UNIVERSIDADE FEDERAL DE MINAS GERAIS
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SIMONE APARECIDA DE ALMEIDA

AVALIAÇÃO DA INFLUÊNCIA DO BACKGROUND GENÉTICO NO REPARO DE LESÃO INTERNA E NA REAÇÃO TIPO CORPO ESTRANHO EM CAMUNDONGOS DIABÉTICOS

Belo Horizonte / MG

Junho de 2018

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Tese apresentada ao curso de Doutorado em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais como requisito parcial à obtenção do título de Doutora em Patologia.

Área de concentração: Patologia Geral

Orientadora: Dra. Mônica A. N. Diniz Ferreira

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UFMG

FOLHA DE APROVAÇÃO

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SIMONE APARECIDA DE ALMEIDA

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em PATOLOGIA, como requisito para obtenção do grau de Doutor em PATOLOGIA, área de concentração PATOLOGIA INVESTIGATIVA.

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Resumo

O diabetes reduz a qualidade de vida ao provocar complicações como neuropatias, amputações de membros, retinopatias e elevado risco de doenças cardiovasculares. Frente a essa realidade uma melhor compreensão dos mecanismos fisiopatológicos do diabetes e suas complicações torna-se imprescindível. Evidências na literatura indicam que fatores exógenos, como os fatores ambientais e fatores endógenos como o background genético, determinam a manifestação de vários processos patológicos. Vários fatores genéticos têm sido associados ao desenvolvimento de diabetes e suas complicações. Entretanto, a influência do background genético na cicatrização interna, envolvendo a angiogênese inflamatória, e na resposta tipo corpo estranho a implantes de biomateriais, não foram investigadas. Neste trabalho estudamos a cicatrização interna e a reação tipo corpo estranho no tecido fibrovascular induzido por implantes de uma matriz sintética de poliéter-poliuretano em camundongos Swiss, C57BL/6 e Balb/c induzidos ao diabetes por estreptozotocina. Os níveis hiperglicêmicos (mg/dl) observados após o tratamento diabetogênico foram de 455,0±15 em camundongos Swiss, 393,0±22 em C57BL/6 e 190,0±10 em Balb/c. A resposta inflamatoria, angiogênica e produção de citocinas, componentes chave, no reparo de lesão interna e na reação tipo corpo estranho ocorreram diferentemente nas 3 linhagens, tanto nos camundongos normoglicêmicos quanto nos hiperglicêmicos de maneira linhagem específica. A angiogênese nos implantes em camundongos Swiss não diabéticos foi maior quando comparados com os implantes das outras linhagens. Nos implantes em Swiss e C57BL/6 diabéticos a maioria dos marcadores inflamatórios estavam aumentados. Entre os animais diabéticos, Todos os

componentes da reação tipo corpo estranho foram maiores nos implantes de camundongos Swiss e C57BL/6 quando comparados com os do Balb/c. Este estudo demonstra a principal contribuição do background genético no padrão dos componentes da angiogênese inflamatória da lesão interna e na resposta do tipo corpo estranho tanto em animais normoglicêmicos quanto hiperglicêmicos e na intensidade da resposta ao biomaterial. Estes resultados evidenciam a importância de considerar a variabilidade de respostas entre as diferentes linhagens na hora da escolha de animais diabéticos e normoglicêmicos para estudos terapêuticos de reparo interno e implantação de dispositivos médico.

Palavras-chave: angiogênese; diabetes; background genético; ferida interna, resposta tipo corpo estranho

Abstract

Diabetes reduces quality of life by causing complications such as neuropathies, limb amputations, retinopathies, and a high risk of cardiovascular disease. Faced with this reality, a better understanding of the pathophysiological mechanisms of diabetes and its complications becomes essential. Evidence in the literature indicates that exogenous factors such as environmental factors and endogenous factors such as the genetic background, determine the manifestation of several pathological processes. Several genetic factors have been associated with the development of diabetes and its complications. However, to what extent this susceptibility influences internal healing involving inflammatory angiogenesis and the foreign body response to a biomaterial implant (glucose sensors, orthopedic implants, catheters, vascular grafts, etc.) has not been fully investigated. In this work we studied the internal healing and foreign body type reaction in the fibrovascular tissue induced by implants of a synthetic polyether polyurethane matrix in Swiss, C57BL/6 and Balb/c mice induced by streptozotocin diabetes. Hyperglycemic levels (mg / dl) following diabetogenic treatment were 455.0 ± 15 in Swiss mice, 393.0 ± 22 in C57BL/6 and 190.0 ± 10 in Balb/c. Inflammation, angiogenesis, and cytokine production (key components) in the internal injury repair process and foreign body type response in both normoglycemic and hyperglycemic animals occurred in a specific lineage manner. Angiogenesis in implants of non-diabetic Swiss mice was higher than in implants of other strains. In the hyperglycemic environment, almost all inflammatory markers increased in implants of Swiss and C57BL/6 diabetic mice. All foreign body type reaction characteristics were higher in Swiss and C57BL/6 mice implants compared to those in Balb/c diabetic implants. This study demonstrates the main contribution

of the genetic background in the inflammatory angiogenesis components of the internal lesion and the foreign body type response in both normoglycemic and hyperglycemic animals and in the intensity of the biomaterial response. This variability may be relevant when considering the animal model of diabetes and therapeutic approaches in internal healing and in therapeutic approaches that use implantable devices in diabetics.

Keywords: angiogenesis; diabetes; genetic background; internal wound, foreign body response

Sumário

LISTA DE ABREVIATURAS E SIGLAS

CAPES Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CEUA Comitê de Ética de Uso Animal

CCL2 Chemokine (C-C Motif) Ligand 2

CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico

CXCL-1 chemokine (C-X-C motif) ligand 1

DNA Ácido desoxirribonucleico

DMT1 Diabetes *melitus* tipo 1

DMT2 Diabetes *melitus* tipo 1

EGF Fator de crescimento epidérmico

FAPEMIG Fundação de Amparo à Pesquisa do estado de Minas Gerais

FGF Fator de crescimento de fibroblastos

GLUT1 *Glucose transporter 1*

GLUT2 *Glucose transporter 2*

H&E Hematoxilina e eosina

Hb Hemoglobina

KC antiga denominação para chemokine (C-X-C motif) ligand 1

MCP-1 *Monocyte chemoattractant protein-1*

MEC Matriz extracelular

MHC Complexo Maior de histocompatibilidade

MMP Metaloproteinases de matiz

MPO Mieloperoxidade

NAG N-acetyl-β-D-glucosaminidase activity

NK *Natural Killer*

NO Óxido nítrico

OMS Organização Mundial da Saúde

PDGF Fator derivado de plaquetas

ROS Espécies reativas do oxigênio

rpm Rotações por minuto

SEM *Standard error of mean*

STZ Estreptozotocina

TGF-α Fator de crescimento transformante alfa

TGF-β Fator de crescimento transformante beta

TNF-α Fator de necrose tumoral alfa

VEGF Fator de crescimento endotelial vascular

Capítulo 1

1- INTRODUÇÃO

1.1 Diabetes

Nas últimas décadas tem ocorrido um aumento alarmante na incidência de diabetes em todo o mundo. Fatores de risco como a predisposição genética, mudanças pronunciadas no ambiente, no comportamento, no estilo de vida e contribuições de mecanismos epigenéticos tem resultado em taxas crescentes de obesidade e diabetes (Zimmet et al., 2001).

Segundo dados da *International Diabetes Federation* a prevalência de diabetes *mellitus* (DM) no mundo foi de 425 milhões de casos em 2017, e há estimativa que este número possa aumentar para 629 milhões até 2045. No Brasil, o número de pessoas com diabetes foi contabilizado em 12,5 milhões, com uma prevalência nacional da doença de 11,4% (*International Diabetes Federation; 2017)*. Estima-se ainda que no Brasil, o número de portadores de diabetes que não foram diagnosticados, chega a 70% (*International Diabetes Federation, 2017).*

O DM é a sexta causa mais frequente de internação hospitalar e contribui para outras causas de intervenção, como cardiopatia isquêmica, insuficiência cardíaca, acidente cardiovascular e hipertensão arterial (Barriere et al., 2018).

O diabetes é uma síndrome de etiologia múltipla, caracterizada pela deficiência relativa ou absoluta da insulina em exercer sua ação sobre órgãos-responsivos-alvo. Assim a glicose permanece em alta concentração no sangue, ocorrendo juntamente com anormalidades no metabolismo de lipídios, proteínas e carboidratos (Brownlee et al., 2001; Xiang et al., 2010). O DM é classificado em dois principais tipos. O tipo 1, também conhecido como diabetes juvenil ou insulinodependente, é uma doença

autoimune que atinge cerca de 10% da população de portadores de diabetes, no qual o próprio organismo destrói as células β pancreáticas, responsáveis pela produção de insulina, levando a deficiência absoluta de insulina. O diabetes tipo 2, atinge aproximadamente 90% desta população e apresenta prejuízos na produção e ação da insulina, resultante de defeito secretório progressivo e/ou resistência à insulina. Outros tipos específicos incluem aqueles que resultam de defeito genético, aqueles induzidos quimicamente por drogas, como no tratamento de HIV/AIDS ou após transplante de órgãos e o diabetes gestacional que é definido como intolerância à glicose durante a gestação (American Diabetes Association, 2017).

A deficiência de insulina leva em primeira instância, a elevação dos níveis glicêmicos que é o sinal patognomônico da doença. A hiperglicemia tem sido identificada como o principal fator de contribuição para a patogênese diabética, seja através de mecanismos diretos ou indiretos. Este estado produz alterações bioquímicas e metabólicas que levam a modificações funcionais e estruturais (Rubinstein et al., 2013).

Em estados não tratados, a hiperglicemia é acompanhada por glicosúria (perda de glicose pela urina), poliúria (diurese aumentada), desidratação, hiperosmolaridade intra e extracelular, polidipsia (aumento da sede), polifagia (aumento da fome) e perda de peso. As intensas alterações metabólicas podem ainda culminar com a produção excessiva de corpos cetônicos, ocasionando acidose metabólica e distúrbios eletrolíticos (Teixeira et al., 1999).

A literatura tem mostrado que o estado hiperglicêmico produz um excesso de espécies reativas de oxigênio (ROS), criando um estado de estresse oxidativo. Existe

uma relação inversa entre os níveis de glicose plasmática e a proliferação de células T e B. A pré-incubação de células do baço e de linfonodo em meio contendo alto nível de glicose leva a uma significativa diminuição tempo e dose-dependente da proliferação de células T e B associada a um aumento do estresse oxidativo (Rubinstein et al., 2013).

1.2 Complicações consequentes ao diabetes

O diabetes reduz a qualidade de vida ao provocar neuropatias, nefropatias, amputações de membros, retinopatias e elevado risco de doenças cardiovasculares representando milhões gastos pelo governo em remédios, internações e aposentadorias precoces (Bell et al., 2001).

Em longo prazo várias complicações do diabetes *mellitus* são caracterizadas por vasculopatia associada à angiogênese aberrante que pode ser deficiente ou excessiva. A angiogênese excessiva desempenha um importante papel na retinopatia e nefropatia diabética. A angiogênese diminuída contribui para uma vascularização colateral deficiente observada por exemplo nas coronárias, vasculopatia embrionária em gestações complicadas devido ao diabetes materno, cicatrização prejudicada de feridas, úlceras de pele e aumento do risco de rejeição de transplantes em pacientes diabéticos (Martin et al., 2003; Kota et al., 2012). Muitas dessas complicações têm sido responsáveis por altas taxas de morbidade e mortalidade associadas à doença (Misra et al., 2014).

1.3 Background genético

O background genético é a coleção de todos os genes presentes em um organismo que influencia uma ou mais características (Doetschman et al., 2009). Evidências na literatura indicam que fatores exógenos como os fatores ambientais e fatores endógenos como o background genético, determinam a manifestação de vários processos patológicos (Marques et al., 2011; Marques et al., 2014).

Já foi demonstrado por exemplo, que a dislipidemia aterogênica, a intolerância à glicose, a tendência trombótica, a inflamação subclínica e a disfunção endotelial são mais elevados em sul-asiáticos do que em caucasianos brancos. Esse fenômeno é parcialmente explicado pelo excesso de gordura corporal, alta porcentagem de gordura intra-abdominal e subcutânea e depósito de gordura ectópica em vários órgãos e locais do corpo em sul asiáticos quando comparados aos caucasianos brancos, o que pode contribuir para várias alterações metabólicas.

Em outro relato, por estudo de metanálise, mostrou a associação de polimorfismos de alguns genes no desenvolvimento do diabetes *melitus* tipo 1 (DMT1), indicando que o polimorfismo do gene KIR2DL1 foi associado à diminuição do risco de DMT1. Através da análise de subgrupo por etnia, houve uma possível associação negativa nos asiáticos, mas não em caucasianos. Além disso, os resultados dessa pesquisa mostraram que o KIR2DS1 foi negativamente associado à suscetibilidade ao DMT1 nos asiáticos, mas não nos caucasianos (Liu et al., 2017).

Diversos genes contribuem com essa predisposição, a maioria relacionada ao complexo principal de histocompatibilidade (ex.: genes HLA, como DR3 e DR4). O peso da influência genética é comprovado pelo fato de 30-70% dos gêmeos

univitelinos serem concordantes com a doença. Foi descrito também na literatura que a frequência de DMT1 entre japoneses, cerca de 20 vezes menor que entre escandinavos, pode ser atribuído ao background genético (Misra et al., 2014; Liu et al., 2012; *International Federation of Diabetes 2015*).

O estudo experimental do diabetes tem sido importante para elucidar a patogênese da doença. Pesquisas com diferentes linhagens de animais de laboratório podem ser extrapoladas com correlação para o estudo de diferentes populações humanas.

Atualmente, a classificação genética das espécies se baseia e está de acordo com os programas de acasalamento utilizados. Estes, definem a forma de transmissão dos caracteres genéticos, constituído de dois grandes sistemas: inbred (ou isogênicos) e outbred (ou heterogênicos) (The Jackson Laboratory, 2018).

Animais *inbred* são também chamados de *isogênicos* por terem por definição que são iguais geneticamente. São obtidos a partir de cruzamentos entre irmãos da mesma geração, por pelo menos, vinte gerações consecutivas em pares monogâmicos permanentes. Isto assegura um alto grau de consanguinidade, cerca de 98,6%, fixando algumas características e perdendo outras. Nesse grupo, encontram-se as linhagens BALB/c e C57BL/6. Por outro lado, os animais heterogênicos são animais de constituição genética variada, por serem obtidos através de cruzamentos aleatórios, evitando que animais em acasalamento sejam parentes próximos, nesta classe encontramos a linhagem Swiss (The Jackson Laboratory, 2018).

Estudos mostram um diferente perfil de predisposição de patologias entre as

linhagens, determinada pela influência do background genético. Os camundongos C57BL/6 constituem a linhagem mais usada entre os animais isogênicos. Esta linhagem apresenta baixa susceptibilidade a tumor, porém alta susceptibilidade à obesidade induzida por dieta, com moderada hiperglicemia e hiperinsulinemia, além de mostrarem uma maior susceptibilidade em desenvolver fibrose pulmonar e intraperitoneal em comparação com outras linhagens (Kolb et al., 2002; Margettes et al., 2013).

Estudos com camundongos BALB/c, também isogênicos, têm mostrado que essa linhagem desenvolve naturalmente altos níveis de colesterol plasmático e altas pressões sanguíneas sistólicas, mas são resistentes à aterosclerose induzida por dieta. Esta linhagem é muito utilizada para estudo de tumor de mama (Oosterlinck et al., 2011).

Os camundongos Swiss, representantes da categoria heterogênicos, são animais albinos, apresentam um perfil para pesquisas relacionadas ao câncer, estudos de toxicidade e doenças infecciosas (Oosterlinck et al., 2011). Experimentos com esses animais são de grande importância, pois esses animais possuem grande variabilidade gênica, devido aos cruzamentos aleatórios, assim como ocorre com a espécie humana, ou seja, produzindo populações naturais.

Os estudos têm comprovado que a angiogênese, a cicatrização e a fibrose também são dependentes do background genético (Misra et al., 2014; Liu et al., 2012; Marques et al., 2011). Rubinstein e cols. 2013 mostraram a influência do background genético no diabetes utilizando duas linhagens geneticamente diferentes de camundongos, Balb/c e C57BL/6. Essas linhagens apresentaram respostas diferentes

para o diabetes induzido pela estreptozotocina, sendo o Balb/c mais resistente, apresentando níveis glicêmicos inferiores comparados à linhagem C57BL/6. Os linfócitos T e B dos camundongos Balb/c apresentaram uma menor proliferação e viabilidade quando colocados em um meio contendo alta concentração de glicose, o que não foi visto nos animais C57BL/6.

Outro estudo mostrou que diferentes linhagens (C57BL/6, 129X1, Balb/c DBA/2 e FVB/N) quando induzidas ao diabetes por dieta hipercalórica apresentavam respostas diferentes quanto aos níveis de glicemia, insulina e de triacilglicerol. A linhagem 129X1 apresentou maiores níveis nesses parâmetros enquanto a Balb/c foi a mais resistente em altera-los (Montgomery et al., 2013).

A influência do background genético no desenvolvimento de hiperinsulinemia em diabetes em distintas linhagens de camundongos também foi relatada, mostrando uma maior função secretora de insulina na linhagem DBA/2 em comparação com a linhagem C57BL/6 (Kulkarni et al., 2003).

Em 2005 Bock e cols. relataram a influência do background genético no tamanho e na estrutura do pâncreas endócrino tais como na massa de ilhotas, massa de células beta e número de ilhotas.

O reparo tecidual assim como a reação do tipo corpo estranho também são influenciados por fatores genéticos. Em vários modelos experimentais foram demostrados que a cicatrização de feridas, a formação de vasos sanguíneos, a inflamação e a fibrose são fenótipos dependentes da coleção de genes presentes em um organismo (Liu et al., 2014; Marques et al., 2011).

No entanto, não encontramos na literatura, qualquer estudo que investigasse a

influência da heterogeneidade genética no processo de reparo de lesão interna em animais diabéticos, o que remete a uma lesão não superficial ou a resposta frente a um implante de biomaterial. Sendo assim, este trabalho foi elaborado de modo a investigar a influência do backgroud genético na cicatrização interna, através da avaliação do processo angiogênico e inflamatório (1ª parte) e na reação tipo corpo estranho (2ª parte) utilizando implante de biomaterial.

1.4 1ª Parte do trabalho

1.4.1 Reparo de lesões crônicas (interna)

No diabético, as lesões evoluem para o estado crônico, ou seja, não conseguem prosseguir através de um processo de reparação ordenada e coordenada para produzir integridade anatômica e funcional do local lesado. As feridas crônicas são raramente vistas em indivíduos saudáveis. De fato, pacientes com feridas crônicas frequentemente sofrem de doenças como diabetes e obesidade (Sem et al., 2009).

Muitas vezes disfarçado de comorbidade, lesões crônicas representam uma epidemia silenciosa que afeta uma significativa parcela da população mundial e representa uma grande ameaça para a saúde e para a economia (Sem et al., 2009; Campos et al., 2008). As ulcerações com falha na reparação em indivíduos com diabetes são uma das principais causas de admissões em hospitais nos países desenvolvidos e é a principal morbidade associada ao diabetes, muitas vezes causando dor, sofrimento e menor qualidade de vida. Estima-se que em 15% dos indivíduos com diabetes ocorrem ulcerações e que 84% das amputações relacionadas

a membros inferiores são realizadas em indivíduos portadores dessa doença (Zimmet et al., 2001).

A compreensão parcial das bases moleculares implícitas no reparo tecidual e em sua deficiência, bem como a falta de modelos animais para testes pré-clínicos que reproduzam corretamente as condições humanas, levou a uma deficiência nas terapias para o tratamento de feridas crônicas ou para acelerar a cicatrização de feridas agudas e no controle da formação de fibrose (Eming et al., 2014).

A cicatrização de feridas envolve extensa comunicação entre os diferentes constituintes celulares dos diversos compartimentos da pele e sua matriz extracelular (MEC). Em condições fisiológicas normais, a restauração de uma barreira epidérmica funcional é altamente eficiente (Eming et al., 2014). A cicatrização de feridas em um indivíduo não diabético consiste em perfeita e coordenada cascata de eventos celulares, moleculares e bioquímicos que se interagem para que ocorra a reconstituição tecidual. Esse processo pode ser dividido em quatro fases: hemostasia, inflamatória, proliferativa ou de granulação e fase de remodelamento ou de maturação (Broughton et al., 2006).

A hemostasia é caraterizada pela acumulação e agregação de plaquetas no local da ferida, dando início à formação de um coágulo e de uma matriz provisória, necessária para a migração celular (Eming et al., 2007; Eming et al., 2014). As plaquetas sofrem a degranulação, liberando vários fatores de crescimento, como o Fator derivado de plaquetas (PDGF), o Fator de crescimento transformante-β (TGF-β), o Fator de crescimento epidérmico (EGF), o Fator de crescimento transformante-α (TGF-α) e o Fator de crescimento de células endoteliais (VEGF); além de

glicoproteínas adesivas como a fibronectina e trombospondina, que são importantes constituintes da MEC provisória (Arnold & West 1991; Streit et al., 2000).

Na fase inflamatória, são produzidos numerosos mediadores vasoativos e fatores quimiotáticos que permeabilizam os vasos sanguíneos, permitindo a migração de leucócitos, como neutrófilos e monócitos (diferenciados em macrófagos no sítio da lesão) ao local da ferida (Singuer, 1999). Os macrófagos produzem vários fatores de crescimento, tais como o PDGF, o TGF-β e o VEGF, que se destacam como as principais citocinas necessárias para estimular a formação do tecido de granulação.

A fase proliferativa, é a fase responsável pelo fechamento da lesão propriamente dito. Envolve epitelização, angiogênese, e formação de uma matriz provisória. Os fatores de crescimento liberados pelos macrófagos estimulam a formação de novos vasos sanguíneos (angiogênese) e fibroplasia, que compõem o chamado tecido de granulação (Eming et al., 2007; Eming et al., 2014). Os fibroblastos produzem a nova matriz extracelular necessária ao crescimento celular enquanto os novos vasos sanguíneos carreiam oxigênio e nutrientes necessários ao metabolismo celular local (Singuer, 1999).

Por fim, na fase de maturação, a matriz provisória resultante da fase de proliferação, composta de colágeno tipo III, proteoglicanos e fibronectina é substituída por uma matriz mais forte e bem organizada composta por colágeno tipo I. O TGF-β é uma citocina predominante na fase de maturação que inibe a produção de MMP, regula positivamente a expressão de inibidores de MMP, tem um papel importante na remodelação da matriz de fibroblastos/colágeno e na organização da MEC (Grinell,

2013). A contração da ferida, que é a última etapa do processo de reparo, é o resultado da atividade das células mesenquimais.

Em pacientes diabéticos, todos os estágios da cascata de cicatrização de feridas estão afetados. O reparo tecidual nestes indivíduos é caracterizado por diminuição da atividade do VEGF e de outros fatores de crescimento que influenciam o desenvolvimento de vasos sanguíneos, cruciais para a cicatrização adequada de feridas. A angiogênese deficiente resulta em um tecido mal perfundido com consequentes alterações em vários componentes do reparo tecidual tais como diminuições da função dos macrófagos, do acúmulo de colágeno, da quantidade de tecido de granulação, da migração e proliferação de fibroblastos e alteração do equilíbrio entre a produção e o remodelamento da matriz extracelular (Fig. 1) (Maruyama et al., 2007; Gibran et al., 2002; Falanga et al., 2005; Galiano et al., 2004; Kota et al., 2012).

Além disso, feridas diabéticas são caracterizadas por deficiência de óxido nítrico (NO), o que tem efeitos negativos sobre a cicatrização, angiogênese, na expressão de fatores de crescimento e a resposta à infecção (Rubinstein et al., 2013). O óxido nítrico endotelial (eNO) é capaz de ativar a mobilização de derivados endoteliais de células progenitoras da medula óssea para a região da ferida. E estas células são importantes no processo de neovascularização (Gallagher et al., 2007).

Ademais, os mecanismos envolvidos nas cicatrizações patológicas, por exemplo, nas cicatrizes hipertróficas e na formação de queloides, são pouco entendidos, e as opções de tratamentos eficazes ainda continuam um desafio (Eming et al., 2014).

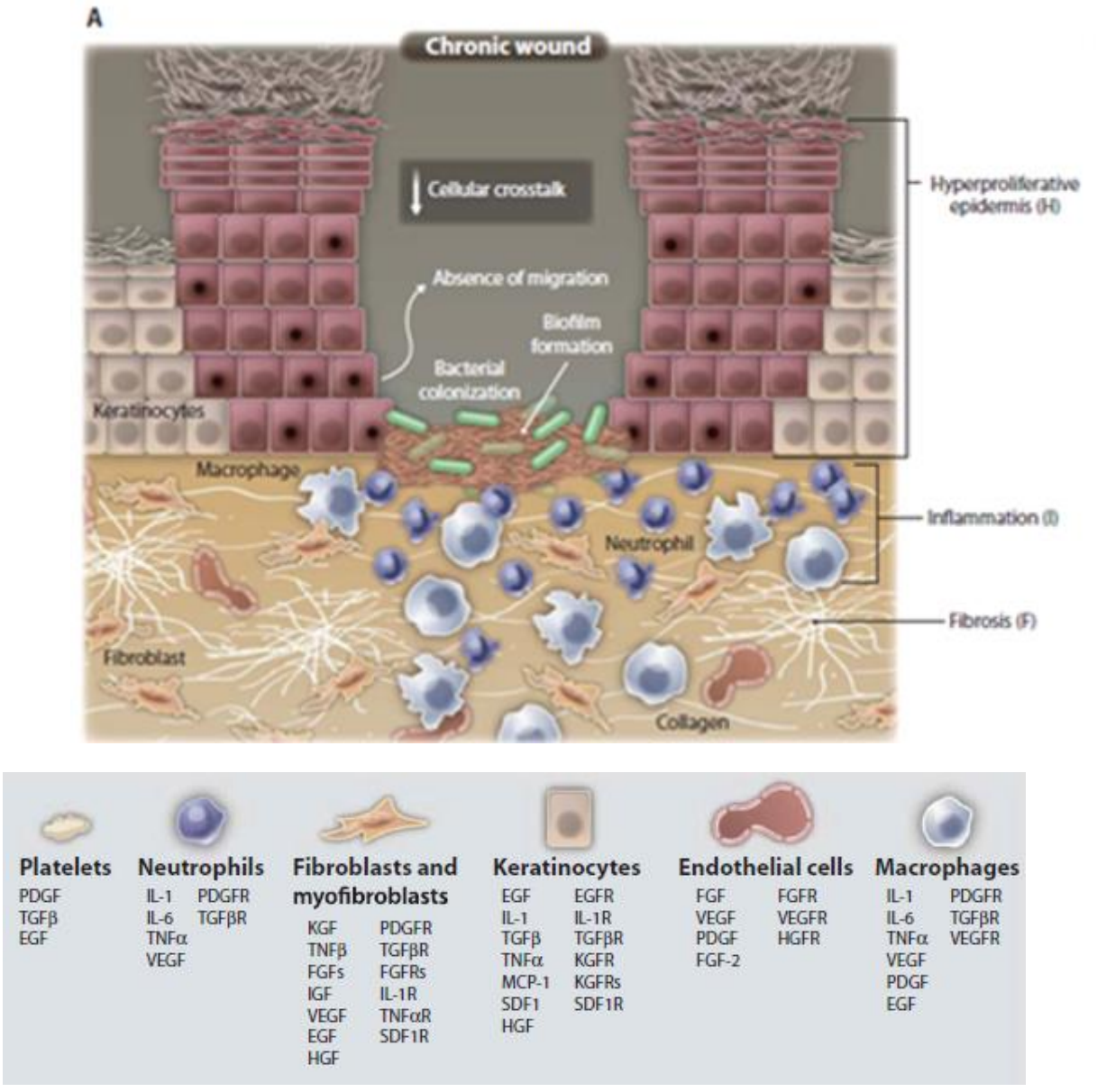

Fig. 1: Patologia molecular das feridas crônicas. Ilustrações mostram mecanismos moleculares e celulares que estão prejudicados em feridas crônicas. (A) As feridas crônicas mostram uma epiderme hiperproliferativa e não migratória, inflamação não resolvida, presença de infecção e formação de biofilme. Embora haja um aumento nas células inflamatórias (neutrófilos e macrófagos), nem todas são adequadamente funcionais (indicado pela alteração morfológica da célula). Produção descontrolada de proteases interferem com os mecanismos de reparo essenciais. Alguns fibroblastos tornam-se senescentes. Em feridas crônicas, há uma redução da angiogênese, do recrutamento e ativação de células tronco, remodelamento da MEC em comparação com a cicatrização normal. (Eming et al., 2014).

Embora existam diferenças etiológicas e clínicas evidentes, as lesões cutâneas e serosas têm eventos comuns como hemostasia, inflamação, proliferação, angiogênese e remodelamento da matriz extracelular (Singer et al., 1999). Estes eventos têm sido estudados de forma exaustiva na pele de indivíduos diabéticos, mas não há informação suficiente em outras localizações anatômicas como no subcutâneo ou na cavidade intraperitoneal, necessárias devido às frequentes complicações na cicatrização que se apresentam neste tipo de pacientes após procedimentos de elevada complexidade como por exemplo no transplante de rim (Roine et al., 2010) ou outras formas de lesões nestes sítios.

1.5 2ª Parte

1.5.1 Resposta tipo corpo estranho

A implantação cirúrgica de materiais e dispositivos biomédicos como *stents* farmacológicos, órgãos artificiais, biosensores, *scaffolds* para engenharia de tecidos e válvulas cardíacas em indivíduos diabéticos tornou-se um procedimento comum para a reparação e/ou substituição de tecidos biológicos e aumentou drasticamente ao longo da última década. Esta tendência deverá continuar com a ampliação da aplicação de biomateriais e a rápida expansão do envelhecimento populacional (Onuki et al., 2008; Socarras et al., 2014).

O biomaterial refere-se a qualquer dispositivo médico projetado para interagir com tecidos/componentes de tecido humano e/ou animal, incluindo dispositivos, terapias celulares, polímeros sintéticos e biopolímeros que são usados como dispositivos para substituir uma parte ou função de um sistema vivo (Park & Lakes 2007). Eles devem ser biocompatíveis e biotoleráveis, ou seja, ter habilidade de

desencadear localmente uma cicatrização normal e integração do tecido, residindo no tecido por longo período com mínima reação inflamatória (Ratner et al., 2016).

O organismo pode reagir a presença destes dispositivos de diferentes formas desde aceitá-los a apresentar reações imunológicas prejudicando o seu funcionamento e trazendo risco para o paciente. A resposta inflamatória persistente a presença continua desse material é denominada “reação tipo corpo estranho” e refere-se à resposta imune não específica a materiais não próprios do hospedeiro que nele são implantados (Fig. 2). Essa reação em um indivíduo normoglicêmico é caracterizada por uma inflamação persistente, infiltração de leucócitos, fusão de macrófagos gerando células gigantes tipo corpo estranho e formação de uma capsula fibrosa em torno do dispositivo (Onuki et al., 2008).

A intensidade e extensão dessa resposta (implante/hospedeiro) depende de características do material e do hospedeiro como tamanho e formato do biomaterial, a constituição química e rugosidade de sua superfície, seu aspecto morfológico e porosidade, o tempo de contato com o tecido hospedeiro, extensão da lesão no procedimento de implantação, o tecido ou órgão onde o dispositivo é implantado e pela extensão da formação provisória da matriz (Onuki, et al., 2008; Anderson, et al. 2008).

A resposta inflamatória aguda a biomateriais, caracterizada principalmente pelo recrutamento de neutrófilos, dependendo da extensão da lesão no local do implante, geralmente dura menos de uma semana. Os mastócitos também são recrutados nesta fase, com degranulação e liberação de histamina, essenciais na adsorção de fibrinogênio e deposição de fibrina na superfície do biomaterial, sendo conhecida por

mediar respostas inflamatórias agudas a biomateriais implantados. As interleucinas IL-4 e IL-13, também liberadas na degranulação de mastócitos, auxiliam na indução da fusão de macrófagos, formando células gigantes do tipo corpo estranho através da regulação dos receptores de manose. Pesquisas também têm demonstrado que a quimiocina CCL2/MCP-1 e transdutores de sinais são importantes requisitos na formação de células gigantes (Kyriakides et al., 2004).

A fase crônica é marcada pela presença de células mononucleares, isto é, macrófagos e linfócitos, no local do implante. Linfócitos Th2 na fase inflamatória crônica transitória participam com a produção de IL-4 e IL-13, que podem induzir a fusão de monócitos/macrófagos para formar células gigantes do tipo corpo estranho. A inflamação crônica também tem sido usada para descrever a reação tipo corpo estranho onde monócitos, macrófagos e células gigantes tipo corpo estranho estão presentes na interface com o biomaterial (Anderson et al., 2008).

Os mecanismos moleculares exatos que levam à fusão de macrófagos não foram completamente elucidados. Kyriakides e cols. 2004 mostraram que a formação de células gigantes do tipo corpo estranho foi reduzida em camundongos CCL-2-null implantados com biomaterial. O bloqueio da CCL2 *in vitro* também reduziu a formação de células gigantes confirmando ainda que CCL2 é um participante crítico na fusão de macrófagos (Kyriakides et al., 2004).

O tecido de granulação formado durante o processo é o precursor da formação de cápsulas fibrosas (Anderson et al., 2008). A fibrose e o encapsulamento extensivos podem resultar em mau funcionamento do biomaterial, extrusão, infecção, trombose e contração de tecidos moles podendo comprometer a saúde do indivíduo (Anderson

et al., 2008).

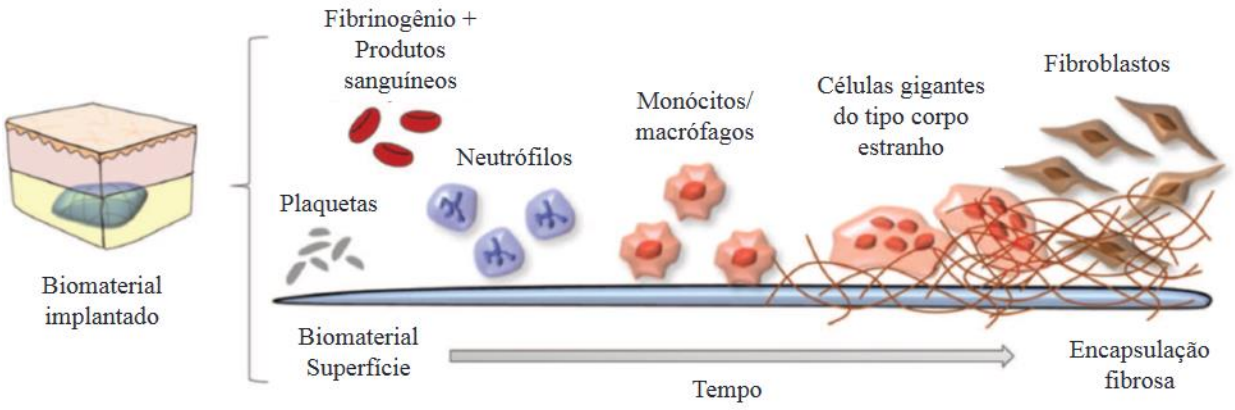

Fig 2. Resposta tipo corpo estranho. A resposta tipo corpo estranho é caracterizada por inflamação persistente, recrutamento e fusão de macrófagos para formar células gigantes tipo corpo estranho. A sinalização inflamatória persistente ativa os fibroblastos secretores de colágeno no sítio do biomaterial, resultando na formação de uma cápsula fibrosa que pode persistir durante a permanência do implante. A fibrose e o encapsulamento extensivos podem resultar em mau funcionamento biomaterial, extrusão, infecção, trombose e contração de tecidos moles (Imagem modificada de Melanie *et al*., 2014).

1.5.2 Reação tipo corpo estranho no diabetes

O diabetes *mellitus* é tido também como um fator agravante na complicação de uma variedade de implantes (Onuki et al., 2008; Socarras et al., 2014).

Embora muitos dos mecanismos alterados na cicatrização de feridas cutâneas em diabéticos tenham sido elucidados, a cicatrização interna de lesões provocadas por dispositivos médicos implantados tem sido pouco investigada. Entender esse processo de reparo é crucial para facilitar o aperfeiçoamento na concepção de dispositivos médico implantável (Le et al., 2011; Onuki et al., 2008; Oviedo-Socarras et al., 2014).

Um estudo que investigou a cicatrização de feridas em babuíno diabético

utilizando implante de poliestireno e outro utilizando dispositivos percutâneos compostos de malha de fibra de titânio em coelhos diabéticos, foi constatado que a formação de tecido de granulação e tecido conjuntivo foram reduzidos comparados com animais não diabéticos (Gerritse et al., 2000; Thomson et al., 2010). Foi também demonstrado pelo nosso grupo de pesquisa que a resposta do tipo corpo estranho após o implante de discos de polieter-poliuretano, no subcutâneo ou intraperitoneal, em ratos diabéticos foi atenuada em comparação com a resposta de animais não diabéticos (Socarras et al., 2014). Entretanto, não foi encontrado nenhum estudo que tenha investigado a influência do background genético na resposta do tipo corpo estranho em camundongos diabéticos.

1.6 Indução do diabetes em camundongos

A pesquisa com animais tem sido fundamental para a compreensão dos mecanismos e complicações desenvolvidas no diabetes. Existem vários modelos que refletem com precisão os aspectos de ambos os diabetes tipo 1 e tipo 2. Alguns protocolos utilizam diferentes estratégias para induzirem o diabetes tipo 1. Eles incluem lesão às células betas do pâncreas usando estreptozotocina ou aloxana; pancreatectomia parcial ou total; hormônios anti-insulínicos e lesões no SNC. Alternativamente, modelos genéticos para o diabetes tipo 2 são produzidos por endogamia seletiva para desenvolver a hiperglicemia e outros traços relacionados à doença, tais como a obesidade, deficiência imunológica, ou a resistência à insulina (Eleazu et al., 2013).

A administração de agentes químicos β-citotóxicos como a aloxana e a estreptozotocina consiste numa maneira eficiente para promover o diabetes *mellitus* insulino dependente tipo 1 e assim, torna-se possível o estudo de mecanismos fisiopatológicos, atividade hipoglicemiante e anti-diabetogênica de certos compostos (Rees et al., 2005).

A estreptozotocina (STZ), isolada de *Streptomyces archromogenes*, é um antibiótico, de natureza glicosamina-nitrosuréia com propriedades tóxicas, tem uma estrutura química semelhante à da glicose, e é captada pelas células β-pancreáticas através de transportadores de glicose GLUT-2 (Fig. 3) (Xiang et al., 2010).

As células β são mais sensíveis a ação da estreptozotocina em relação a outros tipos celulares, especialmente as de roedores devido à baixa expressão gênica a enzimas antioxidantes (Kaneto et al., 2005). Vários mecanismos têm sido propostos para explicar a ação da estreptozotocina sobre danos às células β-pancreáticas. Sugere-se que a estreptozotocina age como agente alquilante sobre a estrutura do DNA por radical •CH3 (Szkudelski et al., 2001; Bolzan et al., 2002). E também atua estimulando a produção de espécies reativas de oxigênio (ERO; O_2-, H_2O_2 e OH), com elevação da peroxidação lipídica e diminuição na atividade das enzimas antioxidantes das células β-pancreáticas (West et al., 2000; Damasceno et al., 2002).

Um dos principais efeitos tóxicos da STZ envolve a alteração da estrutura do DNA, o qual é fragmentado pelas EROs, comprometendo, desta forma, a biossíntese e secreção de insulina (Tabatabaei et al., 2008; Xiang et al., 2010). Assim, Bolzán & Bianchi (2002) relataram que a STZ interfere no metabolismo energético das células

β-pancreáticas, pois inibe o ciclo do ácido cítrico, limitando a produção de ATP mitocondrial, que frequentemente resulta em apoptose celular.

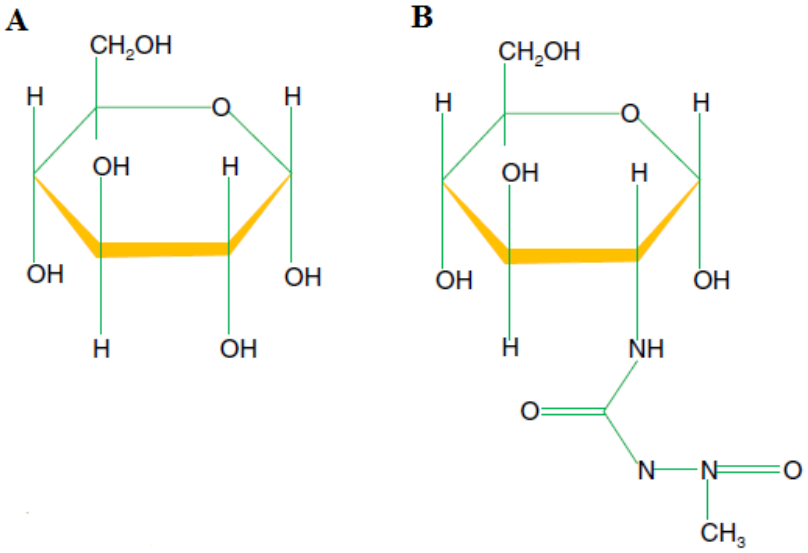

Fig. 3 Estrutura de: A) glicose B) Estreptozotocina (Modificado de Eleazu *et al*., 2013).

1.7 Implante de matriz sintética de poliéter-poliuretano

A esponja de poliéter-poliuretano usada neste estudo é um biomaterial que pode ser implantado no subcutâneo ou na cavidade intraperitoneal como modelo para estudo. O modelo de implantação subcutânea de matriz sintética em animais foi descrito inicialmente por Grindlay e Waugh em 1951 e modificado por Andrade e cols. em 1987. O implante de esponja no animal proporciona um microambiente inflamado e com crescimento de tecido fibrovascular. Nesse ambiente, cada um dos vários componentes desse tecido proliferativo como o recrutamento e ativação de células inflamatórias, angiogênese e deposição de matriz extracelular, podem ser avaliados (Andrade et al., 1997; Almeida et al., 2014; Campos et al., 2008). Além disso, esse modelo tem sido empregado para caracterizar a sequência das alterações histológicas na formação do tecido de granulação e para monitorar a cinética de recrutamento e

proliferação celular. Ele também é particularmente útil, ao permitir a coleta e análise das fases fluida e celular do exsudato inflamatório formado no interior da esponja (Andrade et al., 2009).

1.8 Justificativa

A influência de alterações sistêmicas na formação do tecido fibrovascular induzido por matriz sintética de esponja tem possibilitado caracterizar em animais a susceptibilidade ao lúpus, a tumores sólidos e ao diabetes (Campos et al., 2008; Teixeira et al., 1999). Entretanto, a influência do background genético no reparo de lesões crônicas internas e resposta do tipo corpo estranho, em animais diabéticos, utilizando o modelo de implante de esponja, ainda não havia sido estudada, sendo então, o foco deste trabalho.

2-OBJETIVOS

Avaliar a influência do background genético no reparo de lesão interna e resposta do tipo corpo estranho em animais diabéticos utilizando modelo de implante de esponja em camundongos.

2.1Objetivos específicos

- **Avaliar a influência do backgroud genético na angiogênese inflamatória em lesão interna em camundongos diabeticos.**

  -Avaliar sinais clínicos do diabetes nas diferentes linhagens (Swiss, C57BL/6 e Balb/c).

-Avaliar características histológicas do tecido fibrovascular induzido pelo implante de esponja nas três linhagens.

-Avaliar a influência da hiperglicemia e do background genético nos componentes inflamatório e angiogênico do tecido fibrovascular, induzido pelo implante de esponja.

- **Avaliar o efeito do background genético sobre a resposta do tipo corpo estranho nos implantes das três linhagens diabéticas**

-Avaliar parâmetros fibrogênicos (colágeno tipo I e III).

-Avaliar formação e espessura de cápsula.

-Avaliar recrutamento de mastócitos e formação de células gigantes nas três linhagens diabéticas.

-Avaliar o índice de apoptose.

3- ASPECTOS ÉTICOS

O uso de animais e procedimentos realizados neste projeto foram aprovados pela Comissão de Ética no Uso de Animais (CEUA/UFMG), sob o número 275/2014.

4. MATERIAL E MÉTODOS, RESULTADOS E DISCUSSÃO

Estes tópicos serão apresentados sob a forma de dois artigos científicos elaborados durante o período de doutoramento:

Artigo 1 - publicado

Artigo 2 - submetido para publicação (comprovante de submissão: capítulo 3)

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Capítulo 2

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Original article

Murine strain differences in inflammatory angiogenesis of internal wound in diabetes

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ABSTRACT

Genetic susceptibility is associated with inflammation, neovascularization, and diabetes phenotypes. However, to what extent this susceptibility influences inflammatory angiogenesis in internal injuries in diabetes has not been fully investigated. Using the subcutaneous implantation of a synthetic matrix as an internal wound model in Swiss, C57BL/6 and Balb/c mice, we have studied inflammation, angiogenesis, and cytokine production in the fibrovascular tissue induced by implants in diabetic animals. The hyperglycemic levels (mg/dl) after the diabetogenic treatment were 455.0 ± 15 in Swiss, 393.0 ± 22 in C57BL/6, and 190.0 ± 10 in Balb/c mice. Angiogenesis in Swiss implants from non-diabetic animals were higher than those in the implants from the other strains. However, the angiogenic inducers VEGF and nitric oxide (NO) were higher in implants from non-diabetic Swiss and Balb/c mice. Strain-related differences were also observed in the angiogenic parameters in implants from diabetic mice. Hb content and number of vessels decreased more than 40% in Swiss implants. In contrast, Hb content did not alter in implants from Balb/c diabetic mice and the number of vessels decreased. VEGF levels increased in implants from Swiss and C57BL/6 diabetic mice, but decreased in Balb/c implants. The levels of pro-inflammatory markers intra-implant also varied among the strains in both conditions. In the hyperglycemic environment, almost all inflammatory markers increased in implants from diabetic Swiss mice. These findings demonstrate the major contribution of genetic background in the pattern of inflammatory angiogenesis components of internal injury, in both normoglycemic and hyperglycemic animals.

1. Introduction

Diabetes, a group of metabolic disorders, has common underlying mechanisms associated with hyperglycemia. The duration and intensity of hyperglycemia have been strongly correlated with the rate and progression of diabetes complications (vasculopathy, neuropathy) in both type 1 and type 2 diabetes. The extent of the disease severity and complications varies in each individual, suggesting that genetic background is a relevant factor in the pathological process. In fact, a number of animal studies have demonstrated that mouse strains can differ in their metabolic phenotype. For example, Kooptiwut et al. [1] showed differences in insulin secretory function in two mice models with distinct susceptibility to beta-cell failure. Kulkarni et al. [2] showed the impact of genetic background on development of hyperinsulinemia and diabetes in distinct mice strain. Bock et al. [3] reported the influence of genetic background on the size and structure of endocrine pancreas (variation in islet mass, beta-cell mass, and islet number). Regarding the susceptibility to the diabetogenic treatment (streptozotocin) the mouse strains show inherent differences to the effects of this compound in the development of hyperglycemia [4–6]. Thus, while much is known about the impact of genetic background on constitutive parameters of the metabolic system in diabetes (glucose metabolism, insulin secretion, and disease susceptibility), information on the influence of genetic background on key components (inflammation and

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angiogenesis) of healing processes in the hyperglycemic environment is scarce. Interestingly, these processes have been shown to be influenced by a number of factors, including the genetic trait, in the most frequently used experimental mouse strains (Swiss, C57BL/6, Balb/c, DBA) and models of inflammation and neovascularization. For instance, strain-related differences in angiogenesis signaling in response to hypoxia and to angiogenic factors have been reported [7–9]. We have also demonstrated that the genetic background of Swiss, C57BL/6 and Balb/c mice not only influenced the kinetics of sponge-induced inflammatory angiogenesis, but also the response of distinct mouse strains to pharmacological compounds [10,11].

It has been clearly shown that the diabetes-associated healing impairment, which has been extensively investigated in cutaneous wounds, is characterized by a decreased inflammatory response, amount of fibrosis or fibrogenesis, collagen synthesis, tensile strength, angiogenesis, and altered production of cytokines [12–15]. This pattern is in marked contrast with the healing process in non-diabetic individuals, which is characterized by efficient inflammatory cell recruitment, adequate cytokine production, angiogenesis, matrix formation, and reepitheliazation [16,17]. However, less is known about the healing process of internal injuries in diabetes, as pointed out by Le et al. [18]. In a limited number of studies, using subcutaneous implants in rabbits, baboons, and rats, the formation of granulation tissue and connective tissue ingrowth within and around the devices in diabetic animals were shown to be impaired [19,20]. Although it is thus clear that the angiogenic and inflammatory components of repair processes in diabetes are impaired, no direct comparison of these parameters has been reported in Swiss, C57BL/6 and Balb/c mice. Therefore, our aim in this study to was to determine the effect of mouse strain on the pattern of the inflammatory and angiogenic components of internal fibroproliferative tissue induced by synthetic matrix in diabetic mice. An analysis of the components of the newly formed proliferating fibrovascular tissue might disclose whether genetic background would influence the defective inflammation and angiogenesis in repair processes in diabetic animals. This information would be particularly relevant when considering the animal model of diabetes and therapeutic approaches in healing deficiencies in diabetic individuals.

2. Material and methods

2.1. Animals

All animal care and experimental procedures complied with the guidelines established by our local institutional animal welfare committee. Efforts were made to avoid unnecessary distress to the animals. Female mice Swiss, C57BL/6, and Balb/c were divided into six groups of 10 animals each for biochemical analyses and 5 of each group for histological analyses. The animals were 8–10 weeks old and 25–30 g body weight. The mice were provided by the Central Animal Facility at the Institute of Biological Sciences, Federal University of Minas Gerais number 275/2014. The animals were housed individually and provided with chow pellets and water ad libitum. The light/dark cycle was 12 h/12 h with lights on at 7:00 a.m. and lights off at 7:00 p.m.

2.2. Induction of diabetes mellitus

Streptozotocin (STZ) was obtained from Sigma-Aldrich, St. Louis, MO, USA. STZ was dissolved in a 10 mM sodium citrate buffer, pH 4.5 and always prepared for immediate use within 5–10 min. STZ doses were determined according to the body weight of animals and administered intravenously in injections of 50 mg/kg for 5 consecutive days. This experimental protocol has been shown to induce a model of insulin insufficiency and diabetes type 1 [6,21,22]. The glucose concentration in the blood was measured in fasted state (8 h) in all animals through blood samples taken from the tail vein before the first dose of streptozotocin and on the 20th day after the injections. The measurement was performed using a glucometer Call® On Plus Blood Glucose Meter (ACON Laboratories, Inc.). Animals whose blood glucose levels exceeded 180 mg/dl after treatment were considered diabetic.

2.3. Preparation of sponge discs and implantation

Polyether–polyurethane sponge discs, 5 mm thick × 8 mm diameter (Vitafoam Ltd., Manchester, U.K.) were used as the matrix for fibrovascular tissue growth as previously described [23,24]. The sponge discs were soaked overnight in 70% ethanol and sterilized by boiling in distilled water for 30 min before the implantation surgery. Fifteen days after the last dose of STZ injection following confirmation of diabetes, the animals were anesthetized with a 40 μL mixture of ketamine and xylazine (57 mg/ml and 8.6 mg/ml, respectively). The dorsal hair was shaved and the skin wiped with 70% ethanol. The sponge discs were aseptically implanted inside a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. The incisions were closed with silk braided non-absorbable suture. At 10 days post implantation (25 days after induction of diabetes), the animals were anesthetized with ketamine and xylazine and later killed by cervical dislocation. At this point, the fibrovascular tissue induced by the sponge matrix is composed of well-developed blood vessels containing red blood cells, inflammatory cells, fibroblasts within a mature extracellular matrix. All these features are present in the granulation tissue during healing processes.

The sponge discs were carefully dissected from adherent tissue, removed and weighed. They were then processed as described below for the various assays.

2.4. Hemoglobin extraction

The extent of vascularization of the sponge implants was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin method. At 10 days post implantation, the animals were killed and the sponge implants carefully removed, dissected, cleared of any adherent tissue, and weighed. Each implant was homogenized (T10 basic Ultra-Turrax disperser; S10 N – ST dispersing element, IKA) in 2 ml of Drabkin reagent (Labtest, São Paulo, Brazil) and centrifuged at 12,000*g* for 20 min. The supernatants were filtered through a 0.22-μm Millipore filter. The hemoglobin concentration in the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and comparing it against a standard hemoglobin curve. Hemoglobin content in the implant was expressed as μg Hb per mg wet tissue [10,21].

2.5. Tissue extraction and determination of myeloperoxidase (MPO) and N-acetyl-β-D glucosaminidase (NAG) [25] activities

The number of neutrophils in implants was measured by assaying myeloperoxidase (MPO) activity as previously described [22,26]. The implants were weighed, homogenized in saline sodium phosphate EDTA-HCl buffer (0.1 M NaCl, 0.02 M Na_3PO4, 0.015 M Na_2EDTA; using HCl to adjust pH), pH 4.7, and centrifuged at 12,000*g* for 20 min, 4 °C. The pellets were then re-suspended in 2 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% hexa1,6-bis-decyltrimethylammonium bromide (HTAB, Sigma), homogenized for 30 s, followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was

assayed by measuring the change in absorbance (optical density; OD) at 450 nm. The assay was performed using 25 μl of 3,3′-5,5′-tetramethylbenzidine (TMB, Sigma), at a final concentration of 1.6 mM, diluted in dimethyl sulphoxy (DMSO, Merck); 100 μl of H_2O_2 (0.003% v/v) diluted in 0.05 M of sodium phosphate pH 5.4 and 25 μl of supernatant of the processed samples, followed by incubation at 37 °C for 5 min. The reaction was terminated by the addition of 100 μl of H_2SO_4 (4 M). Results were expressed as a change in OD per g 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) [25] present in high levels in activated macrophages [22,26]. The implants were homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega) and centrifuged (3000 *g*; 10 min at 4 °C). Samples (100 μl) of the supernatant diluted in citrate-sodium phosphate buffer (200 ml citric acid 0.1 M; 310 ml

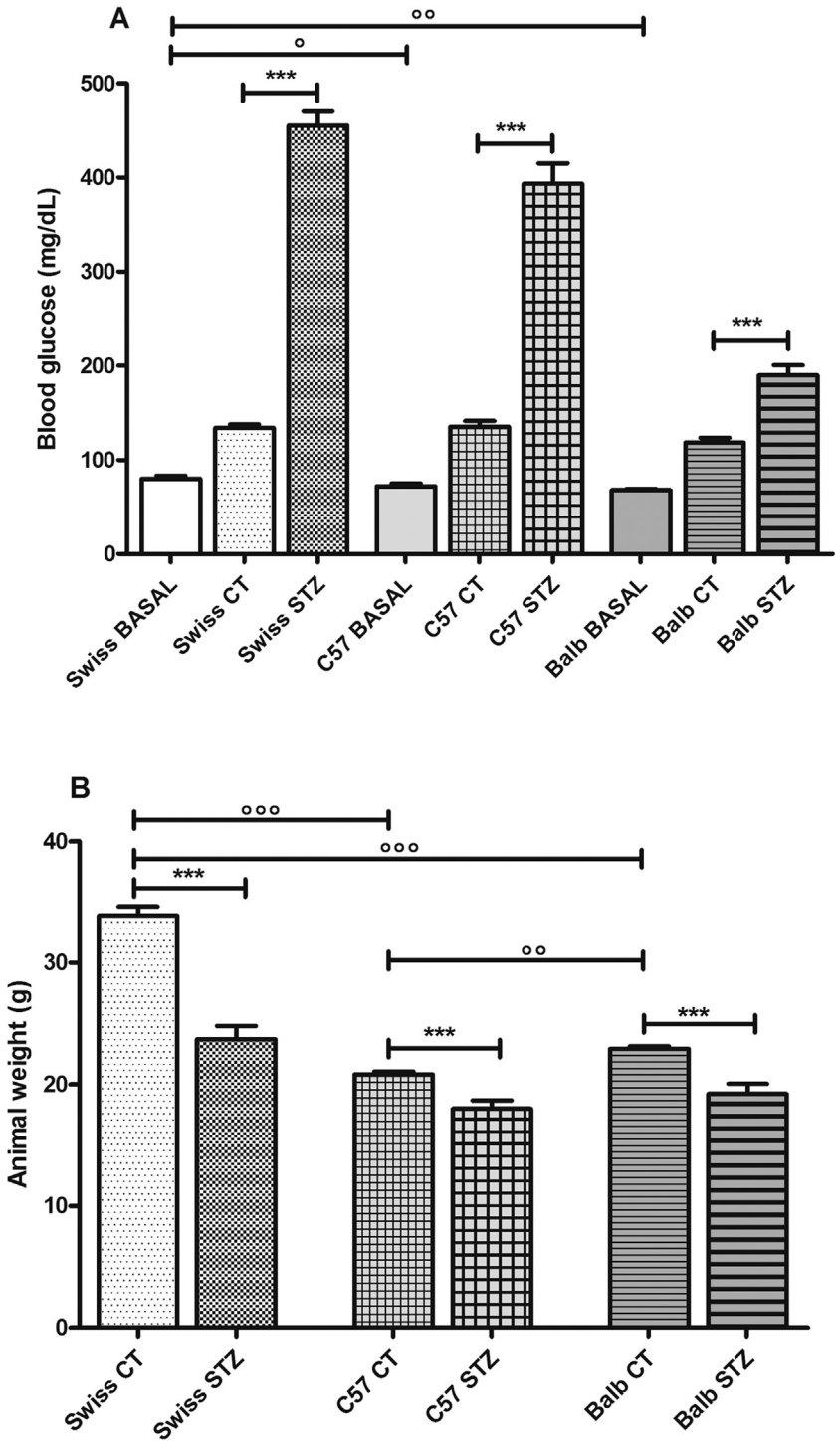

Fig. 1. Blood glucose levels and body weight of non-diabetic and diabetic mice. A significant increase in blood glucose level (A) and decrease in body weight (B) was observed after streptozotocin (STZ) injection in mice as compared with non-diabetic animals. Blood glucose levels increased more than 5-fold in Swiss or C57BL/6 mice and 2.8 fold in Balb/c animals compared with basal levels. Data are expressed as means ± SEM. o Significant difference among the control animals of the strains (ANOVA); *Significant difference between non-diabetic and diabetic in the same strain (Student's *t*-test); o * $P < 0.05$; oo ** $P < 0.01$; ooo ***$P < 0.001$. CT (control non-diabetic); STZ (streptozotocin).

Na_2HPO_4 0.1 M;), pH 4.5 were incubated for 30 min at 37 °C with 100 μl of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) prepared in citrate-sodium phosphate buffer to yield a final concentration of 2.24 mM. The reaction was stopped by the addition of 100 μl of 0.26 M glycine buffer (the same volume of 0.8 M glycine; 0.8 M NaCl; 0.8 M NaOH), pH 10.6. Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. Results were expressed as *p*-nitrophenol nmol/mg wet tissue.

2.6. Measurement of VEGF, TNF-α, CCL2, or CXCL-1/KC production in the sponge implants

The implants removed at 10 days post implantation were homogenized in 1000 μl of sodium phosphate buffered, pH 7.4 containing 0.05% Tween-20 and centrifuged at 10,000*g*, 4 °C for 30 min. The cytokines VEGF, TNF-α, CCL2 and CXCL-1/KC in the supernatant from each implant were measured in 50 μl of the

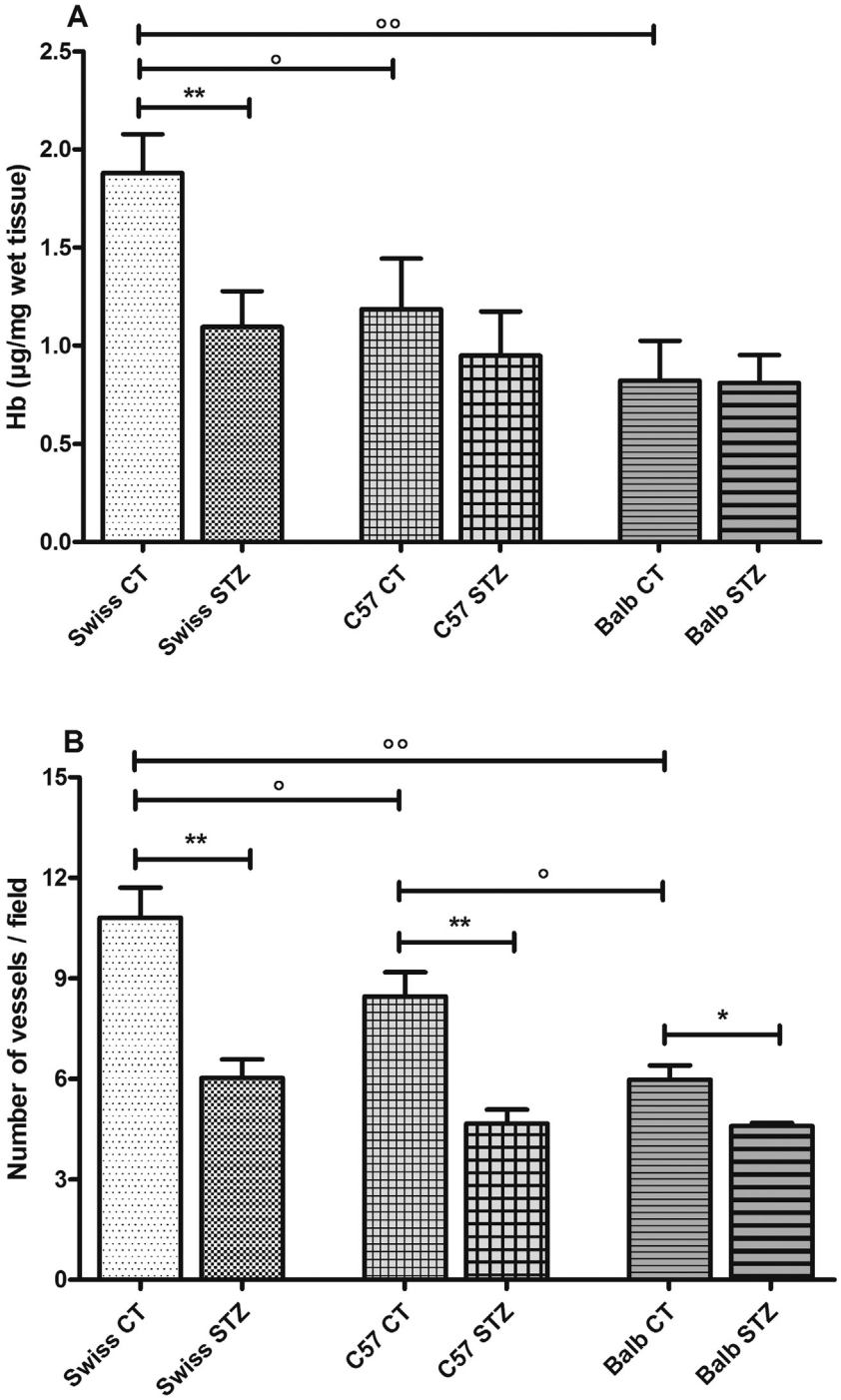

Fig. 2. Hemoglobin (Hb) content (A) and number of blood vessels (B) in 10-day old implants from non-diabetic and diabetic mice. Hb content decreased in implants from diabetic Swiss mice, but did not change in diabetic C57BL/6 or Balb/c implants. Decreased blood vessels were seen in all implants from diabetic animals. Values shown are expressed as mean ± SEM group. o Significant difference among the control animals of the strains (ANOVA); *Significant difference between non-diabetic and diabetic in the same strain (Student's *t*-test); o * $P < 0.05$; oo ** $P < 0.01$. CT (control non-diabetic); STZ (streptozotocin).

supernatant using Immunoassay Kits (R&D Systems, USA) and following the manufacturer's protocol.

Briefly, dilutions of cell-free supernatants 100 μl per well were added in duplicate to ELISA plates blocked and coated with a specific murine monoclonal antibody against the cytokine, followed by the addition of a 100 μl/well of second monoclonal antibody against the cytokine. Streptavidin-HRP and substrate solution (TMB and H_2O_2; 100 μl/well) were added to the wells and incubated for 20 min. The reaction was stopped with 2 N sulphuric acid (50 μl) and the intensity of the color was measured at 540 nm on a spectrophotometer (E-Max, Molecular Devices). Standards were 0.5-log10 dilutions of recombinant murine cytokines from 7.5 pg ml^{-1} to 1000 pg ml^{-1} (100 μl). The results were expressed as pg cytokine per mg wet tissue.

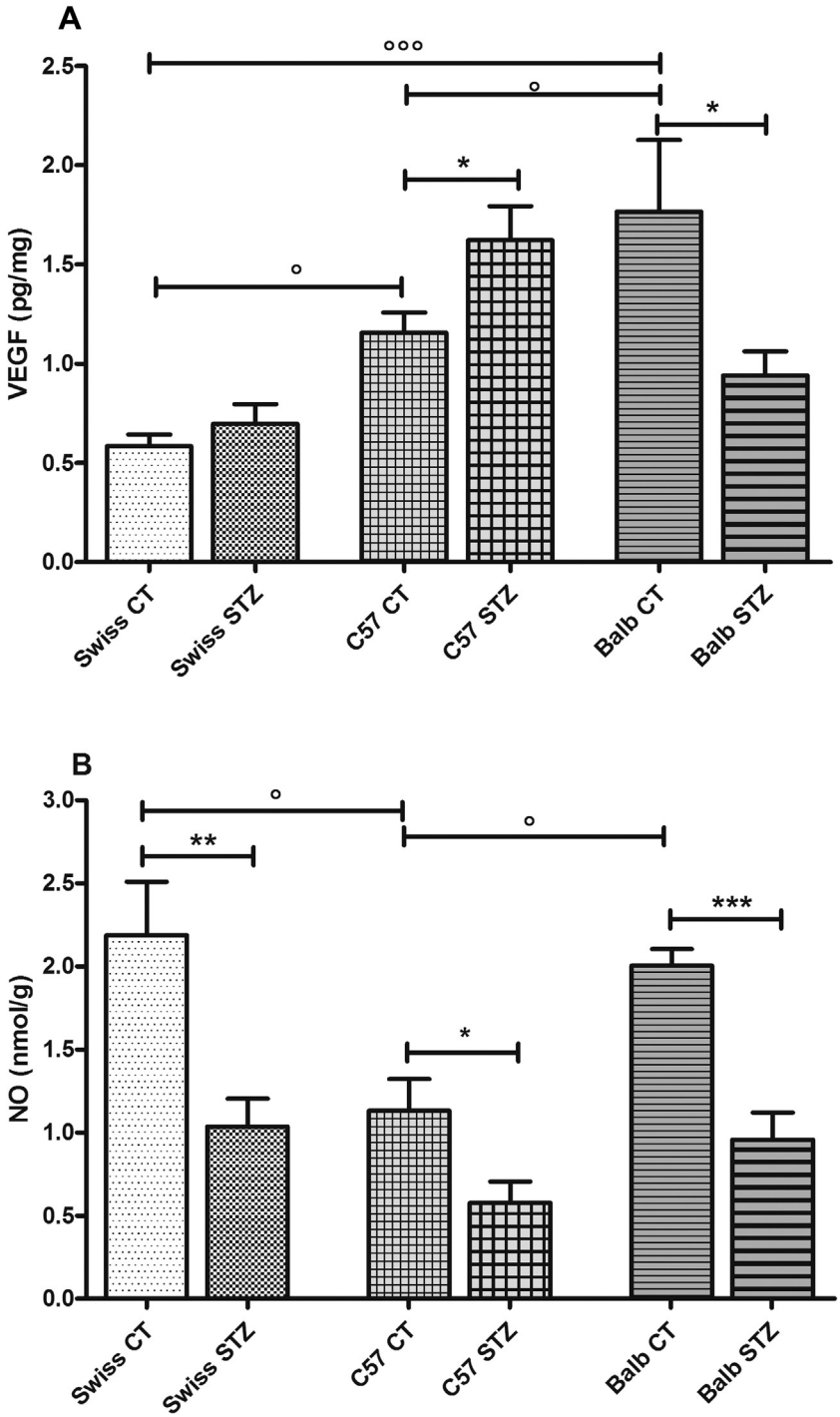

Fig. 3. VEGF and nitric oxide (NO) levels in 10-day old implants from non-diabetic and diabetic mice of different strains. Both markers were higher in implants from Balb/c mice. In hyperglycemic environment, VEGF increased in Swiss implants and C57BL/6, and NO levels fell in implants from the three strains. Values shown are expressed as mean ± SEM. o Significant difference among the strains (ANOVA); *Significant difference between non-diabetic and diabetic in the control animals of the strains (Student's *t*-test); o * $P < 0.05$; oo ** $P < 0.01$; ooo *** $P < 0.001$. CT (control non-diabetic); STZ (streptozotocin).

2.7. Nitric oxide (NO) determination in the sponge implants

For these set of experiments, the implants of non-diabetic and diabetic groups of mice removed 10 days after implantation were weighed and processed for analyses using World precision Instruments (TBR4100 Free Radical Analyzer). NO levels production in the implants were determined by means of carbon monoxide macrosensors, each one gas-permeable. The freshly removed implants were placed in nitrogen and crushed. NO concentrations were determined by calibration curves of known concentrations of s-nitroso-*n*-acetyl-DL-penicillamine (SNAP; 0.2–500 nmol/L) obtained from Sigma [24].

2.8. Histological analysis and staining

The sponge implants from a separate group of mice (n = 5 for each strain) were excised carefully, dissected free of adherent tissue, and fixed in 10% neutral buffered formalin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) and processed for light microscopic studies. To perform morphometric analysis, images of cross sections obtained from 20 fields per slide (8533 μm^2/field) were captured with a plan apochromatic objective (40×) in light microscopy (final magnification = 400×). The images were digitized through a JVC TK- 1270/JCB microcamera and transferred to an analyzer (Kontron Electronics, Carl Zeiss—KS300 version 2). A countable vessel was defined as a structure with a lumen.

2.9. Statistical analysis

All data were analyzed using GraphPad Prism for windows (GraphPad Software Inc.). Results are expressed as mean ± SEM. Comparisons between three or more groups were made using one-way analysis of variance (ANOVA) followed by the Newman-Keuls correction factor for multiple comparisons as a post-test. Unpaired *T*-test was used to compare two groups. Differences between means were considered significant when p values were <0.05.

3. Results

The initial fasting blood glucose (basal) levels (mg/dl) were surprisingly higher in non-diabetic Swiss mice (80.1 ± 3.1) than those in C57BL/6 mice (72.1 ± 2.9) or in Balb/c mice (67.9 ± 1.5). Blood glucose levels increased in non-diabetic mice during the experimental period after implantation (Swiss = 134.0 ± 3.9; C57 = 135.4 ± 6.2; Balb/c = 118.4 ± 5.0) (Fig. 1A). The administration of 50 mg/kg of streptozotocin for five consecutive days in Swiss, C57BL/6 and Balb/c mice induced diabetes type 1 in all treated

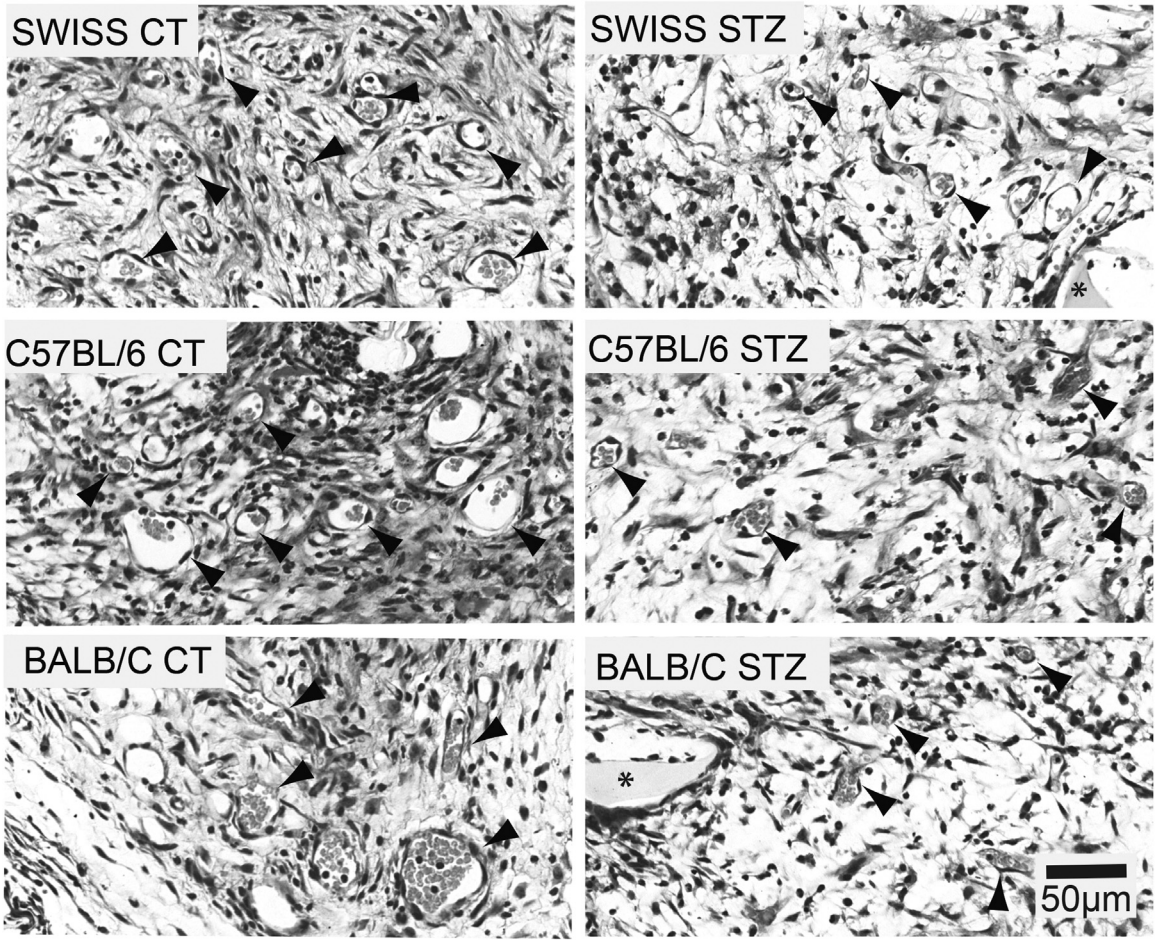

Fig. 4. Representative histological sections (5 μm, stained with H&E) of fibrovascular tissue in 10-day old implants from non-diabetic and diabetic mice of different strains (A and B, Swiss implants; C and D, C57BL/6 implants; E and F, Balb/c implants). Granulation tissue and new extracellular matrix formation can be seen in implant sections, in which the connective proliferating tissue is composed of spindle-shaped fibroblasts, microvessels, and inflammatory infiltrate. Vascularization is more intense in implants from Swiss strain, as compared with that from the other two strains. Collagen deposition is more pronounced in C57BL/6 implants sections compared with the other two strains. Triangular shapes which vary in size due to irregularity in synthetic matrix represent the pores of the sponge matrix. Cellularity and newly formed blood vessels are decreased in implants from diabetic mice (STZ). Microvessels are more dilated in Balb/c or C57BL/6 implants. Bar 50 μm. (*) sponge matrix; Arrowhead (▶) shows blood vessels.

animals, however the mouse strains responded distinctly to the diabetogenic treatment. The blood glucose values, 20 days after diabetes induction, increased to 455.4 ± 14.5 in Swiss mice (5.7 fold increase), to 393.3 ± 21.7 in C57BL/6 (5.5 fold increase), and to 190.0 ± 10.5 in Balb/c (2.8 fold increase). The diabetogenic treatment also distinctly affected the animals' body weight. At the end of the experiment (25 days after diabetes induction), diabetic Swiss mice lost 29.4% of the body weight as compared with non-diabetic animals, whereas weight losses in C57BL/6 and Balb/c mice were 14.3% and 17.4%, respectively (Fig. 1B).

3.1. Measurement of implant angiogenesis

Quantitative measurement of angiogenesis was performed indirectly by hemoglobin content (μg/mg wet tissue) and directly by determining the number of vessels in histological sections of the implants (H&E staining). These parameters were different among the three strains of non-diabetic mice. Implants of Swiss mice had the highest levels of hemoglobin and number of vessels, and Balb/c implants were the least vascularized (Fig. 2A and B). After the diabetogenic treatment, implant vascularization from Swiss mice

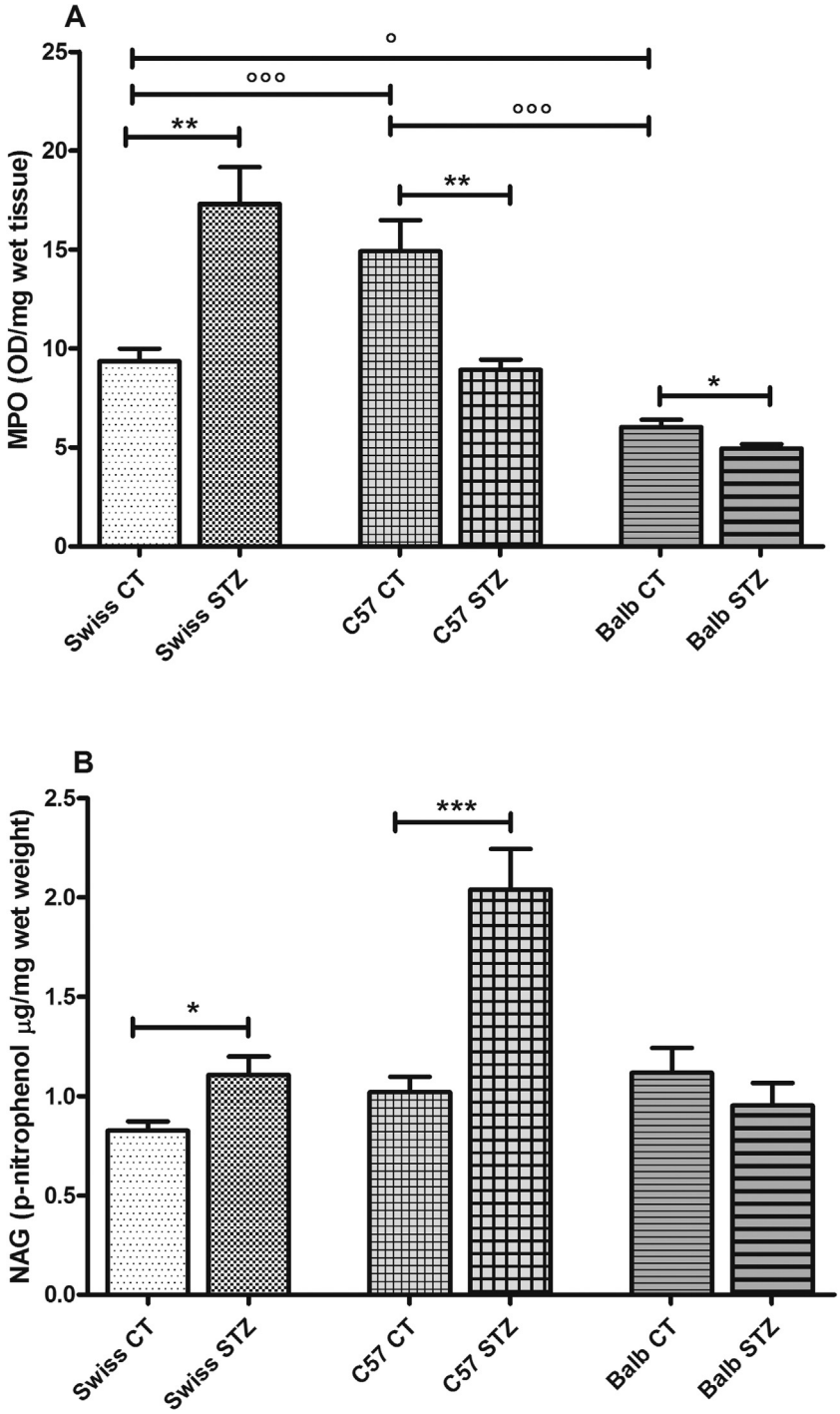

Fig. 5. Inflammatory enzyme activities in 10-day old implants from non-diabetic and diabetic mice of different strains. An increase in the inflammatory reaction was observed after diabetes induction in Swiss implants. MPO—myeoloperoxidase (A); NAG—*n*-acetyl-β-D-glucosaminidase (B); Values shown are expressed as mean ± SEM. o Significant difference among the control animals of the strains (ANOVA); *Significant difference between non-diabetic and diabetic in the same strain (Student's *t*-test); o * $P < 0.05$; oo ** $P < 0.01$; ooo *** $P < 0.001$. CT (control non-diabetic); STZ (streptozotocin).

fell to 44% and from C57BL/6 diabetic animals to 45% in relation to the control groups. Hb content in implants from Balb/c mice was stable and the number of vessels decreased 23% as compared with the values of implants from non-diabetic animals. Measurement of the angiogenic inducers, VEGF, and nitric oxide (NO) revealed strain-related differences in both non-diabetic and diabetic animals (Fig. 3A and B). The highest levels of both inducers in non-diabetic animals were observed in Swiss and Balb/c implants. VEGF levels (pg/mg wet tissue) in the implants from Swiss mice were 0.58 ± 0.06, whereas in Balb/c implants the cytokine production was 1.76 ± 0.4 (Fig. 3A). Fig. 3B shows that NO levels in implants from Swiss mice were (2.2 ± 0.3) and Balb/c (2.0 ± 0.1), which were higher than those from C57BL/6 implants (1.1 ± 0.1).

After diabetes, VEGF increased in Swiss and C57BL/6 implants, but decreased in Balb/c implants. Nitric oxide levels fell in the implants of the three strains after diabetes (Fig. 3A and B).

Histological analysis of the implants from non-diabetic animals of the three strains showed a proliferating stroma occupying the pores of the sponge matrix. Blood vessels, inflammatory infiltrate, and spindle-shaped fibroblasts were seen in all sections, but there were more blood vessels in Swiss implants as compared with C57BL/6 or Balb/c. In diabetic animals, the implants presented a less mature and less dense extracellular matrix with fewer, but more dilated blood vessels (H&E stained sections). This latter feature was clearly more evident in C57BL/6 and Balb/c implants (Fig. 4A–F).

3.2. Inflammation in sponge implants

Intra-implant inflammatory parameters (myeloperoxidase-MPO and *N*-acetyl-β-D-glucosaminidase – NAG activities; and levels of pro-inflammatory cytokines CXCL-1/KC, CCL2 and TNF-α) were markedly influenced by the genetic background in both non-diabetic and diabetic animals (Figs. 5A and B; 6A-C). Balb/c and C57BL/6 implants constitutively presented more MPO activity as compared with Swiss (Fig. 5A). In implants from diabetic animals, there was an increase (69%) in the enzyme activity in implants from Swiss mice, but a 38% decrease in C57BL/6 implants and 38% in Balb/c implants. NAG activity was similar for the implants of the three strains, but after diabetogenic treatment, this activity increased two-fold in C57BL/6 and in Swiss implants, and no changes in implants from Balb/c strain (Fig. 5B).

The levels of inflammatory cytokines (CXCL-1/KC, CCL2, and TNF-α) varied among the strains before and after diabetes induction (Fig. 6A–C). CXCL-1/KC levels were higher in C57BL/6 implants of non-diabetic animals as compared with the levels of the other two strains and increased (130%) in C57BL/6 implants after diabetes (Fig. 6A). A CCL2 level that is recognized for its monocyte/macrophage chemoattractant activity was increased in C57BL/6 as compared with Swiss and Balb/c implants before diabetes, but decreased (11%) in implants of diabetic Balb/c animals (Fig. 6B). Constitutively, the levels of TNF-α were higher in implants from non-diabetic Balb/c mice, as compared with the other two strains (Swiss, C57BL/6). In implants from diabetic Swiss and Balb/c mice, there was a 30% and 35% decrease, respectively, in the levels of this cytokine, whereas in implants from diabetic C57BL/6 mice, a 46% increase was observed (Fig. 6C).

4. Discussion

The major finding of this study was that angiogenesis, inflammation, and cytokine production (key components) of internal injury healing processes in both normoglycemic and hyperglycemic mice occurred in a strain-specific manner. In the first series of data, we have shown that the basal blood glucose levels of the different strains of mice (Swiss, C57BL/6, and Balb/c)

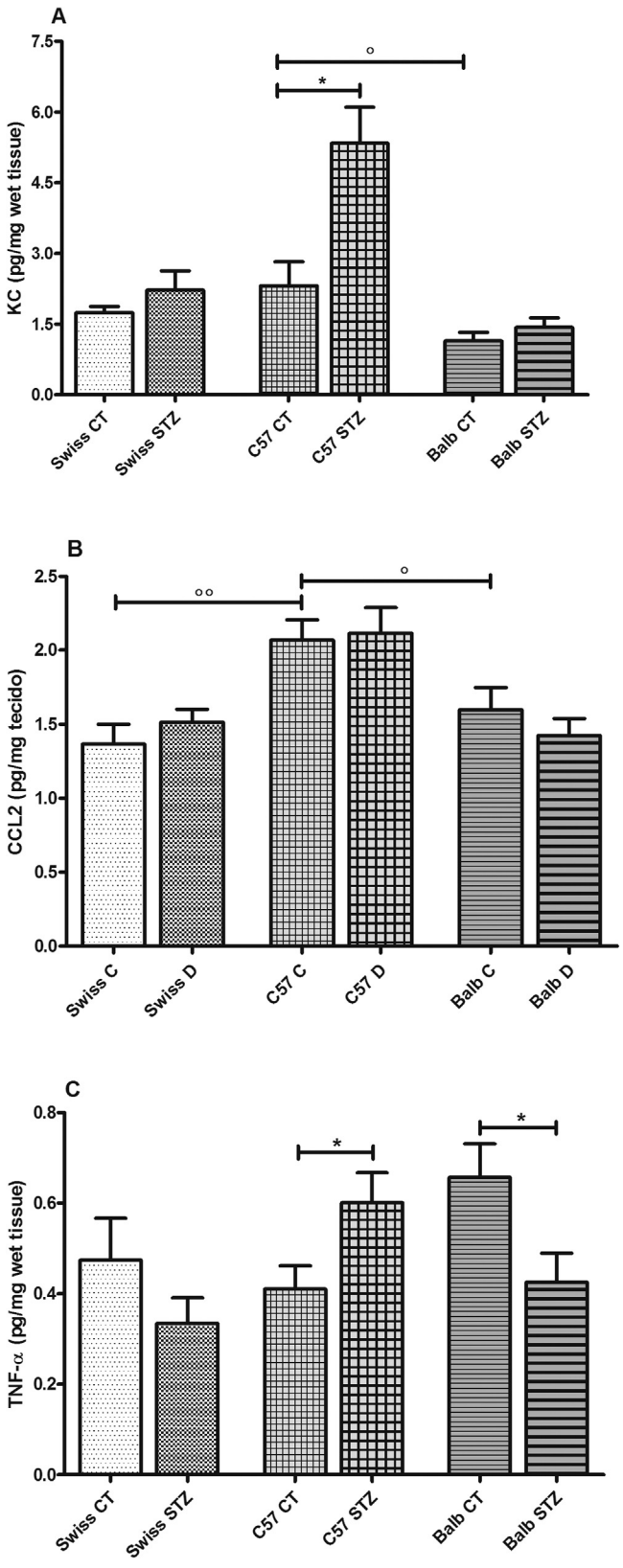

Fig. 6. Levels of inflammatory cytokines CXCL-1/KC (A), CCL2 (B), and TNF-α (C) in 10-day old implants from non-diabetic and diabetic mice of different strains. CXCL-1/KC and CCL2 levels were similar in implants from non-diabetic mice, but TNF-α levels were higher in Balb/c implants. After diabetes, CXCL-1/KC and TNF-α increased in C57BL/6 implants, whereas CCL2 decreased in Balb/c implants. Values shown are expressed as mean ± SEM. o Significant difference among the control animals of the strains (ANOVA); *Significant difference between non-diabetic and diabetic in the same strain (Student's *t*-test); o * $P < 0.05$; oo ** $P < 0.01$. CT (control non-diabetic); STZ (streptozotocin).

varied constitutively and that, after manipulation (surgical procedure for sponge implantation), the blood glucose levels increased markedly in all three strains. Our results are in agreement with various reports that showed that blood glucose levels are affected by surgical procedure and anesthetic compounds, including the one used in our experiments (xylazine/ketamine) [27–29]. However, the effects of the diabetogenic treatment (streptozotocin) resulted in a more pronounced hyperglycemia in all strains.

Furthermore, the same diabetogenic treatment (five consecutive doses of streptozotocin) resulted in a wide variation in blood glucose levels. In Swiss and C57BL/6 mice, the diabetogenic treatment increased blood glucose levels more than 5-fold, whereas in Balb/c mice, this increase was only 2.8 fold. Although blood glucose levels in Balb/c group increased less (approximately 3 fold) after STZ treatment compared with those of the other two strains (more than 5 fold), there were consistent decreases in the animal's body weight and other angiogenic and inflammatory parameters analyzed compared with their non-diabetic counterparts, as discussed below. In a study by Rubinstein et al., 2008, female Balb/cByJ mice that received low doses of streptozotocin (40 mg/kg for 5 consecutive days) were considered diabetic and displayed altered humoral immune responses. In these animals, basal blood glucose level raised from 83 ± 7 to 183 ± 33 mg/dl, after STZ treatment, an increment of 2.2 fold. Our results are in line with previous studies that have shown that susceptibility or resistance to streptozotocin treatment is highly variable among mouse strains and with the demonstration of inter-strain variation regarding insulin resistance, glucose homeostasis, and tolerance test [2,6,30,31].

In diabetes, healing impairment of cutaneous lesions is a prevalent complication and is characterized by a decreased inflammatory response, amount of fibrosis, collagen synthesis, tensile strength, angiogenesis, and altered production of cytokines [12–14]. Whether this altered pattern of repair occurs in internal healing in diabetic animals of distinct background has not been investigated to our knowledge. In our study, we chose the most commonly used laboratory mouse strains, one outbred (Swiss mouse), and two inbred (C57BL/6 and Balb/c strains), and the subcutaneous implantation of a synthetic polyether-polyurethane matrix as a model of internal injury to test our hypothesis.

Hemoglobin content (Hb) and number of vessels (angiogenesis parameters) in implants from normoglycemic Swiss mice were higher than those in the implants from the other strains. This finding is consistent with other reports showing that different mouse strains vary in their ability to develop collateral vessels and form neovasculature in response to angiogenic factors and in chronic inflammatory processes [7,8,10,32,33]. Hemoglobin content and number of vessels in implants from diabetic Swiss mice decreased 42% and 44%, respectively, as compared with the values of non-diabetic animals. Interestingly, the number of vessels, but not Hb content, decreased in implants from diabetic Balb/c mice. This unchanged value in Hb content may be attributed to a more dilated state of the newly formed vessels in the diabetic inbred strain as compared with the outbred, as shown in histological analysis. In fact, vascular dysfunctions, such as increased permeability, vasodilatation, and structural changes are well established abnormalities in the diabetic state in experimental animals and human microvasculature [19,33,34]. Our findings are also in agreement with previous studies that demonstrated reduced angiogenesis in healing processes in diabetes [35,36]. However, the authors are not aware of any study that has established such functional and structural differences in the neovasculature among distinct diabetic mouse strains.

The production of pro-angiogenic markers, VEGF and NO intra-implant, showed strain-specific differences in normoglycemic animals. VEGF and NO were higher in implants from Balb/c mice (the least vascularized implants). Thus, it seems clear that genetic heterogeneity may be involved in the expression and function of angiogenic factors. The variation in NO production among the normoglycemic mice of different strains was expected to occur since cytokine and NO production and the pathways resulting in the induction of iNOS expression have been shown to vary in different cells or species [32,34,37].

VEGF production was also distinctly regulated in the different strains in the hyperglycemic environment. While there was an increase in the levels of this cytokine in implants from diabetic Swiss (19%) and C57BL/6 (40%) mice, there was a decrease (47%) in VEGF levels in implants from Balb/c mice. Conversely, NO production in the implants of all strains was down regulated in implants of the three strains in the diabetic environment. NO production decreased about 50% in implants from Swiss, C57BL/6 and Balb/c mice.

These data indicate that VEGF production is differentially regulated in a hyperglycemic environment, whereas glucose homeostasis plays a decisive role in NO regulation/production. Since bioactivity of NO is critical to the angiogenic processes, such as the survival, proliferation, and migration of endothelial cells [24,34,38]. It may be possible that, at least in part, the decreased angiogenesis in implants from diabetic animals may be attributed to NO deficiency and that, even in presence of high concentrations of VEGF (Swiss and C57B/6 strain), angiogenesis was not improved. This demonstrates that even markers of the same physiological process may vary independently. However, it has been reported that the hypoxic cortex of CD1 mice had the largest neovascularized area, which coincided with increased VEGF expression, as compared with C57BL/6 or Balb/c animals [36]. Thus, regulation of angiogenesis may be different in different tissues and different strains.

We measured five inflammation markers (MPO, NAG, and three cytokines CXCL-1/KC, CCL2, and TNF-α), again with clear, strain-related differences. Constitutively, MPO activity was more pronounced in Balb/c implants as compared with the other two strains. In the hyperglycemic environment, this activity increased by 69% in Swiss implants, whereas it decreased in implants from C57BL/6 or Balb/c implants. NAG activity was similar among all three strains before diabetes induction, but varied after diabetes. It remained unchanged in Balb/c implants, increased 73% in Swiss and in C57BL/6 implants. Our findings are in accordance with the notion of altered inflammatory microenvironment in diabetic skin wounds extending this concept to the internal injury in mice. To some extent, our results are also in agreement with studies performed in subcutaneous and percutaneous implants from diabetic baboons and rabbits, in which persistent infiltration of neutrophils and a reduced number of macrophages were observed in internal wounds [19,35]. These changes have occurred together with alterations in chemokine and growth factor production (increase levels of CXCL-1/KC, CCL2, and TNF-α), but, again without a clear pattern. Alterations in the production of inflammatory cytokines were observed in wounds of type-1 diabetic patients and experimental animals.

The most striking differences were the lowest levels of CXCL-1/KC versus highest levels of TNF-α in implants from normoglycemic Balb/c mice as compared with the other two strains. After diabetes, CXCL-1/KC levels increased 130% in C57BL/6 implants and TNFα increased (46%). In implants from diabetic Swiss and Balb/c, a 28 and 35% decrease, respectively, was observed in TNF-α levels as compared with the normoglycemic control. Our finding that Balb/c implants contained more TNF-α than the other strains was in contrast to that of Muller et al. [39], who reported that TNF-α expression was 50% lower in Balb/c mice as compared with C57BL/6 mice, but these authors used islets to study ex vivo effects of

MLD-STZ. The discrepancy between the levels of the chemokine CCL2 and NAG activity (activated macrophages) in C57BL/6 implants is also in contrast to the correlation between these variables previously observed.

Overall, our results confirmed and extended the differences in the intensity and magnitude of inflammatory processes between mouse strains that have beenreported in various experimental models of inflammation [38,40,41].

However, to the best of the authors' knowledge, this is the first demonstration of strain-related differences in the pattern of the inflammatory and angiogenic components of internal fibroproliferative tissue induced by synthetic matrix in diabetes in mice. Such variability may be relevant when considering the animal model of diabetes and therapeutic approaches in healing deficiencies in diabetic individuals.

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CAPÍTULO 3

Journal of Biomedical Materials Research Part A

Genetic background influences foreign body reaction in diabetic mice

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Keywords:	Genetic Background, Diabetes, Host response, Biomaterial implantation, Fibrosis

Genetic background influences foreign body reaction in diabetic mice

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Abstract

A number of genetic factors have been linked to the development of diabetes, a condition that often requires implantable devices like glucose sensors. In normglycemic individuals, this procedure induces a foreign body reaction (FBR) that is detrimental to bioimplants functionality. However, the influence of the genetic background on this reaction in diabetes has not been investigated. We examined the components of FBR (capsule thickness, collagen deposition, mast cell and foreign body giant cell number) in subcutaneous implants of polyether-polyurethane (SIPP) in streptozotocin-(STZ)-induced diabetes in Swiss, C57BL/6, and Balb/c mice. The fasting blood glucose levels before STZ injections were 133.5 ±5.1 mg/dl, after the treatmentincreased 68.4% in Swiss mice, 62.4% in C57BL/6 and 30.9% in Balb/c mice. All FBR features were higher in implants of Swiss and C57BL/6 mice compared with those in implants of Balb/c. Likewise, the apoptotic index was higher in implants of diabetic Swiss and C57BL/6 mice whose glycemic levels were the highest. Our findings show an association between the severity of hyperglycemic levels and the intensity of the FBR to SIPP. These important strain-related differences in susceptibility to diabetes and the intensity of the FBR must be considered in management using implantable devices in diabetic individuals.

Keywords: Genetic Background, Diabetes, Host response, Biomaterial implantation,Fibrosis.

Introduction

Genetic susceptibility is considered to underlie a number of physiological and pathological conditions in both humans and experimental animals. There is a clear association between genetic background and the development of the diabetes. It has been demonstrated, for example, differences in insulin secretory function in two mice models with distinct susceptibility to beta-cell failure (Kooptiwut et al., 2002). The influence of genetic background on development of hyperinsulinemia and diabetes in distinct mice strains has also been reported (Kulkarni et al., 2003). Bock and cols 2005 reported the influence of genetic background on the size and structure of endocrine pancreas (variation in islet mass, beta-cell mass, and islet number) (Bock et al., 2005). Inherent differences to the effects of streptozotocin in the development of hyperglycemia have also been described in distinct mouse strains (Almeida et al., 2017; Herold et al., 1997; Oosterlinck et al., 2011; Rubinstein et al., 2013). In the context of tissue repair, there is compelling evidence of the influence of the genetic background on healing parameters such as angiogenesis, inflammation and fibrogenesis (Walkin et al., 2013).

Wound healing impairment and additional long term complications of diabetes often requires implantable devices such as glucose sensors, orthopedic implants, catheter, vascular grafts, drug-eluting stents, artificial organs, biosensors, scaffolds for tissue engineering, heart valves, and others (Onuki et al., 2008; Socarras et al., 2014). In normoglycemic individuals, it is well established that host reactions following implantation of biomaterials include injury, blood-material interactions, provisional matrix formation, acute inflammation (neutrophils and mast cells recruitment/activation), chronic inflammation (macrophages and

fibroblasts recruitment/activation), granulation tissue development, the formation of foreign body giant cells (multinucleated fused macrophages) and the development of a dense of layer of fibrotic connective tissue which are detrimental to the implants' function, safety, and biocompatibility (Anderson et al., 2008; Gretzer et al., 2006; Morais et al., 2010). This type of host response to implanted materials has been rarely examined in diabetic animals (Le et al., 2011). In a study that investigated wound healing in diabetic baboon using polystyrene implant and another using percutaneous devices composed of titanium fiber mesh in diabetic rabbits, it was found that granulation tissue formation and connective tissue ingrowth were reduced compared with that of non-diabetic animals (Gerritsen et al., 2000; Thomson et al., 2010). We have also previously reported that the foreign body response after implantation of polyether-polyurethane discs subcutaneously or intraperitoneally in diabetic rats was attenuated compared with the response of non-diabetic animals (Socarras et al., 2014). However, we found no study that has investigated the influence of the genetic background in the foreign body response in diabetic mice. Thus, our aim was to characterize the components of this response (fibrous capsule thickness, collagen deposition, foreign body giant cells and mast cell numbers) in polyether-polyurethane implants of three strains of diabetic animals (Swiss, C57BL6 and Balb/c). We have shown that the pattern of the reaction to the synthetic matrix differs in important ways between the three strains of diabetic mice.

Animals

Female Swiss, C57BL/6, and Balb/c mice (8–10 weeks old; 25–30 g body weight; n=10 for each group) were used in this study. The mice were provided by the

Central Animal Facility at the Institute of Biological Sciences, Federal University of Minas Gerais number 275/2014. Animal care and experimental procedures complied with the guidelines established by our local institutional animal welfare committee. Efforts were made to avoid unnecessary distress to the animals. The animals were housed individually and provided with chow pellets and water ad libitum. The light/dark cycle was 12 hours/12 hours with lights on at 7:00 am and lights off at 7:00 pm.

Induction of diabetes mellitus

Streptozotocin (STZ) was obtained from Sigma-Aldrich, St. Louis, MO, USA. STZ was dissolved in a 10 mM sodium citrate buffer, pH 4.5 and always prepared for immediate use within 5 to 10 min. STZ doses were determined according to the body weight of animals and administered intravenously in injections of 50 mg/kg for 5 consecutive days. This experimental protocol has been shown to induce a model of insulin insufficiency and diabetes type 1 (Castro et al., 2012; Ferreira et al., 2004; Rubinstein et al., 2013). Glucose concentrationin the blood wasmeasuredin all animals through blood samples taken fromthe tail vein before the first dose of streptozotocin and on the 20th day after the injections.Animals whose blood glucose levels exceeded 180 mg/dl after treatment were considered diabetic(Almeida et al., 2017).

Preparation of sponge discs and implantation

Polyether–polyurethane sponge discs, 5 mm thick × 8 mm diameter (Vitafoam Ltd, Manchester, U.K.) were used as the matrix for fibrovascular tissue growth as previously described (Almeida et al., 2014; Orellano et al., 2015). The sponge

discs were soaked overnight in 70% ethanol and sterilized by boiling in distilled water for 30 min before the implantation surgery. Fifteen days after the last dose of STZ injection following confirmation of diabetes, the animals were anesthetized with a 40 μL mixture of ketamine and xylazine (80 mg/ml and 10 mg/ml, respectively). The dorsal hair was shaved and the skin wiped with 70% ethanol. The sponge discs were aseptically implanted inside a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. The incisions were closed with silk braided non-absorbable suture. At 10 days post implantation (25 days after induction of diabetes), the animals were anesthetized with ketamine and xylazine and later killed by cervical dislocation.

The sponge discs were carefully dissected from adherent tissue, removed and weighed. They were then processed as described below for the various assays.

Histological staining and morphometric analysis

The sponge implants (n=5 for each group) from the diabetic animals (Swiss, C57BL/6 and Balb/c) were fixed in 10% buffered formalin (pH 7.4) and processed for paraffin embedding. Sections with 5 μm thickness were stained and processed for light microscopic studies. Hematoxylin/eosin (H&E) staining was used for determining capsule thickness and foreign body giant cells. Toluidine blue staining was used to detect mast cells. Picrosirius-red staining followed by polarized-light microscopy was used to visualize and determine collagen fibers. The presence of apoptosis was investigated by TUNEL (TdT mediated dUTP nick end labeling) using a commercial kit (TdT-FragEL DNA Fragmentation Detection Kit, Cat QIA33; Calbiochem, San Diego, CA, USA). The method was applied

according to the manufacturer's instructions.

Morphometric analyses were performed to quantify capsule thickness, total collagen and types I and III, number of foreign body giant cells, mast cells and apoptosis. For collagen analysis and wall thickness, images were obtained from 10 fields per slide (514,764 μm^2/field) at 20x (final magnification = 200x). To perform such analyses, images of sequential cross sections from each implant were obtained from 20 fields per slide (137,910 μm^2/field) were captured with a plan apochromatic objective (40x) in light microscopy (final magnification = 400x) for number of foreign body giant cells and mast cells. For apoptotic index, images were obtained from 10 representative fields (30,815 μm^2/field) at 100x (final magnification = 1000x). The result of the number of cells in apoptosis is given as Apoptotic Index (percentage of positive labeled cells by the total number of cells per field).

The images were digitized through a JVC TK-1270/JCB microcamera and transferred to an analyzer (software Image-Pro Plus 4.5, Media Cybernetics, Inc. Silver Spring, MD, USA).

Results

The diabetogenic treatment 50 mg/kg of streptozotocin for five consecutive days resulted in diabetes type 1 (insulin-dependent diabetes mellitus)in all treated animals. The mouse strains responded distinctly to the diabetogenic treatment. The initial fasting blood glucose levels (before STZ injections) was 133.5 ±5.1 mg/dl. The values increased to 422.6 ± 20.7 in Swiss mice, to 355.5 ± 23.7 in C57BL/6 and to 193.1 ± 13.4 in Balb/c after the treatment (Fig 1A).Twenty-five days after the diabetogenic treatment, the body weight of the animals of the three

strains decreased. Swiss mice weight was initially 32.9±0.9 g but decreased to 22.1±0.9 g, C57BL/6 mice weight was 20.4±0.16g to 18.7±0.4 and Balb/c mice was 22.4±.6 g to 19.5±0.6 g (Fig.1B).

Histological examination of sponge implants

The sponge matrix was well tolerated by the animals. No signs of infection or rejection were observed in the implant location during the 10-day period post-implantation.

In histological sections of the implants (H&E), the synthetic matrix induced the formation of a fibrovascular tissue that differed among the implants of the three diabetic strains in terms of the intensity of the various components evaluated. The granulation tissue that filled the subcutaneous matrix was composed of spindle-shaped fibroblasts, micro vessels, and a dense inflammatory infiltrate containing macrophages, neutrophils, mast cells and foreign body giant cells embedded in an extracellular matrix. The capsule was thicker in implants of C57BL/6 diabetic mice compared with that of the other two strains (Fig. 2A-D). The implant wet weight of C57BL/6 diabetic mice was also heavier than that of Swiss or Balb/c mice (Fig. 2E).

Morphometric analysis of collagen types in *Picrossirius* staining sections

To characterize collagen deposition in the implants, morphometric analysis of sections stained with *Picrossirius* red was performed. In Figure 3A-D, total collagen and types III and I were evaluated.In implants of Swiss and Balb/c mice, immature collagen (type III) was the predominant type, whereas in C57/BL57

implants there was more type I collagen deposited in the synthetic matrix.

Mast cells and foreign body giant cells in implants

Previous work has established the relevance of mast cell recruitment/activation in mediating foreign body response to various biomaterials (Avula et al., 2014; Egozi et al., 2003; Orenstein et al., 2010). We evaluated the number of this cell population in Toluidine blue staining sections and found 13±2 cells/field in implants of Swiss mice *versus* 12±2 in implant of C57BL/6 mice *versus* 5±1 in implant of Balb/c mice (Fig. 4A-D). The number of foreign body giant cells in H&E staining sections (Fig. 5A-D) was similar between Swiss (6±1 cells/field) and C57BL/6 (4±1) implants, but lower in Balb/c implants (1±1).

Apoptosis in the implants

Dark-brown TUNEL positive nuclei with other morphological features of cellular death (apoptotic bodies, cellular shrinkage, and condensed chromatin) are clearly marked, as shown in Figure 6A-C. In implant sections stained with TUNEL, the number of these positive cells was clearly different among the implants of diabetic mice. Implants of Swiss mice had more positive cells, followed by C57BL/6 implants and by Balb/c implants (Fig. 6D).

Discussion

Genetic susceptibility is considered an important risk factor for many diseases, including diabetes. This condition is associated with greater risks for adverse health outcomes including the need for functional bioengineered substitutes for instance, artificial organs, scaffolds for tissue engineering, heart valves and other

implantable devices like glucose sensors, orthopaedic implants, catheter, drug-eluting stents, biosensors (Le et al., 2011; Onuki et al., 2008). In turn, implantation procedure of biomedical devices often induces an adverse foreign body response which is detrimental to biomaterial functionality (Anderson et al., 2008; Keane and Badylak, 2014; Klopfleisch and Jung, 2017; Morais et al., 2010). However, this response has rarely been examined in diabetic animals (Gerritsen et al., 2000; Le et al., 2011; Thomson et al., 2010)and we found no study that investigated whether this reaction would differ in diabetic animals from different strains.

In this study, we examined the foreign body response to the synthetic matrix of polyether-polyurethane in diabetic mice of three strains (Swiss, C57BL/6 and Balb/c). In the first series of data, we show that the diabetogenic treatment induced by streptozotocin injection induced diabetes type I that varied in intensity among the strains. The hyperglycaemic levels were more pronounced in Swiss mice followed by C57BL/6 and by Balb/c. This finding is consistent with a number of reports, including one from our Laboratory (Almeida et al., 2017), that highlighted inherent genetic differences to the effects of this compound in the development of hyperglycaemia (Herold et al., 1997; Oosterlinck et al., 2011; Rubinstein et al., 2013).

Using the sponge implantation technique, we have been able to identify genetic-related differences in the foreign body reaction in diabetic animals. All the fibrogenic markers examined (capsule thickness, collagen deposition, foreign body giant cell and mast cell numbers) were higher in Swiss and C57BL/6 implants of diabetic mice compared with those of Balb/c implants. We found that the capsule around the implants of diabetic C57BL/6 mice was thicker than that

of Swiss or Balb/c. This result is in agreement with the higher susceptibility of C57BL/6 mice to developing pulmonary and intraperitoneal fibrosis compared with other strains (Kolb et al., 2002; Margetts et al., 2013). Fibrous capsule that results in encapsulation of implantable devices have been implicated clinically in the failure of a wide variety of medical devices (Anderson and McNally, 2011; Anderson et al., 2008; Onuki et al., 2008). It has been proposed that encapsulation is influenced by several factors, including properties of the biomaterial, biomaterial porosity, surface texture, and implantation site (Klopfleisch and Jung, 2017; Morais et al., 2010). Thus, our finding showing that the genetic background influenced the hyperglycemic levels and the degree of encapsulation (capsule thickness), adds other potential factors that influences the complex foreign body response.

Mature collagen (type I) was the predominant type in implants of diabetic C57BL/6 mice. In contrast, in implants of Swiss or Balb/c mice the predominant type was collagen type III. Interestingly, although implants of diabetic Swiss animals had more foreign body giant cells, their fibrous capsule was not the thickest. Our results are in agreement with the report by Kyriakides et al, 2004 showing that foreign body giant cells are not critical for progression of encapsulation process (Kyriakides and Bornstein, 2003). Furthermore, Iizuka et al. 2002 found that colony-stimulating factor-1-deficient mice formed smaller foreign body giant cells but the fibrotic response was not affected (Iizuka et al., 2002). It has been proposed that macrophages are the key cell type in the development of the foreign body response, especially fibrosis. We have previously shown that in implants of diabetic rats macrophage activation was lower compared with that of normglycemic animals (Socarras et al., 2014).

Some previous work have established the importance of mast cell responses in controlling phagocyte chemotaxis to the site of biomaterial implantation and in mediating wound-healing response to implanted biomaterials in normglycemic and in diabetic animals (Avula et al., 2014; Klueh et al., 2010; Socarras et al., 2014; Tang et al., 1998). In a model of continuous glucose monitoring, mast cells associated with the margins of the device were reported to be responsible for controlling local tissue reaction to the implanted sensors. In addition, it was demonstrated that the foreign body reaction decreased the sensor function and lifespan increased in mice that were genetically deficient in mast cells(Klueh et al., 2010). Here, we show that the number of mast cells at the site of the implants was a genetic trait. A three-fold difference in the number of mast cells was observed in implants of diabetic Swiss and of C57BL/6 mice compared with the number in implants of diabetic Balb/c mice. The implants of these animals had also the lowest number of foreign body giant cells. This cell type is considered a hallmark of the foreign body response and is persistent as long as the biomaterial is detected in the subcutaneous tissue (Klopfleisch and Jung, 2017). Thus, it is possible that increased number of both cell types (mast cells and foreign body giant cells) in implants of diabetic Swiss and C57BL/6 mice have contributed to the more pronounced foreign body reaction observed in these two strains.

In addition, the apoptotic index was also lower in implants of diabetic Balb/c mice. Significant increase in apoptosis in diabetic wounds with poorly controlled blood sugar and microangiopathy in humans has been reported (Rai et al., 2005).

Thus, our finding showing lower apoptotic index in implants of diabetic Balb/c mice whose glycemic levels were the lowest, is consistent with the above-mentioned work and with the notion that processes that interfere with sufficient

number of cells involved in the various phases of repair, such as fibroblast apoptosis, impair wound healing (Rai et al., 2005; Socarras et al., 2014).

Collectively, our measurements of foreign body reaction would indicate that Balb/c mice provided the lowest level of response to the synthetic matrix of polyether-polyurethane implants, so this strain would not be a suitable model to study this response; in contrast to the other two strains Swiss and C57BL/6 that would be better models to study the foreign body reaction in diabetes. Furthermore, our study reveals an association between the severity of hyperglycemic levels and the intensity of the foreign body response (capsule thickness, foreign body giant cell and mast cell number) to subcutaneous implantation of synthetic matrix of polyether-polyurethane.

Our findings may be relevant in understanding biomaterial integration and performance in distinct genetic backgrounds and in varying hyperglycemic environments.

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Figure legends

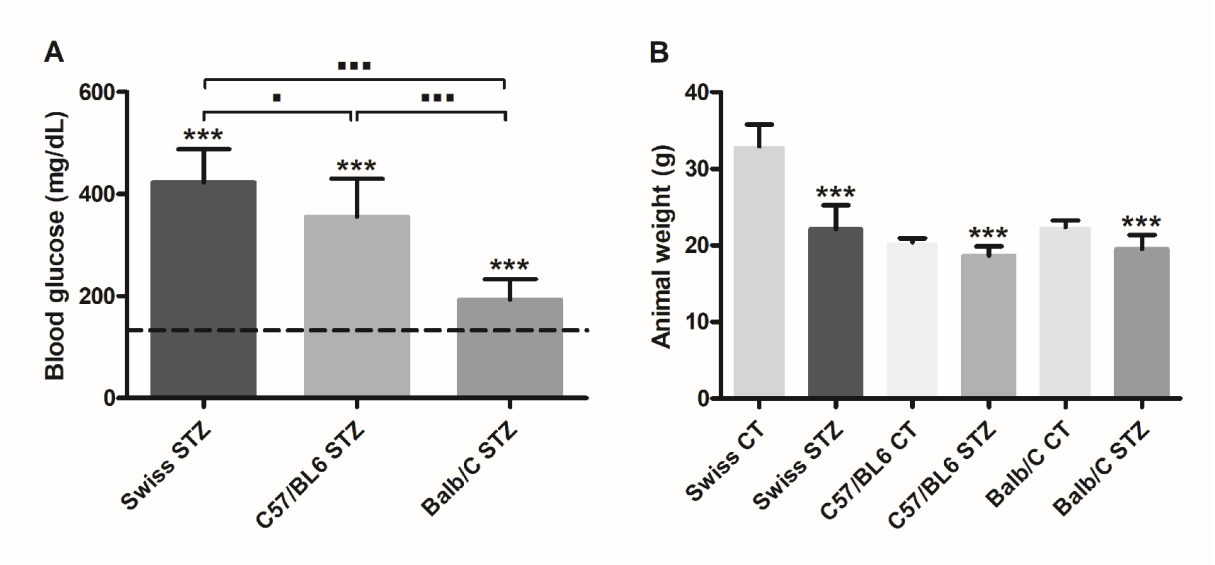

Figure 1. Blood sugar levels and body weight of Swiss, C57BL/6 and Balb/c mice before and after injections of streptozotocin. A significant increase in glycemic levels and decrease in body weight is observed after the diabetogenic treatment. Data are expressed as mean ± SEM. Significant difference in glycemic levels before (dashed line), ***p<0.001 and after streptozotocin injections (bars) of Swiss, C57BL/6 and Balb/c mice, ▪p<0.05; ▪▪▪p<0.001; ANOVA.

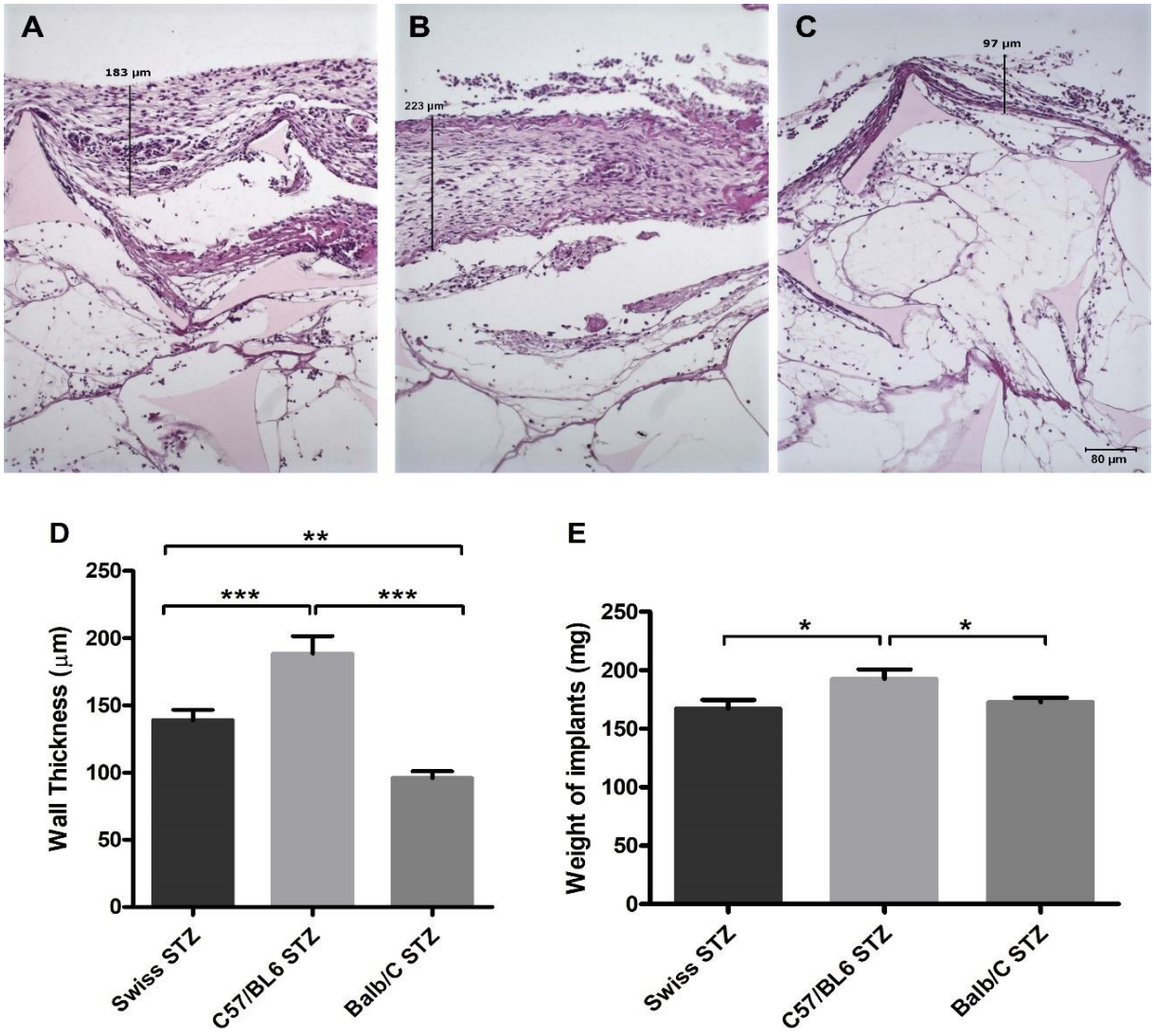

Figure 2. Histological analysis of 10-day old implants of diabetic Swiss (A), C57BL/6 (B) and Balb/c (C) mice in H&E-stained sections. Representative implant sections showing the newly formed proliferating tissue in an extracellular matrix. The fibrous capsule thickness measurements as shown in D are bigger in implants of C57BL/6 mice, followed by Swiss and Balb/c. In E, implant wet weight. Data are expressed as mean ± SEM. Significant difference between implants of Swiss, C57BL/6 and Balb/c mice, *p<0.05; **p<0.01; ***p<0.001; ANOVA. Final magnification 200x.

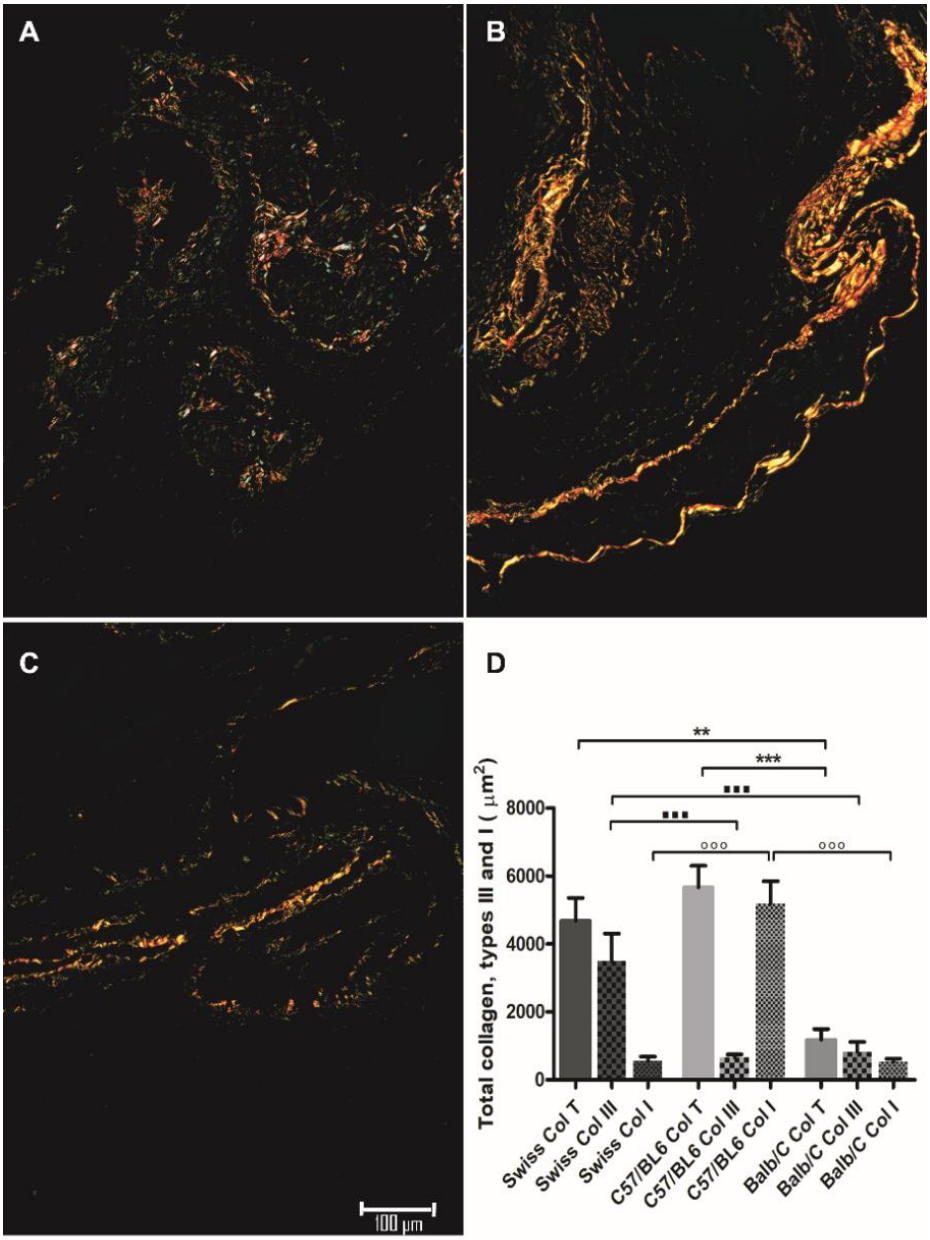

Figure 3. Collagen deposition in 10-day old implants of diabetic Swiss (A), C57BL/6 (B) and Balb/c (C) mice. Representative histological sections (*Picrossirius*-red staining) of implants of the three groups of animals showing distinct types of collagen in the implants. In D, the amount of collagen was increased in implants of Swiss and C57BL/6 as compared with that of Balb/c mice. Values are expressed as mean ± SEM. Significant difference between strains, **p<0.01; ***p<0.001 (Total collagen); ■■■ p<0.001 (Collagen type III); °°° p<0.001 (Collagen type I); ANOVA. Final magnification 200x.

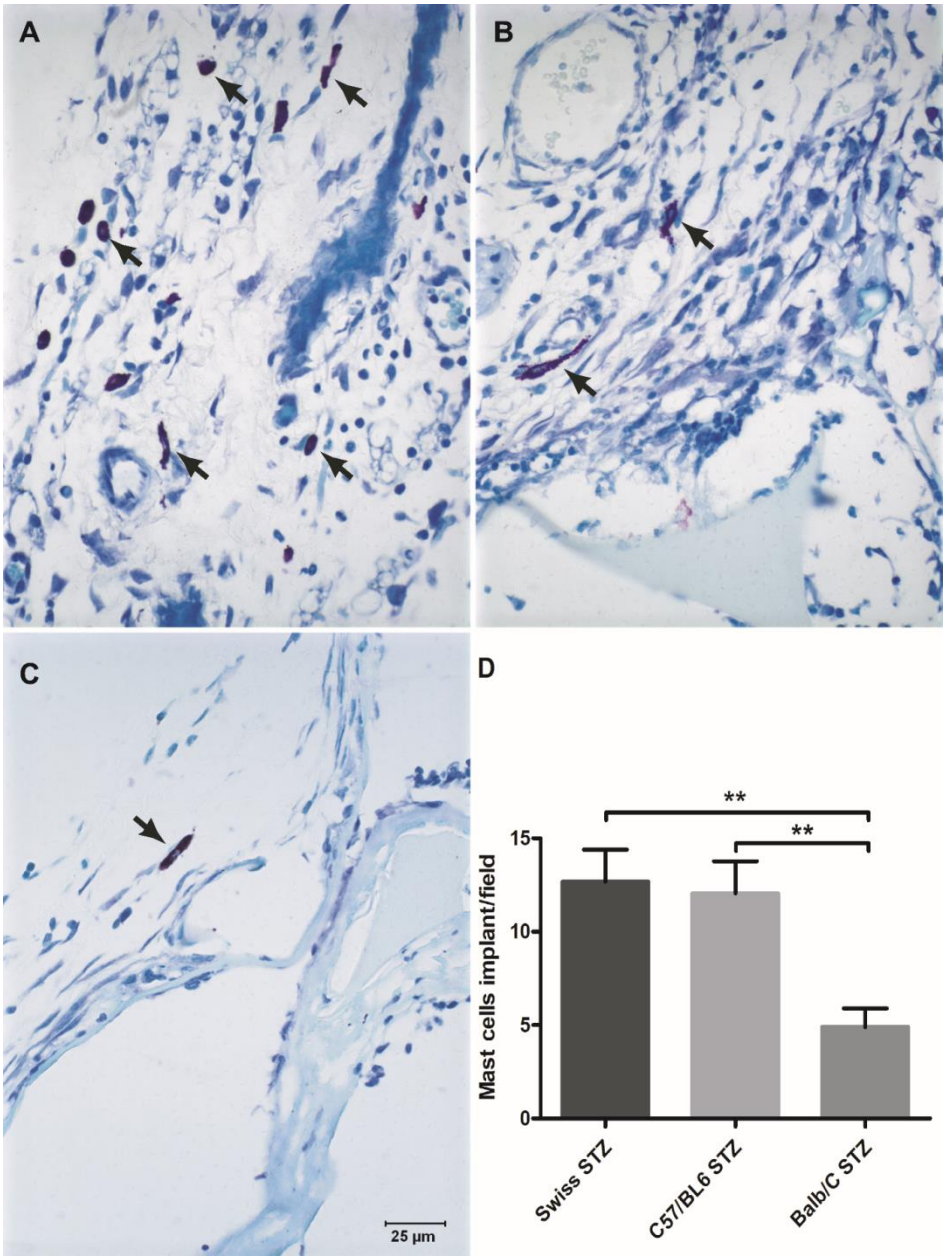

Figure 4.Histological analysis of mast cells in 10-day old implantsof Swiss (A), C57BL/6 (B) and Balb/c (C) mice. Representative histological sections stained with Toluidine blue show that mast cells in implants of Swiss and C57BL/6 mice are higher compared with that of Balb/c mice. In D, the graph showing the mast cells number for field. Data are expressed as mean ± SEM. Significant differences between the strains as shown, **p<0.01; (ANOVA). Final magnification 400x.

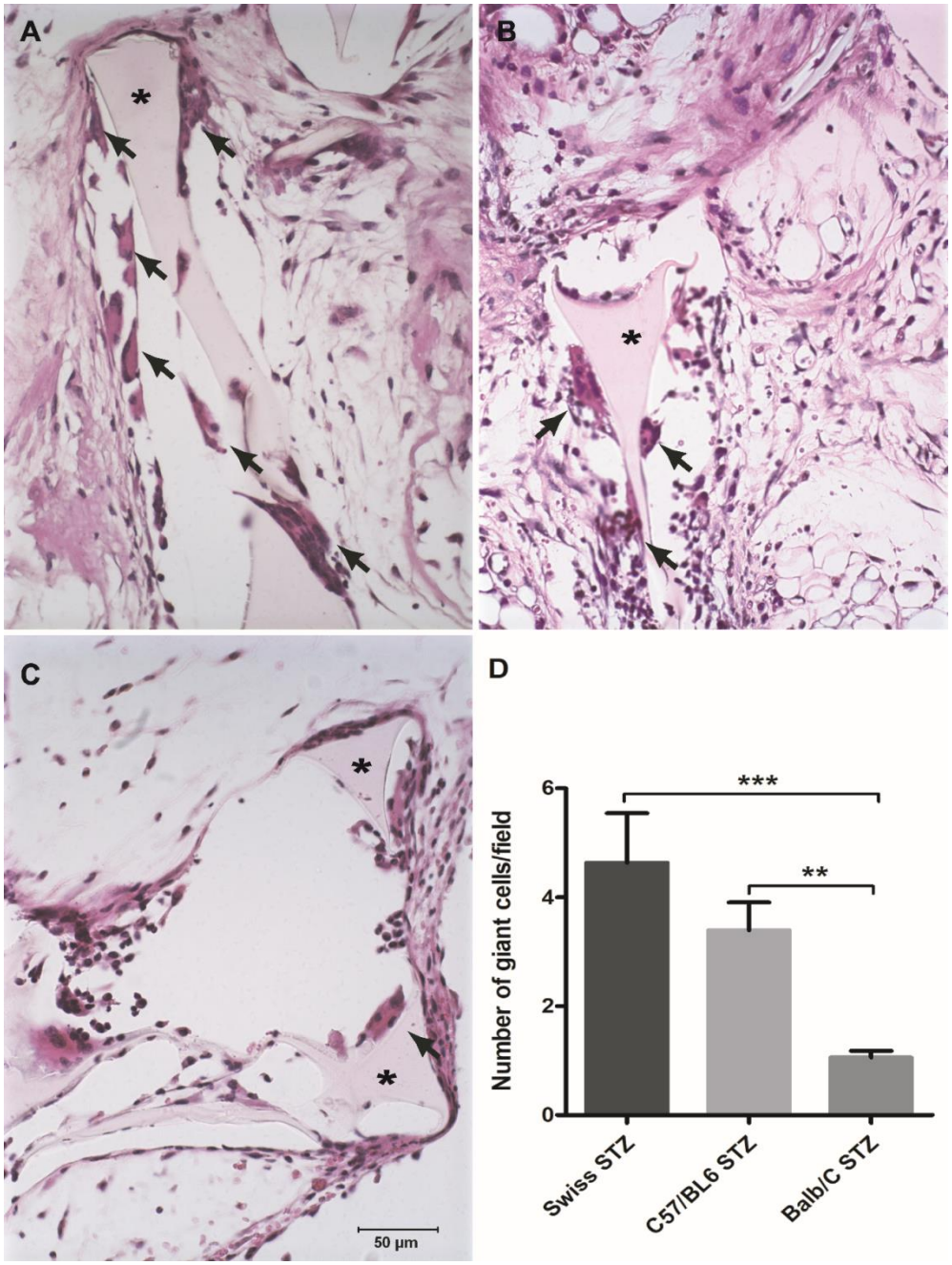

Figure 5. Histological characteristics of multinucleated giant cells in 10-day old implant Swiss (A), C57BL/6 (B) and Balb/c (C) mice. Representative histological sections (H&E staining). The number of giant cells was higher in implants of Swiss and C57BL/6 mice compared with that of Balb/c mice. In D, graph showing the number of giant cells around the synthetic matrix. Data are expressed as mean ± SEM. Significant differences the strains, **p<0.01; ***p<0.001; (ANOVA). Final magnification 400x.

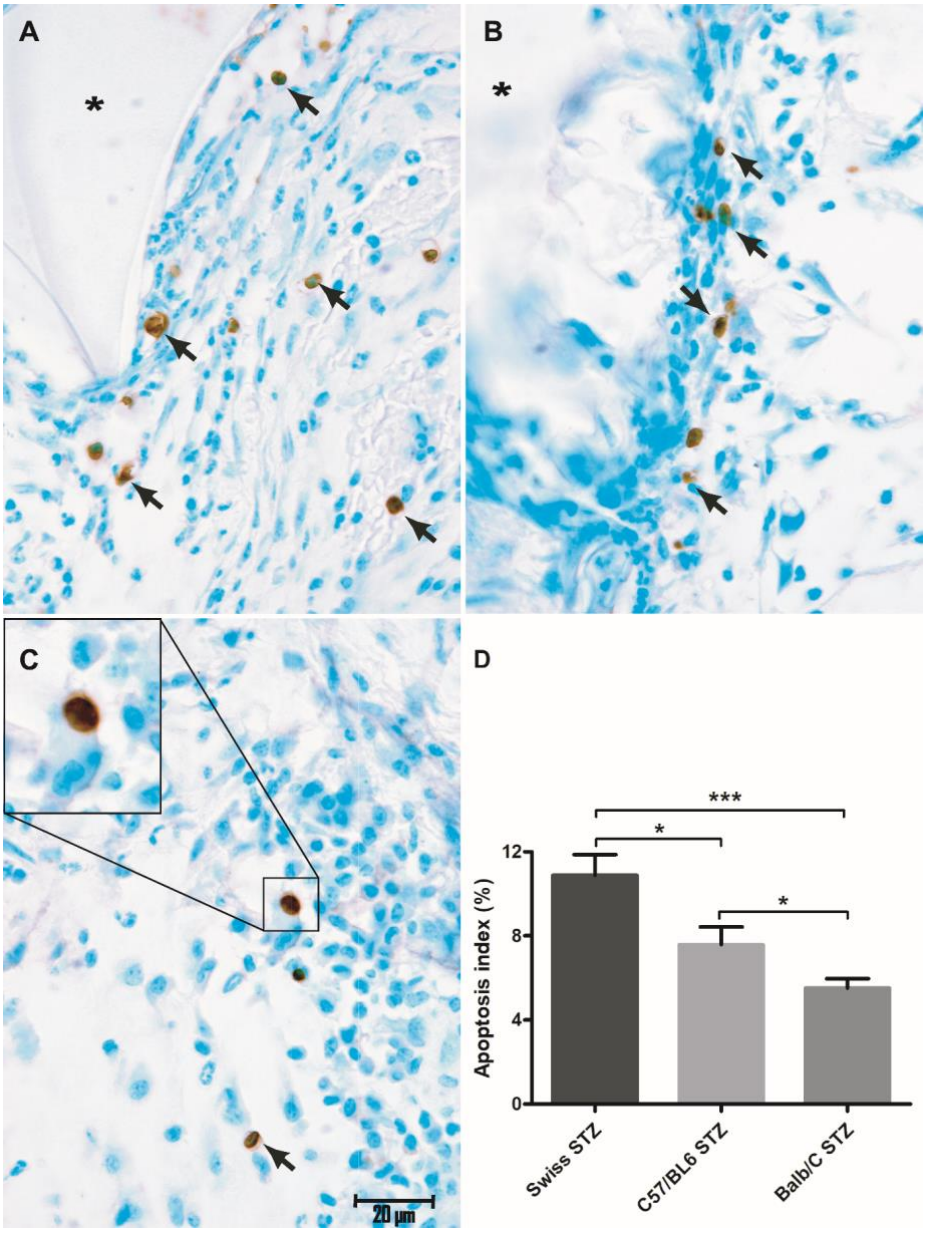

Figure 6. Apoptotic index in 10-day old implants of diabetic Swiss (A), C57BL/6 (B) and Balb/c (C) mice. Representative histological sections (TUNEL staining) of the fibrovascular tissue induced by sponge implants at 10 post implantation show apoptotic cells. Apoptotic index (D) was reduced in implants of diabetic Balb/C mice compared with the indices in implants of diabetic Swiss and C57BL/6 mice. Values shown are expressed as mean ± SEM. Significant difference the strains, *p<0.05; *** p<0.001; (ANOVA). Final magnification 1000X.

8- CONSIDERAÇÕES FINAIS

Nos últimos anos, a implantação cirúrgica de materiais e dispositivos biomédicos como sistema de infusão contínua de drogas como a insulina, órgãos artificiais, biossensores, cateteres, arcabouços para engenharia de tecidos, válvulas do coração em indivíduos diabéticos tornou-se um procedimento comum para a reparação e/ou substituição de tecidos biológicos e aumentou drasticamente ao longo da última década. Esta tendência deverá continuar com a ampliação da aplicação de biomateriais e a rápida expansão do envelhecimento populacional. No entanto, após a implantação de tais dispositivos, o corpo desencadeia uma série de eventos que culmina com a reação de corpo estranho (composta de macrófagos e células gigantes de corpo estranho) que é a fase final das respostas inflamatória e cicatricial ante estes dispositivos. Estas reações podem resultar em diminuída eficácia do dispositivo implantado impedindo-o de interagir com os tecidos circundantes.

Neste contexto, estudos experimentais em modelos animais de diabetes (induzida quimicamente) têm contribuído para elucidar bases fisiológicas, patológicas e moleculares da doença.

Pesquisas têm demonstrado que o background genético e fatores ambientais determinam a manifestação de vários processos patológicos. Em vários modelos experimentais foram demonstrados que a cicatrização de feridas, a angiogênese e a fibrose são fenótipos dependentes da coleção de genes presentes em um organismo. Entretanto, este estudo foi o primeiro a avaliar a influência do background genético sobre a lesão interna (subcutânea) e a reação tipo corpo estranho induzida pela implantação de matriz sintética em

camundongos diabéticos.

Neste estudo, nós mostramos que a inflamação, angiogênese e a produção de citocinas, componentes chave, no processo de reparo de lesão interna, nos animais normo e hiperglicêmicos ocorreram de maneira linhagem específica (Tabela 1 e 2).

Tabela 1: Percentual de variação dos parâmetros angiogênicos nas 3 linhagens de camundongos após o tratamento diabetogênico.

Parâmetros angiogênicos após o diabetes	Hb (µg/mg)	Número de vasos	VEGF (pg/mg)	NO (nmol/g)
Swiss	**42↓**	**44↓**	**19↑**	**55↓**
C57	**20↓**	**45↓**	**40↑**	**47↓**
Balb/c	**1↓**	**23↓**	**47↓**	**52↓**

Tabela 2: Percentual de variação dos parâmetros inflamatórios nas 3 linhagens de camundongos após o tratamento diabetogênico.

Parâmetros inflamatórios depois do diabetes	MPO (OD/mg)	NAG (p-nitrophenol nmol/ml.mg^{-1})	CXCL-1/KC (pg/mg)	CCL2 (µg/mg)	TNF-α (µg/mg)
Swiss	**69↑**	**73↑**	**28↑**	**11↑**	**30↓**
C57	**38↓**	**73↑**	**131↑**	**2↑**	**47↑**
Balb/c	**38↓**	**2↓**	**25↑**	**11↓**	**35↓**

Os marcadores fibrogênicos examinados, espessura da cápsula, deposição de colágeno, célula gigante tipo corpo estranho e número de mastócito, também foram modulados de maneira linhagem específica (tabela 3).

Tabela 3: Relação dos marcadores fibrogênicos nas 3 linhagens de camundongos após o tratamento diabetogênico.

Parâmetros fibrogênicos depois do diabetes	Peso do implante (mg)	Espessura de cápsula (μm)	Colágeno total (μm^2)	Células gigantes/ campo	Mastócitos/ campo	Índice de apoptose
Swiss				↑	↑	↑
C57	↑	↑	↑		↑	
Balb/c	↓	↓	↓	↓	↓	↓

Em nossa pesquisa, também vimos uma clara associação entre a severidade dos níveis de hiperglicemia e na intensidade da reação tipo corpo estranho.

Todos os parâmetros avaliados foram mais intensos em implantes da linhagem Swiss e C57BL/6 comparados aos implantes da linhagem Balb/c.

Esses achados demonstram que o background genético influencia a reação tipo corpo estranho e o reparo tecidual devido a implante de biomateriais em ambiente hiperglicêmico

Diante dos resultados obtidos, acreditamos que essa variabilidade possa ser relevante quando se considera o modelo animal de diabetes e abordagens terapêuticas em feridas internas em estados hiperglicêmicos e que esses dados possam ser relevantes experimental e/ou clinicamente no desenvolvimento de estratégias que busquem melhorar o desempenho e função de biomateriais implantados em pacientes diabéticos.