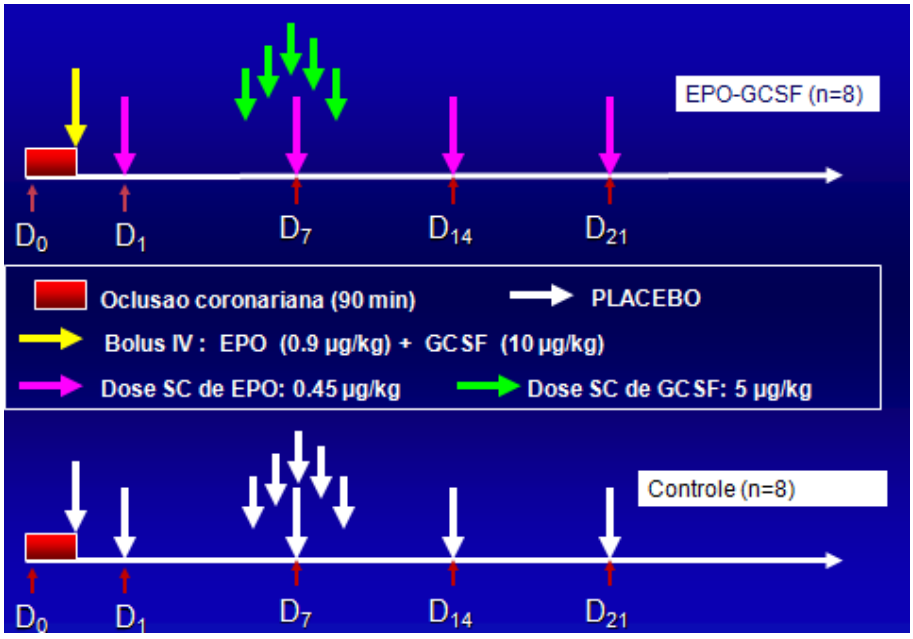

Figura 1- Delineamento do estudo. Protocolo farmacológico administrado. EPO, eritropoetina; GCSF, fator estimulante da colônia de granulócitos; IV, intravenoso; SC, subcutâneo; D, dia.

4.3.2 Indução do IAM

Todos animais receberam beta-bloqueador (atenolol, 25 mg via oral) um dia antes do infarto e diariamente após o terceiro dia da criação do mesmo. Anestesia geral foi induzida com injeção intramuscular de ketamina (20 mg/kg), xylazina (2 mg/kg) e atropina (0.04 mg/kg) e mantida com isoflurane a 2% administrado pelo tubo endotraqueal. Monitorizarão continua da pressão arterial, saturação de oxigênio e telemetria foi realizada durante todos os procedimentos.

IAM foi induzidos através da oclusão por 90 minutos da coronária descendente anterior esquerda conforme previamente publicado por nosso grupo (Figura 2)[54]. Brevemente, a artéria femoral foi canulada usando um cateter guia 6-French 'hockey-stick' (diâmetro, 0.75 mm). Após administração sistêmica de heparina (100 U/kg), o balão de angioplastia (3.0 × 15 mm) foi posicionado sobre a corda guia no meio da artéria, distalmente ao segundo grande ramo diagonal. O balão foi inflado por 90 minutos usando 4 a 5 atmosferas de pressão, até completa oclusão da mesma. Completa oclusão arterial após balão inflado, patência coronariana após reperfusão e previamente ao término do estudo foram confirmadas angiograficamente. Lidocaína (1 mg/min IV) foi iniciada antes da oclusão pelo balão seguido de Amiodarona (75 mg IV ao longo de 10 min) para prevenção de arritmias ventriculares[54]. Doses adicionais de lidocaina (1 to 3 mg/kg IV) foram administradas a discrição do cardiologista com vistas a prevenir ou tratar arritmias ventriculares.

Após plena recuperação, os animais foram transportados as suas celas e monitorados clinicamente ao longo do estudo.

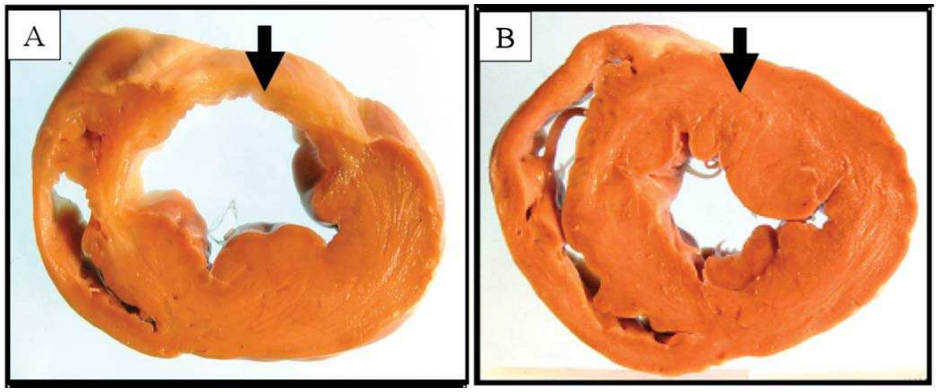

Figura 2 - Representação patológica do infarto. Fatias representativas originarias de animal após 6 semanas do infarto. (B) Animal controle não infartado. O tecido infartado (setas) apresenta-se descolorido e a espessura da parede esta diminuída (A) em comparação ao controle (B).

4.3.3 Avaliação clínica e laboratorial

Os animais foram monitorados durante toda a duração do estudo. Atitude, comportamento, excreções, sinais de estresse respiratório, perda de função motora, e edema de extremidades foram avaliados diariamente. Patência coronariana, e medidas de pressão arterial foram monitoradas pré-infarto, 2 horas, 1 semana e 6 semanas após a oclusão coronariana.

Coletas sanguíneas, através de acesso venoso femoral ou auricular, foram realizadas antes da criação do IAM, duas horas após a reperfusão, e semanalmente até o final do estudo. Hemograma completo com diferencial, Creatinina, Proteína C-reativa, transaminase glutâmico oxalacética, transaminase glutâmico pirúvica , e D-dímeros foram mensurados por laboratório animal especializado (IDEXX Laboratories, Sacramento, CA).

Infarto enzimático foi avaliado através da mensuração da creatina quinasse (fração-MB) e troponina I em quatro pontos no tempo (pré-infarto, 2 horas, 1 semana e 6 semanas após o IAM) por laboratório animal especializado (IDEXX Laboratories, Sacramento, CA).

4.3.4 Avaliação da função cardíaca

Função cardíaca foi avaliada via ecocardiografia e medidas de pressão e volume conforme protocolo validado pelo nosso grupo[54]. Brevemente, os animais foram anestesiados conforme protocolo acima detalhado e a avaliação da função cardíaca foi realizada no inicio do estudo, uma e seis semanas após a criação do infarto.

O estudo ecocardiográfico transtorácico (Acuson 128XP, Siemens, Malvern, PA) foi realizado através da aquisição de imagens padrão utilizando sondas transdutoras S3 (1 a 3 MHz) e S8 (3 a 8 MHz). Volumes ventriculares ao final da sístole e diástole e o índice de mobilidade das paredes ventriculares (*wall motion índex*) foram calculados utilizando os métodos padrão descritos pela Sociedade Americana de Ecocardiografia[55]. Para todos os parâmetros, pelo menos três ciclos cardíacos completos foram analisados e os resultados foram apresentados como a média da leitura dos mesmos. A variabilidade interobservador expressada utilizando o coeficiente de variação das medidas de volume diastólico e sistólicos finais foram de 4.3 +/- 5.7 mL e 1.4 +/- 2.9 mL, respectivamente, correspondendo a uma variabilidade absoluta na fração de ejeção de 2 +/- 3%. A variabilidade intraobservador para o índice de mobilidade das paredes ventriculares foi de 1.9%, enquanto a variabilidade interobservador foi de 2,5%.

Sinais de pressão e condutância do ventrículo esquerdo foram adquiridos usando um cateter 5-French de pressão e volume com 12-eletrodos (Millar Instruments, Houston, TX). Os dados foram coletados antes, uma e seis semanas após a criação do infarto. O cateter foi conectado no console (CFL512, CD Leycom, Zoetermeer, Holanda) através da unidade de pressão-controle (TC510, Millar) e o modulo do paciente (CD Leycom). Dados contínuos foram adquiridos pré e após a oclusão temporária da veia cava inferior (Figura 3)[54].

(A) (B)

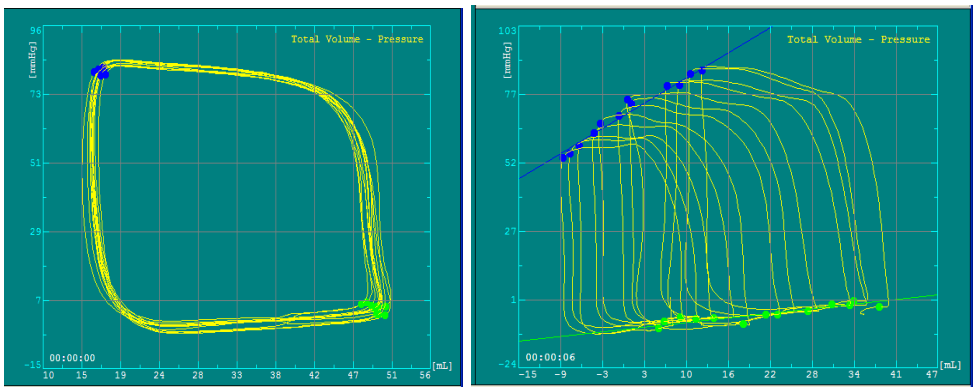

Figura 3 – Exemplos de curvas de pressão e volumes adquiridos. (A) Curva de pressão pré-oclusão. (B) Curvas de pressão e volume durante oclusão temporária da veia cava inferior.

O número de batimentos cardíacos, a máxima razão de pressão do ventrículo esquerdo desenvolvida durante sístole (dP/dt_{max}), o declínio durante relaxamento isovolumétrico (dP/dt_{min}), a pressão-volume do final na diástole do ventrículo esquerdo e a pressão-volume no final da sístole foram gravados durante o estudo. O volume sistólico (do inglês *stroke volume)*, o output cardíaco, a fração de ejeção do ventrículo esquerdo, e o trabalho sistólico foram então calculados.

Adicionalmente, os dados coletados durante a oclusão da veia cava inferior foram utilizados para calcular a relação linear de pressão-volume no final da sístole [também chamada elastância (E_{es})], a intercepção do volume "morto' (V_d), a chamada relação linear entre trabalho sistólico e volume diastólico final (do inglês *preload recruitable stroke work,* PRSW),[56] o consumo de oxigênio do miocárdio estimado pela área de pressão-volume, a eficiência mecânica, e a elastância arterial. A função diastólica foi avaliada durante o estado de repouso através da medida o tempo de relaxamento isovolumétrico constante (τ)[57] e dP/dt_{min} [58], conforme descrito previamente. A relação de volume e pressão na diástole foi avaliada através das mudanças em β, do chamado coeficiente de *stiffness (rigidez)* conforme previamente descrito[59].

4.3.5 Histologia e avaliação imunohistoquímica

Os animais foram sacrificados seis semanas após a criação do IAM. Área de infarto e miocárdio viável, vascularização e hipertrofia foram avaliadas conforme previamente descrito por nós[54] e outros[60, 61].

Brevemente, o coração foi extraído da caixa torácica em diástole (após injeção letal de potássio), pesado e avaliado para determinar a presença de anormalidades na sua superfície. A razão entre o peso do ventrículo esquerdo (VE) e o peso corporal foi utilizada para determinar a massa do VE. Os ventrículos foram fatiados paralelamente ao sulco atrioventricular posterior, iniciando pelo ápice até atingir a base e obedecendo intervalo aproximado de 1-cm entre as fatias (Figura 4A).

As dimensões de cada fatia foram medidas e gravadas. Imagens digitais foram obtidas para análise morfométrica da área do VE, tamanho do infarto, e grossura das paredes usando o software IPLab (Scanalytics, Rockville, MD). Desta forma, a área total de necrose (cicatriz colágena) foi definida como o percentual da área total do VE e, secundariamente, como o percentual da área de necrose versus a área de tecido não infartado ou viável (Figura 4B).

(A) (B)

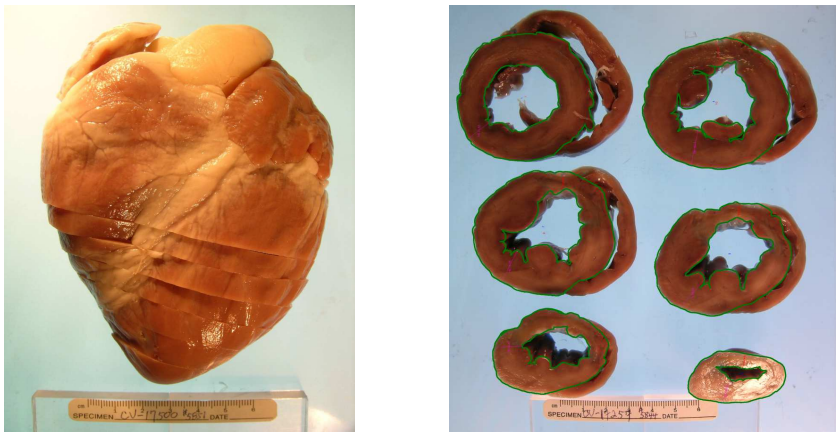

Figura 4- Técnica de preparo do tecido cardíaco. (A) Ventrículo fatiado paralelamente ao sulco atrioventricular posterior. (B) Análise morfométrica utilizando software IPLab.

As peças foram, então, cortadas circunferencialmente em 16 áreas transmurais conforme descrito anteriormente [61]. As mesmas foram transferidas em solução de sucrose a 15%, desidratadas em álcool, e embebidas em parafina. As peças foram posteriormente seccionadas (4 a 5 µm), posicionadas em lâminas, e submetidas à coloração com hematoxilina-eosina, Masson tricrômio, Vermelho Sírio, e Movat pentacrômio. As áreas de fibrose dos 16 segmentos foram somadas e apresentadas como percentual do total da área VE (Figura 5).

(A)

(B)

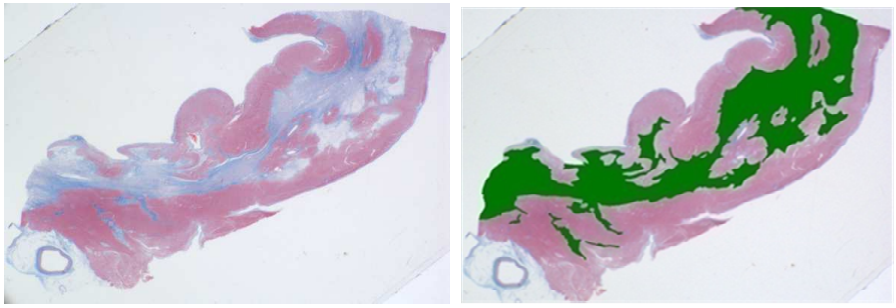

Figura 5- Avaliação das áreas de fibrose. (A) Área de fibrose (azul) utilizando Masson tricrômio. (B) Área de fibrose calculada com ajuda de software em verde.

A densidade vascular e o diâmetro dos cardiomiócitos (hipertrofia) foram medidos em áreas provenientes da base, centro e do ápice do VE. As mesmas foram seccionadas circunferencialmente em quatro áreas transmurais incluindo: área central do infarto, duas áreas adjacentes ao infarto (definida como área localizada a 1.2 mm para medida dos capilares e a 2.0 mm para a medida das arteríolas), e área não infartadas (controle).

Hipertrofia foi determinada pela medida do diâmetro dos cardiomiócitos. As lâminas foram submetidas à coloração com Prata de Gomori para visualização individual dos miócitos. Usando um software para análise de imagens (ImagePro, MediaCybernetics, Bethesda, MD), a hipertrofia concêntrica será medida como a área do corte transversal passando pelo núcleo dos miócitos (média de 75 miócitos por animal). A densidade de miócitos foi calculada pelo número médio de miócitos por área de tecido.[62]

Avaliação imunohistoquímica com anticorpo biotinilizado de lectina (Dolichos biflorus, DBA, Sigma, St Louis, MO) foi utilizada para identificação dos capilares Figura 6). O anticorpo monoclonal contra o clone 1A4 da actina do músculo liso (diluição 1:2000, Sigma) foi utilizada para identificação do músculo liso vascular das artérias e arteríolas. A densidade vascular será medida na região ventricular média em 6 a 9 campos por secção. A densidade capilar

(magnificação de 200 x), arterial e arteriolar (100 x) foi medida e expressa como o número médio de vasos por mm2. A área total de tecido mensurada foi corrigida pelo espaço intersticial remanescente. A vascularização também foi determinada pela razão capilar/miócito (densidade capilar dividida pela densidade de miócitos) e comparada entre diferentes regiões e grupos.[63]

(A) (B)

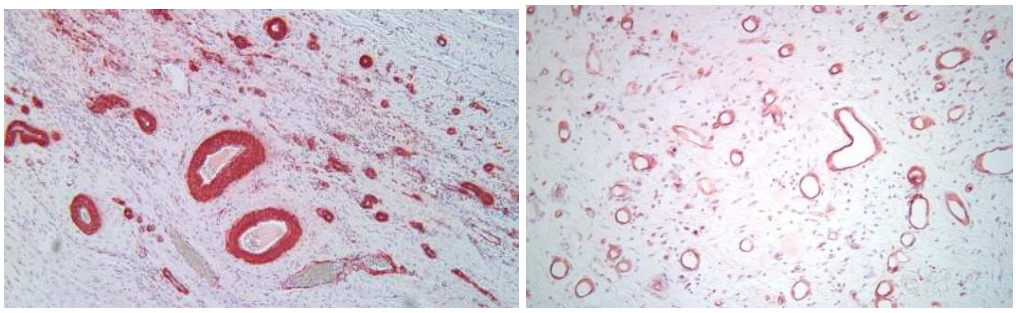

Figura 6- Avaliação da densidade vascular. (A) Arteríolas: áreas representativas submetidas à coloração com anticorpo anti-actina do músculo liso (rosa), magnificação de 100 x. (B) Capilares: áreas representativas submetidas à coloração com anticorpo contra lectina (rosa), magnificação de 200 x.

5 ANÁLISE ESTATÍSTICA

Os resultados foram expressos em forma de média ± erro padrão das médias. Modelo de medidas repetidas de ANOVA foi utilizada para testar as respostas dos parâmetros analisados (medidas de base, a uma e seis semanas) nas variantes experimentais (tratamentos).

Os dados foram comparados usando o método não ajustado de Holm–Sidak (valores observados comparados nos diferentes pontos no tempo). A significância das diferenças entre os grupos (Controle e EPO+GCSF) foi realizada através do teste T (não pareado). Análise histórica utilizando medidas repetidas de ANOVA foi utilizada na avaliação dos diferentes tratamentos (EPO, GCSF, EPO+GCSF). Correlações foram realizadas usando o método de Person. Os dados foram computados através do uso do software SigmaStat 3.5 (Systat

Software, San Jose, CA). Significância estatística foi definida por um valor P menor ou igual a 0.05.

6 ASPECTOS ÉTICOS

A presente pesquisa foi aprovada pela Comissão Coordenadora do Programa de Pós- Graduação em Medicina e Ciências da Saúde da Faculdade de Medicina da PUC, sob o Oficio 763/11-PG (ANEXO 2), e pelo Comitê de Ética em Pesquisa da Universidade da Califórnia, São Francisco, Estados Unidos (ANEXO 3), aonde o presente estudo foi conduzido.

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ANEXO 1

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Cytokine Combination Therapy with Erythropoietin and Granulocyte Colony Stimulating Factor in a Porcine Model of Acute Myocardial Infarction

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Abstract
Purpose Erythropoietin (EPO) and granulocyte colony stimulating factor (GCSF) have generated interest as novel therapies after myocardial infarction (MI), but the effect of combination therapy has not been studied in the large animal model. We investigated the impact of prolonged combination therapy with EPO and GCSF on cardiac function, infarct size, and vascular density after MI in a porcine model.
Methods MI was induced in pigs by a 90 min balloon occlusion of the left anterior descending coronary artery. 16 animals were treated with EPO+GCSF, or saline (control group). Cardiac function was assessed by echocardiography and pressure-volume measurements at baseline, 1 and 6 weeks post-MI. Histopathology was performed 6 weeks post-MI.
Results At week 6, EPO+GCSF therapy stabilized left ventricular ejection fraction, (41±1% vs. 33±1%, $p<0.01$) and improved diastolic function compared to the control group. Histopathology revealed increased areas of viable myocardium and vascular density in the EPO+GCSF therapy, compared to the control. Despite these encouraging results, in a historical analysis comparing combination therapy with monotherapy with EPO or GCSF, there were no significant additive benefits in the LVEF and volumes overtime using the combination therapy.
Conclusion Our findings indicate that EPO+GCSF combination therapy promotes stabilization of cardiac function after acute MI. However, combination therapy does not seem to be superior to monotherapy with either EPO or GCSF.

Key words Erythropoietin · Granulocyte colony stimulating factor · Myocardial infarction · Cardiac remodeling · Cytokine

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Introduction

Myocardial infarction (MI) is a leading cause of morbidity and mortality in Western countries [1]. Despite advances in the management of MI, the number of patients with congestive heart failure continues to grow and remains associated with increased risk of death [1]. Novel therapeutic approaches targeted to repairing myocardial damage have been the focus of intense research over the recent years [2–5].

Recently, granulocyte colony stimulating factor (GCSF) and Erythropoietin (EPO) have emerged as promising candidates for treatment of acute ischemic heart disease. Despite promising pre-clinical data using GCSF [6, 7], human clinical trials in acute MI patients, while generally reassuring in terms of safety, have been disappointing from the standpoint of clinical benefit, raising questions about the adequacy of GCSF monotherapy. Nevertheless, we have recently shown that GCSF therapy mobilizes bone marrow cells, enhances neovascularization, and prevents further

deterioration of LV function in a porcine model of MI with lower LVEF [8]. In line with our results, a recent meta-analysis suggests that GCSF may be potentially beneficial in patients with larger infarcts who have a lower LVEF (<50%) [9].

EPO has been shown to improve myocardial contractility [10], reduce cellular damage and apoptosis [11], and increase neovascularization, leading to reduced infarct size and improved cardiac function in rodent models of MI [12–14]. For the first time, our group has recently shown that in a large animal MI model, prolonged therapy (4 weeks) with EPO decreases infarct size, mobilizes bone marrow cells, enhances neovascularization and results in improvements in ventricular remodeling and function in a porcine model of acute MI [15].

Given the diversity of cytokines and their overlapping functions [16–18] and the beneficial effects of EPO and GCSF therapy post MI, we hypothesized that combination therapy with EPO and GCSF would enhance angiogenesis, and decrease infarct size and, therefore, would result in concomitant improvements in ventricular remodeling and function in a porcine model of acute MI with reperfusion. The current manuscript builds on the previous work and examines whether the combination of EPO and GCSF would be safe and effective in improving the cardiac function post-MI. To our knowledge, no prior study has examined the effect of EPO+GCSF combination therapy after MI in the large animal model. Of note, since limited funding was secured for completion of this combination cytokine study, the current results were compared to historical and previously published EPO and GCSF monotherapy arms [8, 15] by our group. To keep this comparison appropriate, all aspects of the study amongst the groups were performed similarly and by the same operators.

Methods

Animals

This study was carried out in accordance with the guidelines of the National Institutes of Health with a protocol approved by the Institutional Animal Care and Use Committee of University of California San Francisco (UCSF). Eighteen Yorkshire-Landrace pigs weighing 35–43 kg were obtained (Pork Power, Turlock, CA) for this study.

Induction of MI

MI was induced by a 90 min balloon occlusion of the mid left anterior descending coronary artery (LAD) as previously published by our group [8, 15, 19]. Briefly, general anesthesia was induced by intramuscular injection of ketamine (20 mg/kg), xylazine (2 mg/kg) and atropine (0.04 mg/kg) then maintained with 2% isoflurane. The levels of anesthesia were kept the same at all study time points. Continuous blood pressure, oxygen saturation, and telemetry monitoring were performed during all procedures. A 6F sheath was placed in the femoral artery and after systemic heparinization (100–200 U/kg), the coronary artery was selectively engaged with a 6F HS0.75 guide catheter. A standard guide wire was placed in the LAD, and a 2.5 to 3.5× 15 mm coronary angioplasty balloon delivered to the mid LAD just distal to the second diagonal branch. Balloon inflation (~4 atm) for 90 min was performed to induce MI. Complete occlusion with balloon inflation and LAD patency after balloon deflation was confirmed angiographically. Intravenous (IV) amiodarone (75 mg over 10 min) and lidocaine (1 mg/kg IV bolus, followed by 1 mg/min infusion) were started prior to balloon occlusion, with additional lidocaine (1–3 mg/kg IV bolus) given at the discretion of the operator for significant ventricular arrhythmias. Animals were medicated with atenolol (25 mg orally) daily starting 3 days post-MI. Prior to sacrifice, all animals underwent repeat coronary angiography.

Treatment protocol

Animals were assigned to one of two treatment groups using the same protocol previously published by our group testing EPO and GCSF monotherapy [8, 15]: 1) Long-acting EPO analog (Aranesp, Amgen, Thousand Oaks, CA) was given as IV bolus at the time of reperfusion (0.9 ug/Kg), then as weekly SC injections for 4 weeks (0.4 ug/Kg), and GCSF (Neupogen, Amgen, Thousand Oaks, CA) was given as IV bolus at time of reperfusion and then daily SC injections from day 5 to 9 post MI; and 2) control group (normal saline in equivalent volume given IV and SC to match the administration of the EPO therapy group). EPO dosages were selected following clinical data showing safety and feasibility with a single bolus of a fixed dose of darbepoetin [20], clinical studies addressing safety of chronic use of darbepoetin in patients with chronic renal failure [21], and pre-clinical data showing improvement in cardiac function and neovascularization using prolonged EPO therapy in a dose that did not increase the hematocrit [14]. GCSF dosages were selected following clinical data showing safety and feasibility of similar dose post MI [9].

Animal monitoring

Behavior, excreta, attitude (alertness, responsiveness, appetite), signs of respiratory distress (respiratory rate, respiratory pattern), motor weakness, and swelling extremities were monitored daily for the length of study. Arterial blood

pressure was monitored at baseline, immediately post reperfusion, one, and 6 weeks post MI.

Blood sampling and laboratory analysis

Whole blood samples were collected at baseline (prior to MI), at 2 h, on week one to four after MI induction, and then at sacrifice. Hemoglobin, hematocrit, white blood count, creatinine levels, creatine kinase MB fraction (CK-MB), and troponin I (TnI) were measured by the animal core laboratory (IDEEX, Sacramento, CA).

Echocardiography

Transthoracic echocardiography (TTE) was performed at baseline, 1 and 6 weeks after MI using an Acuson 128XP machine with S3 (1–3 MHz) and S8 (3–8 MHz) probes (Siemens, Malvern, PA) as previously published by our group [8, 15, 19]. Long- and short-axis parasternal views and 4- and 2- chamber apical views were acquired. Left ventricular end-diastolic volume (LVEDV), end-systolic volume (LVESV), and ejection fraction (LVEF) were measured using the area/length method. The wall motion index (WMI) was calculated, using the method previously described by the American Society of Echocardiography [22], by grading the standard 17 myocardial segments (normal=1, hypokinesis (reduced endocardial motion and wall thickening in systole) =2, akinesis (absence of inward endocardial motion or wall thickening in systole) =3, dyskinesis (outward motion or "bulging" of the segment in systole, usually associated with thin, scarred myocardium) =4, aneurysm=5), and dividing the sum of the scores by the number of segments visualized. For all above parameters, at least three loops per scan were selected and the results presented as an average of the readings. Readings were made by blinded operators. The inter-observer variability (made from different readings of recorded loops) expressed using coefficient of variation in the measurement of LVEDV and LVESV was 4.3 +/- 5.7 mL and 1.4 +/- 2.9 mL, respectively, corresponding to variability in absolute LVEF of 2 +/- 3%. The intraobserver variability for WMSI (mean difference between *measures 1* and *2)* was 1.9%, while the interobserver variability (mean difference between *observers 1* and *2*) was 2.5%.

Left ventricular pressure-volume (PV) data were collected at baseline, 1 week, and 6 weeks after MI as previously published by our group [8, 15, 19]. Conductance and pressure signals were acquired using a dual field 5F 12-electrode pigtail PV catheter (Millar Instruments, Houston, TX) connected to a Leycom CFL-512 console (CD Leycom, Zoetermeer, Netherlands) via a TC-510 (Millar) pressure control unit and a patient module (CD Leycom) as previously described [19].

Pressure volume measurements

The ventricular end-systolic and end-diastolic pressure-volume relationships are considered as gold standards in the characterization of intrinsic ventricular pump properties [23–25]. In this study, the PV catheter was inserted in the long axis of the left ventricle and oriented with segment one in the apex and segment seven in the aortic outflow as previously reported [8, 15, 19]. Inferior vena cava (IVC) occlusion was performed with a 7F, 34 mm Amplatzer sizing balloon (AGA Medical, Plymouth, MN) introduced via the femoral vein, inflated for 6–10 s to achieve ~50% drop in arterial blood pressure. Continuous data were acquired at a sampling frequency of 250 Hz during the steady state and IVC occlusion.

PV data were analyzed offline using the Conduct NT software (CD Leycom) by a blinded operator with a 10 Hz filter as previously described [19, 26]. Briefly, data were

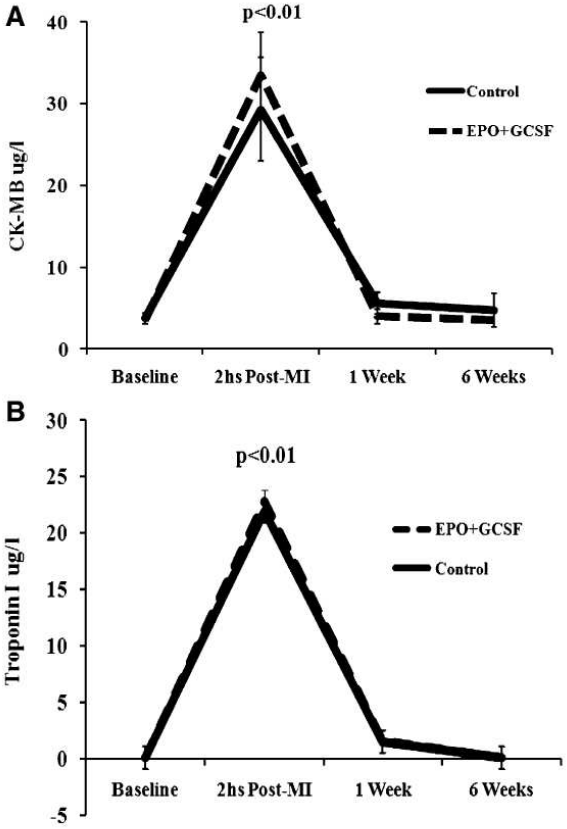

Fig. 1 Enzymatic curve for CK-MB and Troponin I. **a** CK-MB (ug/ml); each line represents the mean of one experimental group. **b** Troponin I (ug.ml); each line represents the mean of one experimental group. Both enzymes were significantly increased 2 h post MI, returning to baseline at 6 weeks. There were no differences between EPO+GCSF and Control in each one of the time-points

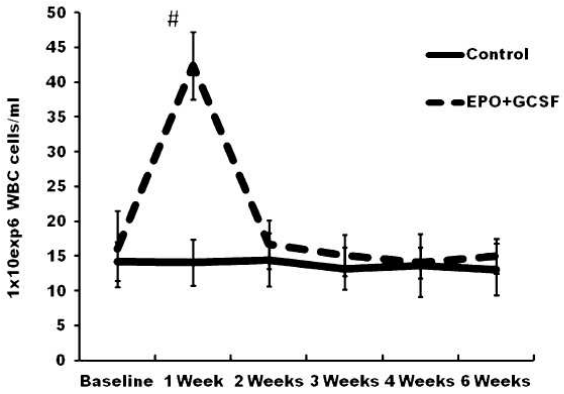

Fig. 2 Leukocyte response to cytokine therapy. EPO+GCSF combination therapy induced a significant increase in WBC 1 week after MI compared to baseline and Control group (both, # $P<0.01$)

calibrated for parallel conductance (V_c) and alpha (α) based on volumes derived from transthoracic echocardiographic images collected at the beginning of each case. Using conductance data, and the α and V_c calculations, the Conduct NT software calculated ventricular volumes as previously described [27]. Steady state data included heart rate (HR), maximum rate of pressure change in systole (dP/dt_{max}), decline with relaxation (dP/dt_{min}), left ventricular end-diastolic pressure (LVEDP), end-systolic pressure (LVESP), LVESV, and LVEDV. Stroke volume (SV) was recorded as LVEDV—LVESV, cardiac output (CO) as SV×HR, LVEF as SV/LVEDV, and stroke work (SW) as the area enclosed by the PV loop.

Diastolic function was evaluated during steady state by the time constant of isovolumic relaxation, τ, and the dP/dt_{min}. τ was computed as described by Raff and Glantz using pressure recorded during the isovolumetric relaxation period which is the period from the time of dP/dtmin to the time when left ventricular pressure falls to 5 mmHg above the end-diastolic pressure of the following beat [28]. τ was calculated as the negative inverse of the linear slope of dp/dt vs. pressure during this period.

Data obtained during IVC occlusion were used to calculate the linear end-systolic pressure-volume relation (characterized by the slope; also called end-systolic elastance (E_{es}) and an intercept, V_0, and the preload recruitable stroke work (PRSW, or slope of SW versus LVEDV curve) [29].

Table 1 Echocardiographic parameters over time

Parameter	Control (n=8)	EPO±GCSF (n=8)	t Test[a]
LVEF (%)			
Baseline	55.2 (1.2)	56.8 (1.3)	NS
1 Week post MI[b]	41.3 (2.1)**	41.1 (1.6)**	NS
6 Weeks post MI[b]	33.2 (1.5)**, ***	41 (1.2)**	$p<0.01$
Repeated measures ANOVA (main effect)	$p<0.01$	$p<0.01$	
LVEDV (mL)			
Baseline	52.2 (1.9)	53.2 (0.9)	NS
1 Week post MI[b]	63.5 (2)**	62.5 (1.5)**	NS
6 Weeks post MI[b]	73.5 (2.5)**, ****	67.9 (2)**, ****, ****	$p=0.04$
Repeated measures ANOVA (main effect)	$p<0.01$	$p<0.01$	
LVESV (mL)			
Baseline	23.6 (1.2)	22.9 (0.9)	NS
1 Week post MI[b]	37.2 (1.8)**	36.8 (1.2)**	NS
6 Weeks post MI[b]	49.5 (1.9)**, ****	40.7 (1.5)**	$p=0.01$
Repeated measures ANOVA (main effect)	$p<0.01$	$p<0.01$	
WMI			
Baseline	1	1	
1 Week post MI[b]	1.7 (0.1)**	1.6 (1)**	NS
6 Weeks post MI[b]	1.9 (0.1)**, ****	1.6 (1)**	$p<0.01$
Repeated measures ANOVA (main effect)	$p<0.01$	$p<0.001$	
HR (bpm)			
Baseline	79 (4)	84 (7)	NS
1 Week post MI[b]	84 (6)	87 (4)	NS
6 Weeks post MI[b]	87 (6)	83 (6)	NS
Repeated measures ANOVA (main effect)	NS	NS	

The values are expressed as the mean±standard error (in parentheses)

LVEF left ventricle ejection fraction, *LVEDV* LV end-diastolic volume, *LVESV* LV end-systolic volume, *WMI* wall motion index, *HR* heart rate

NS Non significant

[a] Significance of differences "between groups" was tested by an unpaired t test

[b] Change from baseline value; significance of *post-hoc* test in repeated measures ANOVA design

*$p<0.05$ vs. baseline, ** $p<0.01$ vs. baseline, *** $p<0.05$ vs. 1 week, **** $p<0.01$ vs. 1 week

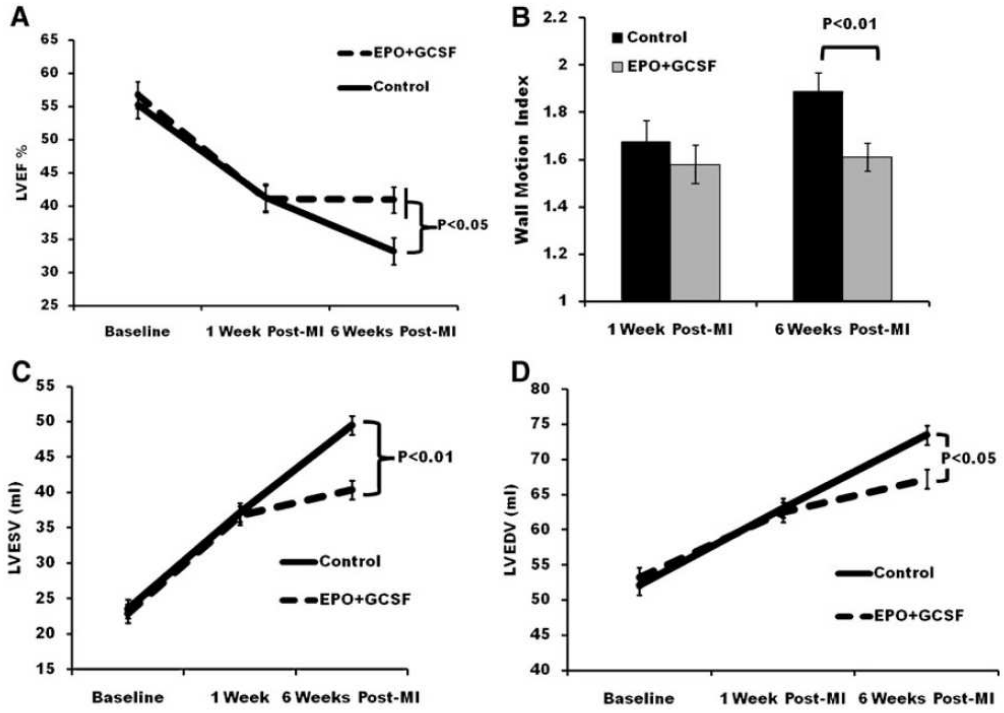

Fig. 3 Changes in LV function and volumes over time following myocardial infarction by echocardiography. **a** Left ventricular ejection fraction (*LVEF*); each line represents the mean of one experimental group. LVEF continue to decrease in controls, while combination therapy stabilizes LVEF. **b** Wall motion index does not differ at 1 week, but is better on the EPO+GCSF therapy compared to control at 6 weeks **c** End systolic volume and **d** end diastolic volume at 6 weeks post-MI. Data are shown as mean±SEM

Histological and immunohistochemistry analysis

All animals were sacrificed 6 weeks after MI and their hearts excised, weighed, and any gross surface abnormalities recorded. Histological and immunohistochemistry was performed in a blinded manner by CV Path Institute, Inc, MD. The ventricles were serially sliced at approximately 1 cm intervals parallel to the posterior atrioventricular sulcus from the apex to the base as previously describe by our group [8, 15, 19]. The thickness of each slice was measured and recorded. Digital images were taken for morphometric analysis of LV area, infarct size, and thickness, using IPLab software (Scanalytics, Rockville, MD). Infarct size was defined as a thinned and pale region of the anterior LV wall [30] and did not account for areas of viable tissue. Myocardial tissue for paraffin embedding was taken from the basal, mid, and apical-cavity levels. The three levels were then divided clockwise, beginning with the interventricular groove, into sixteen segments as described before [31]. Myocardial sections were transferred to 15% sucrose, dehydrated in a graded series of alcohols, and embedded in paraffin. Sections (4-5 μm) were mounted on charged slides and stained with hematoxylin and eosin, Masson's Trichrome to evaluate for fibrosis using the IPLab software. The fibrotic areas from the 16 ventricle segments were summed and presented as a percent of the total LV area. The infarct zone (IZ) was defined as the arc of the left ventricle containing scar tissue.

For vessel density, myocardial tissue for paraffin embedding was taken from the basal, mid, and apical levels, and sectioned circumferentially into 4 adjacent transmural areas to include the central area of infarction, 2 adjacent border areas (border zone for capillary and arteriole measurements were defined at sites 1.2 mm and 2.0 mm outside the zone of infarction), and a control non-infarcted region (remote zone).

Immunohistochemical staining with a biotinylated lectin antibody (Dolichos biflorus, DBA, Sigma, St. Louis, MO) was used for the identification of capillaries. A monoclonal antibody against smooth muscle actin clone 1A4 (dilution 1:2000, Sigma) was used for the identification of vascular smooth muscle cells for identification of arteries and arterioles. Vascular density was measured at mid-ventricle region, in six to nine high power fields per section. Capillary (200X magnification), artery and arteriolar (100X) density were measured and expressed as the mean

Table 2 Conductance catheter measurements over time

Parameter	Control (n=8)	EPO±GCSF (n=8)	t Test[a]
HR (bpm)			
Baseline	80 (3)	86 (5)	NS
1 Week post MI[b]	83 (3)	85 (4)	NS
6 Weeks post MI[b]	88 (5)	92 (3)	NS
Repeated measures ANOVA (main effect)	NS	NS	
MAP(mmHg)			
Baseline	82 (4)	85 (3)	NS
1 Week post MI[b]	80 (6)	88 (4)	NS
6 Weeks post MI[b]	86 (8)	85 (5)	NS
Repeated measures ANOVA (main effect)	NS	NS	
LVESP (mmHg)			
Baseline	86 (2)	89 (5)	NS
1 Week post MI[b]	90 (4)	93 (3)	NS
6 Weeks post MI[b]	76 (3)*, ****	84 (4)	NS
Repeated measures ANOVA (main effect)	p<0.02	NS	
LVEDP (mmHg)			
Baseline	3.1 (0.3)	2.9 (0.5)	NS
1 Week post MI[b]	9.9 (1.4)**	8.8 (1.1)**	NS
6 Weeks post MI[b]	6 (0.5)*, ****	4.3 (1.1)****	NS
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	
SV (mL)			
Baseline	28.6 (1)	30.4 (0.7)	NS
1 Week post MI[b]	26.3 (1.5)	25.7 (1.3)*	NS
6 Weeks post MI[b]	21.4 (0.7)**, ****	27.7 (1.4)	p<0.01
Repeated measures ANOVA (main effect)	p<0.01	p=0.03	
Ees (mmHg/ml)			
Baseline	1.7 (0.2)	1.4 (0.2)	NS
1 Week post MI[b]	1.2 (0.3)	1.8 (0.3)	NS
6 Weeks post MI[b]	1.6 (0.3)	1.7 (0.2)	NS
Repeated measures ANOVA (main effect)	NS	NS	
Vo intercept (ml)			
Baseline	-37.2 (7)	-42.9 (10)	NS
1 Week post MI[b]	-25.9 (6)	-28 (5)	NS
6 Weeks post MI[b]	11.4 (5)**, ****	-11 (6)**	p<0.01
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	
PRSW (mmHg)			
Baseline	53 (3)	51 (1)	NS
1 Week post MI[b]	41 (2)**	40 (2)**	NS
6 Weeks post MI[b]	34 (1)**, ***	41 (2)**	p<0.05
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	
dP/dtmax(mmHg/s)			
Baseline	1270 (65)	1255 (42)	NS
1 Week post MI[b]	1035 (53)**	1020 (36)**	NS
6 Weeks post MI[b]	842 (38)**, ****	1038 (20)**	p<0.01
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	
dP/dtmin(mmHg/s)			
Baseline	- 1156 (18)	-1168 (20)	NS
1 Week post MI[b]	- 1024 (37)**	-1068 (23)**	NS
6 Weeks post MI[b]	- 890 (30)**, ****	-1035 (21)**	p<0.01

Table 2 (continued)

Parameter	Control (n=8)	EPO±GCSF (n=8)	t Test[a]
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	
τ (ms)			
Baseline	57 (1)	56 (1)	NS
1 Week post MI[b]	69 (4)**	64 (2)**	NS
6 Weeks post MI[b]	62 (1)*, ***	56 (1)****	p<0.01
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	

The values are expressed as the mean± by standard error (in parentheses)

HR heart rate, *MAP* mean arterial pressure, *LVESP* left ventricular end-systolic pressure, *LVEDP* left ventricular end-diastolic pressure, *SV* stroke volume, *Ees* linear end-systolic pressure-volume relation or end-systolic elastance, V_0 Volume zero Ees intercept, *PRSW* preload-recruitable stroke work, dP/dt_{max} maximum rate of change of left ventricular pressure with time, dP/dt_{min} peak of pressure decay, τ time constant of isovolumic relaxation, *MI* myocardial infarction

NS Non significant

[a] Significance of differences "between groups" was tested by an unpaired t test

[b] Change from baseline value; significance of *post-hoc* test in repeated measures ANOVA design *p<0.05 vs. baseline, **p<0.01 vs. baseline, ***p<0.05 vs. 1 week, ****p<0.01 vs. 1 week

(± SEM) number of vessels per mm^2. The measured total tissue area was corrected for remaining interstitial space.

Statistical analysis

All results are expressed as means±standard error of the mean (SEM). A repeated measures ANOVA model (SigmaStat 3.5, Systat Software, San Jose, CA) was used to test the responses of examined parameters (measured at baseline, week1 and 6) in the experimental variants (Control, EPO+GCSF). The within-subject design included an overall F test of the main effects and then a *post-hoc* pairwise comparison of the values measured at 1 week and 6 weeks against the baseline, and 6 weeks against 1 week, using the Holm-Sidak method. Significance of differences between groups (Control, EPO+GCSF) was tested by an unpaired t test. A historical analysis using repeated measures ANOVA model (SigmaStat 3.5, Systat Software, San Jose, CA) was used to test the responses of examined parameters (measured at baseline, week1 and 6) in all experimental variants (EPO+GCSF). Correlations were performed using the Person method. A p<0.05 was considered statistically significant in all the employed tests.

Results

Two animals died during creation of the MI model, leaving sixteen animals for the study. Patency of the coronaries was confirmed by angiography post reperfusion in all remaining animals. Mean arterial pressure immediately after reperfusion remained stable compared to baseline and did not differ between groups (mean 83±4 at baseline vs. 81±4 after reperfusion, p=NS). No adverse clinical events related to the drugs were noted during the study, including thromboembolic events and hypertension. CRP levels remained stable over time in both groups. As shown on Fig. 1, CK-MB and TnI levels did not differ between groups and, as expected, were significantly elevated two hours after the induction of the MI, returning to baseline thereafter (mean CK-MB 3.8±1.9 to 29.2±7.1 μg/L, p<0.01; and mean TnI 0.48±0.07 to 22.5±2.3 μg/L, p<0.01). All animals underwent repeat coronary angiography prior to sacrifice at the end of the study and LAD patency was documented. No differences in collateral circulation compared to baseline angiography or amongst the groups were present.

Cytokine therapy mobilizes bone marrow cells

EPO+GCSF group had significantly increased WBC counts at 1 week post-MI (Fig. 2). The mononuclear fraction count (lymphocytes plus monocytes) were also significantly higher at 1 week post-MI on the EPO+GCSF group compared to baseline (p<0.01) and to Control (respectively, 12.6±0.5 vs. 8.7±0.7 10^6 cells/ml), p<0.01 At week 4 post-MI, EPO+GCSF therapy induced a significant increase in the hemoglobin levels compared to baseline (respectively, 10.8±0.4 to 13.8±0.6 g/dl; p<0.01).

Echocardiographic parameters: EPO+GCSF preserves LVEF and prevents LV dilation over time

Echocardiographic parameters are shown in Table 1. At 6 weeks post-MI, EPO+GCSF group stabilized LVEF, while the control group demonstrated a statistically significant further deterioration of function (0±1 vs. -7±1%, p<0.01 vs. control) compared to week 1 post MI (Fig. 3a). The wall

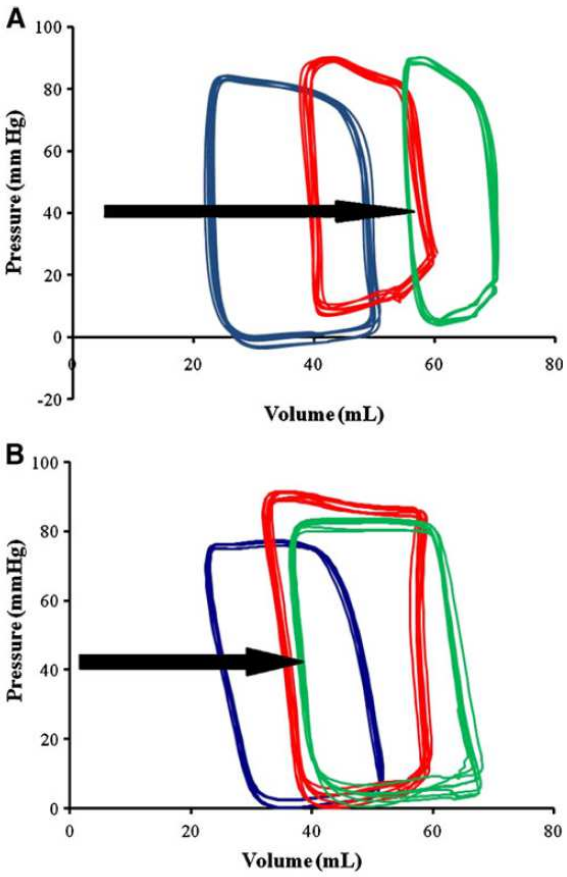

Fig. 4 EPO+GCSF preserves hemodynamics over time. Representative steady-state PV loops from one animal at baseline (*blue*), 1 week post-MI (*red*) and 6 weeks post-MI (*green*) from Control **a** and EPO+GCSF combination therapy **b**. After infarction, the PV loops narrowing is more evident in the Control animals compare to the EPO+GCSF group, indicating reduction in stroke work, and shift rightward due to increasing volumes (*black arrows*)

motion score was better in the EPO+GCSF group compared to the control (Fig. 3b), corroborating the LVEF findings. LVESV and LVEDV were also lower in the EPO+GCSF group at 6 weeks compared to the control (Fig. 3c and d).

PV-loop parameters: Cytokine therapy prevents further impairment of systolic function after AMI

The hemodynamic parameters are summarized in Table 2. At 6 weeks, there was a significant increase in SV in the EPO+GCSF group compared to control (27.7±1.4 vs. 21.4±0.7, $p<0.01$) (Fig. 4). Moreover, the peak positive dP/dt in the EPO+GCSF group was higher than the control group. The linear Ees was unchanged overtime and did not differ between groups, but there was a significant difference in the rightward movement of the V_0, indicating increased heart size in the control group in comparison to EPO+GCSF therapy (Fig. 4). As previously reported by us and others [19, 32, 33], the non-significant changes in the slope of the Ees could be a result of changes in loading conditions, LV dimensions, and regional morphological changes which are difficult to evaluate with the conductance catheter method over time but these reflect what we expect to see in clinical settings. On the other hand, the slope of the PRSW is a reasonable linear, afterload-independent relationship, and a well-described contractility index in intact animal models [29]. In our study, the

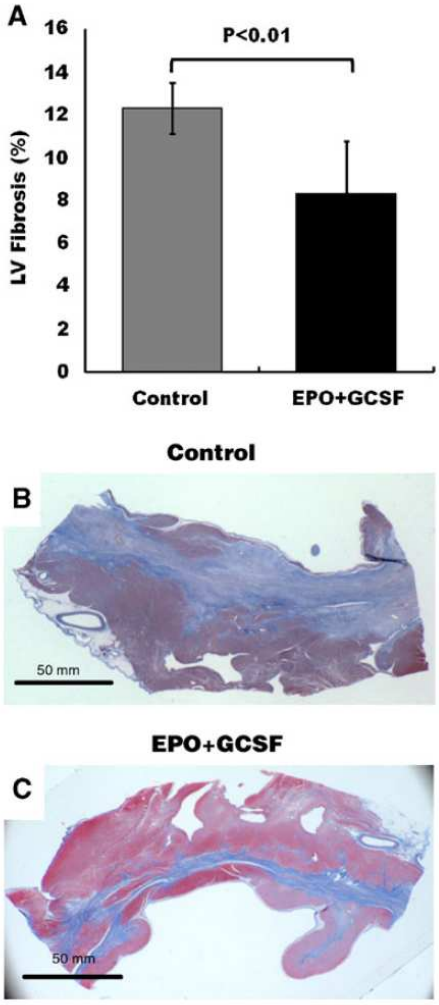

Fig. 5 Extent of fibrosis following myocardial infarction. **a** EPO+GCSF treatment is associated with decreased fibrosis of the left ventricle compared to the control group. Representative infarct zone regions (*arc of the left ventricle containing scar tissue*) stained with Masson Trichrome (*fibrosis=blue*) in sections of **b** Control, **c** EPO+GCSF. Data are mean±SD

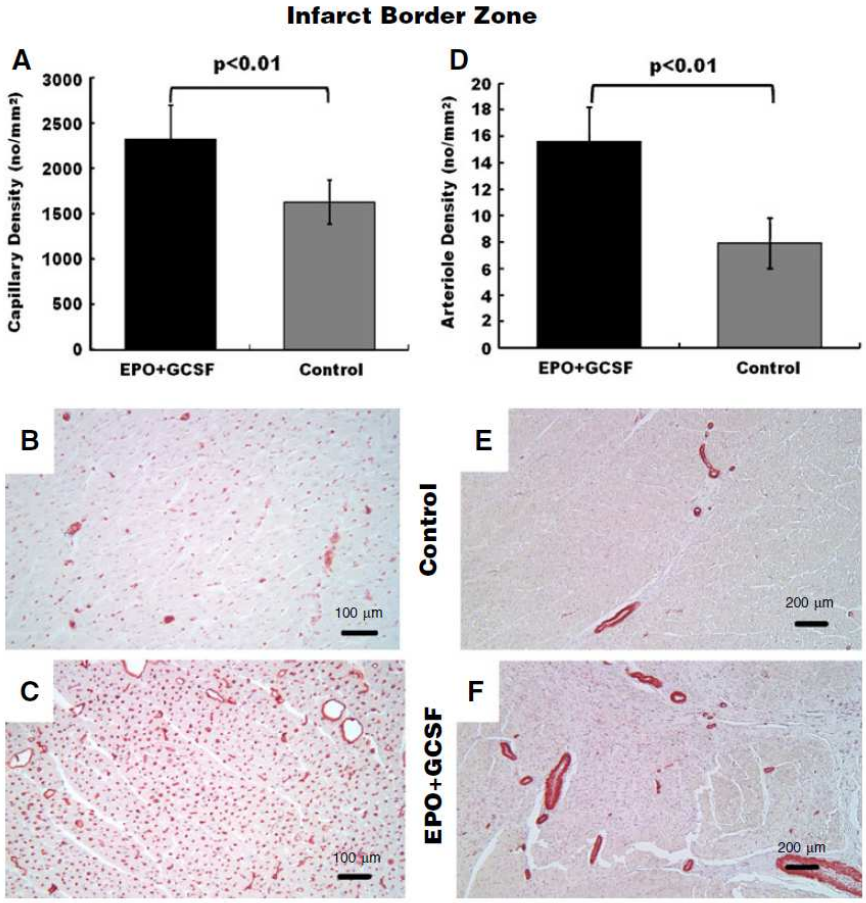

Fig. 6 Effect of cytokine therapy on vascular density. **a** EPO+GCSF therapy resulted in increased capillary density in the infarct border zone compared to control. Representative infarct border zone areas stained with antibodies against lectin (*pink*) in sections of **b** control vs. **c** EPO+GCSF. Scale bar=200 µm. **d** EPO+GCSF results in increased arteriole density at the infarct border zone compared to control. Representative infarct border zone areas stained with antibodies against smooth muscle actin (*pink*) in sections of **e** control vs. **f** EPO+GCSF treated pigs. Scale bar=100 µm

PRSW was significantly decreased at 1 weeks after MI in the control group compared to EPO+GCSF therapy.

EPO+GCSF positively impacts diastolic function

As summarized in Table 2, peak negative dP/dt was significantly lower in the EPO+GCSF group compared to the control. Also, the Tau constant was smaller in the EPO+GCSF group compared to the control pointing towards a beneficial effect of the EPO+GCSF cytokine therapy on diastolic function.

Cytokine therapy leads to more viable myocardium

Measurements derived from gross images of serial myocardial slices suggested that compared with the control group, treatment with EPO+GCSF did not lead to a reduction in the infarct size (16±4 vs.15±3%, p=ns). However, histological evaluation by Masson's Trichrome staining revealed an overall decrease in scar and fibrosis (Fig. 5a, $p<0.01$) in the EPO+GCSF group compared to the control, and therefore, more viable myocardium. The differences between the two techniques rely on the fact that the gross pathology evaluation does not take into account areas of viable tissue within the infarct zone, which are prevalent in this ischemia reperfusion model and contribute to the myocardial wall motion.

Cytokine combination therapy increases vascular density

As shown in Fig. 6a-c, capillary density at the infarct border zone was increased in EPO+GCSF treated animals compared to control ($p<0.01$) as was the arteriolar density ($p<0.01$, Fig. 6d-e).

Table 3 Post hoc analysis-nonsuperiority of combination versus monotherapy over time

Parameter	EPO±GCSF (n=8)	EPO (n=8)	GCSF (n=8)	ANOVA F test
LVEF (%)				
Baseline	56.8 (1.3)	57.7 (0.8)	56.6 (0.6)	NS
1 Week post MI[a]	41.1 (1.6)*	43.8 (0.7)*	42.8 (1.5)*	NS
6 Weeks post MI[a]	41 (1.2)*	39.3 (2.5)*, ***	38.4 (2.1)*, ***	NS
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	p<0.01	
LVEDV (mL)				
Baseline	53.2 (0.9)	53.2 (2)	49.6 (1.2)	NS
1 Week post MI[a]	62.5 (1.5)*	63.5 (2.5)*	60.7 (2.1)*	NS
6 Weeks post MI[a]	67.9 (2)*, **	70.8 (2.1)*, ***	70.5 (1.7)*, ***	NS
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	p<0.01	
LVESV (mL)				
Baseline	22.9 (0.9)	22.6 (0.9)	21.3 (0.4)	NS
1 Week post MI[a]	36.8 (1.2)*	35.6 (1.6)*	34.4 (1)*	NS
6 Weeks post MI[a]	40.7 (1.5)*	43.1 (3.2)*, ***	44 (2.3)*, ***	NS
Repeated measures ANOVA (main effect)	p<0.01	p<0.01	p<0.01	

The values are expressed as the mean± by standard error (in parentheses)

LVEF left ventricle ejection fraction, *LVEDV* LV end-diastolic volume, *LVESV* LV end-systolic volume

NS Non significant

[a] Change from baseline value; significance of *post-hoc* test in repeated measures ANOVA design

*p<0.01 vs. baseline, **p<0.05 vs. 1 week, ***p<0.01 vs. 1 week

EPO+GCSF therapy increased the capillary density and arteriolar density at infarct zone compared to control (respectively, 1058±259 vs. 508±56 capillaries/mm^2, p< 0.01; 27.5±6.8 vs.13.7±6 arterioles/mm^2, p<0.01). Capillary density at the infarct zone and LVEF at 6 weeks also showed significant correlation (r=0.84; p<0.01).

Historical analysis shows non-superiority of EPO+GCSF combination therapy over monotherapy

In a historical analysis comparing monotherapy with EPO and GCSF recently published by our group [8, 15], and EPO+GCSF combination therapy, there was no significant differences in the LVEF and volumes overtime (Table 3). Therefore, although combination therapy seems to be safe and effective, it does not seem to be superior to monotherapy with either EPO or GCSF.

Discussion

The major findings of this study are: [1] EPO+GCSF combination therapy is safe; [2] EPO+GCSF stabilizes systolic function and prevents further deterioration of diastolic cardiac function post-MI; [3] EPO+GCSF therapy induces bone marrow cell mobilization; [4] EPO+GCSF leads to more viable myocardium and increased vascular density compared to the control group; [5] EPO+GCSF combination therapy does not seem to be superior than EPO or GCSF monotherapy in a historical post hoc analysis.

To our knowledge, this is the first report of EPO+GCSF combination therapy in the large animal acute MI model. In this study, we have used doses that our group tested as monotherapy in the same animal model [8, 15], and that have been approved for clinical use, demonstrating that combination therapy was safe and effective. The mild increase in the hemoglobin level at 4 weeks (peak of 13.8 g/dl) was not associated with thromboembolic events and the use of EPO was not associated with hypertension in the treated animals.

In our model, we found that EPO+GCSF combination therapy stabilized systolic function, and reduced post-MI remodeling by diminishing LV diastolic dilatation and pressures over time. Combination therapy also resulted in more viable myocardium and the better wall motion score corroborated this finding, demonstrating preservation of the wall motion at the infarcted area. In addition, EPO+GCSF combination therapy was associated with increased capillary and arteriolar density. This ability to promote revascularization may partly explain the results as previously demonstrated by us and others [12, 34, 35].

Interestingly, when we performed a historical comparison between combination therapy and our previous reports using EPO or GCSF as monotherapy [8, 15], all three therapeutic strategies were superior compared to the control arm. However, we could not demonstrate a clear and significant additive or synergistic effect on cardiac function with EPO+GCSF combination therapy over monotherapy.

EPO+GCSF combination therapy has proven useful in the treatment of patients with refractory anemia due to myelodysplastic syndrome by a synergistic inhibition of progenitor cell apoptosis, [36] and possibly by enhancement of stem cell mobilization [37]. We have recently evaluated the mobilization of Lin–/Sca-1+/c-kit+ cells from

the bone marrow into the circulation post-MI in the eGFP+ chimeric mouse model, and demonstrated that combination of EPO+GCSF therapy resulted in significantly increased mobilization of $Lin^{-}/Sca\text{-}1^{+}/c\text{-}kit^{+}$ cells into the circulation at 6 days post-MI compared with either EPO or GCSF monotherapy, or to control [38]. However, similar to this current report in the large animal MI model, EPO and GCSF combination therapy did not seem to have an additive benefit of combination therapy over monotherapy with either agent in the rodent MI model either [38].

There are a number of limitations that need to be pointed out in this study. Given the difficulties with housing large animals at our facility and the cost associated with such studies, the duration of follow-up and the number of animals/group had to be limited and only selected doses of the agents used could be studied. The follow-up time point in the current study was chosen based on previous pre-clinical investigations that demonstrated functional improvements by this time which plateau thereafter and to keep the design of the experiment consistent with our prior reports with the monotherapy arms [8, 15] to allow direct comparison [39]. We recognize that 6 weeks may be too short to encompass the complete evolution of cardiac remodeling and heart failure but longer study durations are very challenging to undertake in the large and growing porcine animal model.

In addition, the doses of these agents were chosen because of the safety profile in clinical settings. Clearly, different doses and combinations thereof could be studied but the cost of such a study in large animals would be prohibitive. Importantly, our study has limited ability to define the intrinsic mechanisms responsible for the improved cardiac function with EPO+GCSF combination therapy vs. control. These detailed mechanistic questions are difficult to answer in large animal models and are outside the scope of this current report. Notably, we could not overcome the lack of well recognized porcine antibodies to characterize bone marrow progenitor cells both in the circulation and also in the heart. Finally, given the large size of the porcine hearts and the infarcted regions, accurate analyses for differential apoptosis are difficult to make in this model and as such were not undertaken in this study.

In conclusion, we report that prolonged combination therapy with EPO+GCSF in a large animal model of acute MI with reperfusion has beneficial effects on left ventricular function and structure. EPO+GCSF combination therapy after acute MI led to an increase in viable myocardium, increased vascular density, and promoted stabilization of LV global function and improved indices of LV remodeling. In follow-up to a previous report from our laboratory using EPO or GCSF monotherapy post-MI in the large animal model, combination cytokine therapy with EPO+GCSF does not seem to be superior to monotherapy with either agent alone.

Acknowledgments We thank Gina Orcino for animal care and technical support, and Petros Minasi for administrative assistance.

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ANEXO 2

Carta de aprovação da Comissão Coordenadora do programa de Pós-Graduação em Medicina e Ciências da Saúde da Faculdade de Medicina da PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
FACULDADE DE MEDICINA
PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE

Of. 763/11-PG

Porto Alegre, 26 de dezembro de 2011.

A Pós-Graduanda
Franca Stedile Angeli
N/Faculdade

Prezada Pós-Graduanda:

Comunicamos que a proposta de dissertação intitulada "Terapia combinada com eritropoetina e do fator estimulante de colônia de granulócitos em um modelo de infarto agudo do miocárdio" **foi aprovada** pela Comissão Científica do Programa de Pós-Graduação em Medicina e Ciências da Saúde.

A mesma deverá ser encaminhada ao Comitê de Ética em Pesquisa ou Comissão de Ética no Uso de Animais, através do setor de **Pesquisas e Estágios**, 2º andar do Hospital São Lucas/PUCRS. Após aprovação do CEP ou CEUA entregar cópia na secretaria do Programa. Em anexo, cópia da avaliação.

Atenciosamente,

Prof. Dr. Henrique Luiz Staub
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ANEXO 3

Carta de aprovação do Comitê de Ética em Pesquisa da Universidade da Califórnia, São Francisco, Estados Unidos

INSTITUTIONAL ANIMAL CARE AND USE
COMMITTEE
Office of Research, Box 0547
University of California San Francisco

APPROVAL LETTER

July 17, 2008

Franca S. Angeli M.D.
Box 0103

APPROVAL NUMBER: AN075645

Approval Date: July 17, 2008

Title: A Placebo-Controlled Study to Evaluate the Efficacy of Adjunctive Therapy with Granulocyte Colony-Stimulating Factor (GCSF) and Aranesp (Erythropoietin - EPO) on Cardiac Function and Infarct size after Myocardial Infarction in Pigs.

The IACUC approval number should be used for ordering animals and should be included in any correspondence regarding this study. This approval letter supersedes all previous approvals.

All individual participants must read the final approved protocol which must be followed exactly as written. Any modifications to this protocol must be submitted using the RIO online system. Modifications may not be implemented until reviewed and approved by the IACUC.

Non-compliance with any of these conditions violates federal and state laws and regulations, and University policies and guidelines governing the care and use of laboratory animals. Violations may have serious consequences for the welfare of your animals, your laboratory's ability to conduct animal research, and may impact funding from NIH.

UCSF requires health & safety review of all animal use protocols. Refer to OEH&S review letter for details. You may not work with hazardous materials in animals until OEH&S authorizations are current.

If you have questions, contact the IACUC Office at 476-2197 or email iacuc@ucsf.edu.

Animals Approved

Rat - USDA Type D, Acquired - 44

Chair
Institutional Animal Care and Use Committee