UNIVERSIDADE FEDERAL DO PARANÁ
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ALINE MARIA STOLF

EFEITOS DA SILIMARINA SOBRE A ANGIOGÊNESE E ESTRESSE OXIDATIVO EM CAMUNDONGOS NORMOGLICÊMICOS E DIABÉTICOS

CURITIBA
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Tese apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná como requisito parcial para a obtenção do título de Doutor em Farmacologia.

Orientadora: Profa. Dra. Alexandra Acco

Co-orientadora: Profa. Dra. Cibele Campos Cardoso

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **ALINE MARIA STOLF**, intitulada: "**EFEITOS DA SILIMARINA SOBRE A ANGIOGÊNESE E ESTRESSE OXIDATIVO EM CAMUNDONGOS NORMOGLICÊMICOS E DIABÉTICOS**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua Aprovação.

Curitiba, 04 de Novembro de 2016.

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NOTA EXPLICATIVA

Esta tese é apresentada em forma de artigos para publicação, de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná. A tese consta de uma revisão de literatura, objetivos do trabalho e dois artigos científicos, sendo o primeiro um artigo de revisão e o segundo abordando os experimentos realizados, com resultados, discussão e conclusões. Os artigos foram formatados conforme normas propostas por periódicos de circulação internacional.

Dedico esta tese às pessoas que mais me apoiaram e incentivaram neste período: meu pai e meu marido. A vocês, minha eterna gratidão.

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RESUMO

A silimarina é o extrato proveniente da planta *Silybum marianum,* comercializada com indicação para o tratamento e a prevenção de doenças hepáticas. No entanto, atualmente tem sido testada para o tratamento de várias doenças, entre elas o diabetes. Estudos prévios evidenciaram que a silimarina pode ter efeitos benéficos nestes pacientes. No presente trabalho, avaliamos o efeito do tratamento com a silimarina sobre a angiogênese, além da resposta inflamatória e do estresse oxidativo em pâncreas, fígado e rins de camundongos normoglicêmicos e diabéticos. Para tanto, camundongos Swiss machos de 6 semanas de idade foram utilizados nos experimentos. O diabetes foi induzido pela administração de estreptozotocina (STZ) via intraperitoneal (i.p.) na dose de 80 mg/kg. Animais incluídos nos grupos normoglicêmicos receberam veículo i.p.. Os animais que receberam estreptozotocina e mantiveram a glicemia acima de 250 mg/dL foram considerados diabéticos. Após 14 dias da injeção de STZ ou veículo, esponjas de poliuretano (Vitafoam Ltd., Manchester, UK) foram implantadas cirurgicamente no dorso dos animais. Em seguida, esses animais foram tratados com silimarina, em dose alométrica (10,4 mg/kg) ou dose 10 vezes maior, ou água. Após 10 dias de tratamento os animais foram anestesiados com cloridrato de xilazina e cetamina (i.p.), foram colhidas as esponjas, sangue e os órgãos de interesse. Posteriormente foram dosados parâmetros inflamatórios (Mieloperoxidase, MPO; N-acetilglucosaminidase, NAG; e Nitrito), colágeno e hemoglobina nas esponjas; nos órgãos foram investigados parâmetros de estresse oxidativo (Catalase, Cat; Superóxido dismutase, SOD; Glutationa reduzida, GSH; glutationa-S-transferase, GST; e Peroxidação lipídica, LPO), e de inflamação (TNF-α). Ainda, análises histológicas de órgãos, ensaios de respiração mitocondrial em fígado e bioquímica plasmática foram realizados. Os resultados evidenciaram que o diabetes ocasionou uma disfunção nas defesas antioxidantes, fato demonstrado principalmente pela redução da SOD pancreática e Cat no fígado e rins. O diabetes provocou alterações no fluxo de oxigênio mitocondrial através das enzimas NADH oxidase e succinato oxidase. Houve também um aumento da produção de TNF-α e infiltração de células inflamatórias no tecido pancreático. Redução no número de vasos nos implantes dos animais diabéticos também foi observada. O tratamento com silimarina atenuou estes danos, restaurando enzimas antioxidantes e reduzindo a concentração de TNF-α no pâncreas e o infiltrado de células inflamatórias nas ilhotas pancreáticas. Porém, a silimarina não restabeleceu a angiogênese. Assim, o tratamento com silimarina reduziu o estresse oxidativo e a inflamação em alguns órgãos de camundongos com diabetes induzido pela estreptozotocina, mas não alterou a angiogênese. Conclui-se que a silimarina, mesmo não tendo restabelecido a glicemia, pode ser um fármaco útil para tratar algumas complicações relacionadas ao diabetes.

Palavras-chave: Diabetes, silimarina, estreptozotocina, angiogênese, estresse oxidativo, inflamação.

ABSTRACT

Silymarin is the extract obtained from *Silybum marianum,* used to treat and prevent liver diseases. However, it has been tested to treat other diseases, including diabetes. Previous studies showed that silymarin can have beneficial effects in these patients. In the present study we evaluated the effect of silymarin treatment in angiogenesis, and also investigated its effects in oxidative stress and inflammatory parameters in pancreas, liver and kidney of normoglycemic and diabetic animals. For that, male Swiss mice with six weeks old were used in the experiments. Diabetes was induced by administration of 80 mg/kg of streptozotocin intraperitonially (i.p). Mice included in normoglycemic groups received vehicle i.p. Animals who received streptozotocin and had glycemia above 250 mg/dL were considered diabetic. Polyether-polyurethane sponge (Vitafoam Ltd., Manchester, UK) was surgically implanted on the back of mice, 14 days after diabetes induction. After that, animals were treated with silymarin alometric dose (10.4 mg/kg), silymarin 10 times higher dose or water. Mice were anaesthetized for collecting sponges, blood and organs after 10 days of treatment. Myeloperoxidase (MPO), N-acetyl glucosaminidase (NAG), collagen, hemoglobin and nitrite were measured in sponges, while oxidative stress (catalase, Cat; superoxide-dismutase, SOD; reduced glutathione, GSH; glutathione-S-transferase, GST; and lipid peroxidation rate, LPO), and inflammatory (TNF-α) parameters were measured in organs. Mitochondrial enzymatic activity of liver, histological analysis of pancreas, liver and kidney, and plasmatic biochemistry were also performed. Diabetes leads to impairment of antioxidant defenses demonstrated mainly by reduction of pancreatic SOD and hepatic and renal Cat. An alteration in oxygen flux through NADH oxidase and succinate oxidase was observed in liver mitochondria. Inflammatory reaction occurred in diabetic mice, observed by augment in pancreatic TNF-α and infiltration of inflammatory cells in islets, observed in histological analyses. The number of vessels in implants was reduced. The treatment with silymarin was capable to attenuate these damages, restoring antioxidant enzymes and reducing pancreatic TNF-α and inflammatory cells infiltration. In other hand, silymarin treatment did not modify the number of vessels. So, silymarin treatment reduced oxidative stress and inflammation in organs of diabetic mice, but did not alter angiogenesis. In conclusion, even the silymarin has not reestablished normal glycemia, it can be helpful to treat some of the diabetes complications.

Key words: Diabetes, silymarin, streptozotocin, angiogenesis, oxidative stress, inflammation.

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LISTA DE ABREVEATURAS

ALT: Alanina aminotranferase

ANOVA: Análise de variância

AST: Aspartato aminotransferase

Bax/Bcl-2: bcl-2-like protein 2

Bcl-2: B-cell lymphoma 2

Cat: Catalase

CD68: Cluster of Differentiation 68

CYP: Citocromo P

DM1: Diabetes mellitus tipo 1

DM2: Diabetes mellitus tipo 2

EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ERK: Extracellular signal–regulated kinases

GPx: Glutationa peroxidase

GSH: Glutationa reduzida

GST: Glutationa–S-transferase

GLUT2: Glucose transporter 2

GLP-1: Glucagon-like peptide-1

HaCat: Aneuploid immortal keratinocyte cell line from adult human skin

H_2O_2: Peróxido de hidrogênio.

H_3PO_4: Ácido fosfórico

HE: Hematoxilina/ eosina

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1α: Hypoxia-inducible factor 1-alpha

HPLC: High performance liquid chromatography

HMVECad: Human Microvascular Endothelial Cells, adult dermis

IβI/S: Silibinin-β-ciclodextrin

ICAM-1: Intercellular Adhesion Molecule 1

IL: Interleucina

I.P.: Intraperitoneal

LPO: Concentração de hidroperóxidos

MDA: Malondialdeído

MPO: Mieloperoxidase

NPH: Protamina Neutra Hagedorn

NADH: Nicotinamida adenina dinucleotídeo reduzida

NAG: N-acetil glucosaminidase

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

$O2^{\bullet -}$: Ânion superóxido

O_2: Oxigênio singleto

ROS: Reactive oxigen species

$ROO^{\bullet}$: Radical peroxil

RNS: Reactive nitrogen species

S.C.: Subcutânea

SEM: Standard error of the mean

SOD: Superóxido dismutase

STZ: Streptozotocin

TNFα: Tumor necrosis factor alpha

UI: Unidades internacionais

V.O: Via oral

VEGF: Vascular endothelial growth factor

SUMÁRIO

1. Revisão da literatura

1.1. Diabetes mellitus: epidemiologia e complicações

O diabetes mellitus é uma disfunção metabólica caracterizada por hiperglicemia crônica (Alberti e Zimmet, 1999; Skliros *et al.*, 2016). Segundo Danaei e colaboradores (2011), em um estudo realizado em 199 países e territórios, o aumento da glicemia de jejum está ocorrendo no mundo todo. O número de adultos com diabetes mais que dobrou nas últimas três décadas e este aumento está relacionado ao estilo de vida, além do envelhecimento e aumento da população (Danaei *et al.*, 2011).

Estima-se que em 2015 o número de pessoas no mundo vivendo com a doença era de 415 milhões, sendo 5 milhões o número de mortos em decorrência da doença. Em 2040 o número de diabéticos poderá chegar a 642 milhões. Com relação ao custo financeiro, foram gastos em 2015 entre 673 e 1.197 bilhões de dólares no tratamento desta doença (International Diabetes Federation, 2015).

O Brasil é o 8º país com maior prevalência, segundo a Organização Mundial de Saúde (OMS, 2014). Em 2014 estimou-se que havia cerca de 11,9 milhões de pessoas com a doença no país, número que pode aumentar para 19,2 milhões em 2035. A prevalência varia com a faixa etária, sendo de 0,6% para a faixa de 18 a 29 anos e 19,9% para a de 65 a 74 anos (Diretrizes da Sociedade Brasileira de Diabetes, 2015-2016).

Há dois principais tipos de diabetes mellitus, o tipo 1 (DM1) e o tipo 2 (DM2). No DM1 ocorre a deficiência da produção de insulina, com necessidade de administração de insulina exógena (Alberti e Zimmet, 1999). É uma doença inflamatória crônica, em que há destruição das células β pancreáticas. Também ocorre o aparecimento de células T autorreativas e de autoanticorpos nas ilhotas do pâncreas, que pode ser desencadeado por fatores genéticos e ambientais. É mais comum em crianças e adolescentes e sua incidência tem aumentado nas últimas décadas (Achenbach *et al.*, 2005). Fatores associados ao DM1 incluem a ingestão de leite bovino na infância (Lamb *et al.,* 2015), deficiência de vitamina D (Savastio *et al,* 2016) e infeccções virais (de Beeck e Eizirik, 2016)

O DM1 está subdividido nas formas autoimune e idiopática. Na forma autoimune os autoanticorpos formados são os autoanticorpos anti-ilhota ou

antígenos específicos da ilhota, e incluem os anticorpos anti-insulina, antidescarboxilase do ácido glutâmico, antitirosina-fosfatases e antitransportador de zinco. Esses anticorpos podem ser detectáveis meses ou anos antes da fase clínica. A forma idiopática é a de menor incidência. Não há uma etiologia conhecida nem marcadores de autoimunidade. Os indivíduos acometidos podem desenvolver cetoacidose (Diretrizes da Sociedade Brasileira de Diabetes, 2015-2016).

No DM2 ocorre deficiência na resposta à insulina (Alberti e Zimmet, 1999). É a forma predominante, atingindo 90 a 95% dos pacientes diabéticos. Existe uma predisposição genética, mas o padrão não está claramente estabelecido. O risco de desenvolvimento aumenta com o sedentarismo, a idade e a obesidade, sendo que o diagnóstico é mais frequente após os 40 anos e boa parte dos indivíduos acometidos são obesos. Nesta forma, a hiperglicemia pode ser gradual, permanecendo o paciente assintomático por algum tempo. A secreção de insulina pode ser normal ou aumentada, mas é insuficiente para compensar a resistência à insulina. A resistência insulínica pode melhorar com a redução de peso ou tratamento farmacológico, mas dificilmente retorna ao normal. O paciente não depende de insulina endógena para sobreviver, mas esta pode ser necessária para um controle glicêmico mais adequado. A cetoacidose ocorre raramente e quando ocorre pode estar associada a outros fatores (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997; Diretrizes da Sociedade Brasileira de Diabetes, 2015-2016).

Há também o DM Gestacional, que se trata de qualquer intolerância à glicose, de magnitude variável, com início ou diagnóstico durante a gestação. Essa condição causa complicações durante a gestação e embora a maioria dos casos seja reversível, algumas pacientes desenvolvem DM2 anos após o parto. Existem ainda outros tipos expecíficos de diabetes, que são formas menos comuns e de etiologia conhecida. Dentre elas estão os defeitos genéticos na função das células beta (exemplos: diabetes neonatal transitório ou permanente, diabetes mitocondrial), defeitos genéticos na ação da insulina (Síndrome de Rabson-Mendenhall, DM lipoatrófico) e doenças do pâncreas exócrino (pancreatite, fibrose cística) (Diretrizes da Sociedade Brasileira de Diabetes, 2015-2016).

O diabetes causa inúmeros prejuízos à saúde, como transtornos de humor, representados por depressão e ansiedade (Golden *et al.*, 2016), disfunção sexual

(Kizilay *et al.*, 2016) e neuropatia diabética, que tem como um dos sintomas a dor neuropática, que causa extremo sofrimento ao paciente (Schreiber *et al.*, 2015). No entanto, dentre as consequências mais preocupantes do diabetes estão as complicações macro e microvasculares, como a aterosclerose, doenças coronarianas, trombose e doenças vasculares periféricas (Beckman *et al.*, 2002; Beckman *et al.*, 2013; Paneni *et al.*, 2013). Pacientes com DM2 podem ter um alto risco de complicações vasculares mesmo antes de desenvolverem sintomas clássicos da doença (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). As complicações microvasculares incluem as retinopatias, nefropatias e o atraso na cicatrização de feridas cutâneas por danos na microvasculatura do tecido cutâneo e subcutâneo (Ekmektzoglou e Zografos, 2006). Esse prejuízo cicatricial ocorre também em órgãos e tecidos internos, dentre eles os ossos, vasos sanguíneos, nervos, intestino delgado, cólon e coração (Minossi *et al.*, 2014).

Outra complicação vascular do diabetes, que ocorre em alguns tecidos devido ao comprometimento da habilidade de produzir novos vasos, é a isquemia de membros, que é a principal causa de morbidade nesta enfermidade (Johannesson *et al.*, 2009). Os mecanismos envolvidos na redução da angiogênese no diabetes incluem a redução na produção do VEGF (fator de crescimento endotelial vascular) (Rivard *et al.*, 1999; Tuk *et al.*, 2014), redução na atividade quimiotáxica dos monócitos (Waltenberger *et al.*, 2000), redução no número e atividade das células progenitoras epiteliais (Fadini, 2014) e redução na hemeoxigenase-1, uma enzima que degrada o grupo heme e possui propriedades citoprotetivas e pró-angiogênicas (Kozakowska *et al.,* 2015).

Existe uma associação entre o diabetes e o câncer de pâncreas. Isso ocorre devido a alguns fatores: (a) altos níveis de insulina, que estimulam o crescimento das células e dos vasos sanguíneos pancreáticos, favorecendo o desenvolvimento de neoplasias; e (b) influência dos mediadores da inflamação, que estão associados com expressão de oncogenes, alterações no ciclo celular e silenciamento de genes supressores de tumor (Biadgo e Abebe, 2016). A infiltração de células inflamatórias no pâncreas causa a destruição das células β, comprometendo a produção da insulina, especialmente no DM1 (Diana e Lehuen, 2014). Igualmente, em animais de experimentação a indução do diabetes leva a modificações da morfologia do

pâncreas. Análises histológicas demonstram dano celular nos ácinos e nas ilhotas, com degeneração das células β e formação de vacúolos assimétricos (Ahmed *et al.*, 2015). Com a evolução da doença, as funções exócrinas do pâncreas também ficam reduzidas, com deficiência na produção de enzimas como a amilase e a elastase (Dias *et al.*, 2016; Kangrga *et al.*, 2016).

Além do pâncreas, vários órgãos sofrem alterações no diabetes, incluindo rins e fígado. A nefropatia diabética é uma complicação frequente, na qual ocorre hipertensão e destruição dos glomérulos, com comprometimento da filtração (Faulkner *et al.*, 2015). As alterações histológicas nos rins decorrentes do diabetes incluem deposição de cristais, infiltração de hemácias, destruição dos glomérulos, formação de pigmentos (lipofuscina), hipertrofia e proliferação celular (Ahmed *et al.*, 2015; Pourghasem *et al.*, 2015).

O principal fator desencadeador dos danos hepáticos em pacientes diabéticos é a resistência à insulina, que pode ser agravada pelo estresse oxidativo e resposta inflamatória exacerbada. Em alguns casos pode ocorrer acúmulo de gordura, levando à esteatose não alcoólica, que pode evoluir para cirrose e insuficiência hepática. As alterações histológicas hepáticas observadas são esteatose micro e macrovesicular, infiltrados inflamatórios, fibrose, necrose, tumefação e vacuolização (Mohamed *et al.*, 2016).

1.2. Tratamento do diabetes

O tratamento do DM1 baseia-se no controle da glicemia através da administração de insulina. Pacientes acometidos pelo DM2 também podem precisar de insulina para um controle glicêmico mais adequado. Os tipos de insulina utilizados são: a) as de longa duração (Detemir, Glargina), com início da ação em duas horas e 36 ou mais horas de duração; b) insulina de ação intermediária (Protamina Neutra Hagedorn-NPH) com início da ação em 1 a 2 horas, pico de ação de 6 a 10 horas e atividade de 10 a 16 horas; c) as insulinas de ação rápida (Insulina Humana Regular) com início da ação em 30 a 60 minutos, pico de ação de 2 a 4 horas e duração total de 6 a 8 horas (Ahmad, 2014); d) insulina de ação ultrarrápida (Glulisina, Lispro, Asparte), com início de ação em 10 a 15 minutos, pico de ação entre 1 a 2 horas e duração de 3 a 5 horas; e) pré-misturas, que posuem diferentes

combinações de insulinas de ação rápida e intermediária. (Sociedade Brasileira de Diabetes, 2016)

O tratamento do DM2 também inclui dieta, exercícios e outros fármacos adjuvantes, como as sulfoniluréias e meglitinidas, que estimulam a produção de insulina pelo pâncreas; as biguanidas que reduzem a produção de glicose pelo fígado; as tiazolidinedionas, que aumentam a sensibilidade à insulina no músculo, hepatócitos e adipócitos; os inibidores das alfa-glicosidases, que reduzem a absorção intestinal de carboidratos; o agonista do peptídeo semelhante ao glucagon (GLP-1), que mimetiza a ação de incretinas; os inibidores da dipeptitil peptidase 4 que aumentam o nível de GLP-1, com aumento da síntese e secreção de insulina, além da redução de glucagon e os inibidores do co-transportador sódio glucose 2, que ajudam na eliminação da glicose (Skliros *et al.*, 2016).

1.3. Modelo da indução do diabetes

Considerando a alta incidência, morbidade e mortalidade do diabetes, alguns modelos *in vivo* de diabetes quimicamente induzida foram desenvolvidos para investigar aspectos fisiopatológicos e farmacológicos desta enfermidade, como pela utilização de aloxano ou estreptozotocina. Mais recentemente, alguns modelos abordando indução autoimune espontânea foram desenvolvidos (King, 2012), mas abordaremos somente o modelo utilizado neste estudo, referente à utilização da toxina estreptozotocina.

A ação diabetogênica da estreptozotocina (STZ) foi descoberta em 1963 por Rakieten *et al* (Rakieten *et al.*, 1963). A administração de estreptozotocina destrói as células β pancreáticas, causando necrose e fagocitose, levando a uma redução drástica da produção de insulina. A seletividade da estreptozotocina pelas células β pancreáticas é maior que a do aloxano (Junod *et al.*, 1969).

A estreptozotocina é um componente isolado do fungo *Streptomyces achromogenes* e que possui atividade antibiótica e antineoplásica. Essa droga entra nas células pancreáticas por meio de transportadores GLUT2, levando à formação de radicais superóxido, peróxido de hidrogênio e hidroxil, além de liberação de óxido nítrico. É um agente alquilante, capaz de interferir no transporte de glicose, na

função da glucoquinase e danificar o DNA, levando a múltiplas quebras dos filamentos, metilação, síntese não programada, micronúcleo e aberrações cromossômicas (Bolzan e Bianchi, 2002; Rees e Alcolado, 2005), causando necrose das células (Szkudelski, 2001), com consequente estabelecimento da doença. Desta forma, as funções pancreáticas ficam comprometidas, dentre elas a produção de insulina, induzindo assim o diabetes experimental tipo.

1.4. Angiogênese

O termo vasculogênese refere-se ao processo de formação de novos vasos a partir de células progenitoras endoteliais, enquanto a angiogênese e arteriogênese referem-se a essa formação a partir de vasos pré-existentes, estabilização destes vasos e formação de uma rede vascular (Carmeliet, 2003).

A angiogênese desempenha um papel essencial para a cicatrização de feridas. A sua regulação é bastante complexa, envolvendo a participação de diferentes células, fatores de crescimento e quimiocinas (King *et al.*, 2014). A angiogênese inflamatória envolve o recrutamento e a ativação de células inflamatórias, e ativação, proliferação e migração de células precursoras endoteliais provenientes da medula óssea e fibroblastos (Marques *et al.*, 2011). Deficiências no processo de angiogênese levam ao desenvolvimento de feridas crônicas (King *et al.*, 2014).

A hipóxia é um dos estímulos mais importantes para a expansão do leito vascular. Inicialmente, as células são oxigenadas por difusão, mas o crescimento tecidual desencadeia a sinalização para que ocorra a transcrição de genes angiogênicos. Um dos mais marcantes é a indução do fator de crescimento endotelial vascular (VEGF). Este fator, quando produzido pelo organismo em condições fisiológicas ou patológicas, estimula a angiogênese, de maneira concentração-dependente (Carmeliet, 2003).

Várias enfermidades estão relacionadas com um aumento na angiogênese, dentre elas podemos citar câncer (Eskander e Tewari, 2014; Stifter e Dordevic, 2014), doença inflamatória intestinal (Im, 2014), osteoartrite (Henrotin *et al.*, 2014) e a formação de quelóides (Zhang *et al.*, 2014). A angiogênese é crucial para a

progressão das neoplasias, pois a chegada dos nutrientes e oxigênio necessários para o crescimento tumoral é dependente do aporte sanguíneo. A corrente sanguínea também permite que as células cancerosas invadam outros tecidos (Bruno *et al.*, 2014), portanto, o bloqueio da angiogênese representa uma das estratégias de tratamento para impedir a progressão tumoral. Adicionalmente, a angiogênese está envolvida em várias doenças inflamatórias, como asma e artrite, já que os leucócitos produzem fatores angiogênicos, interleucinas e proteinases. Os fatores angiogênicos amplificam o processo inflamatório recrutando leucócitos e alterando suas funções (Carmeliet, 2003).

Existem também doenças relacionadas diretamente com a redução da angiogênese, como, por exemplo, a pré-eclâmpsia (Escudero *et al.*, 2014) e a distrofia muscular Duchenne (Shimizu-Motohashi e Asakura, 2014). Ainda, em pacientes diabéticos, como comentado anteriormente (item 1.1.), a neovascularização está prejudicada, dificultando o processo cicatricial (Desposito *et al.*, 2014). A cicatrização de feridas inicia-se com a hemostasia, fase em que ocorre a agregação plaquetária e a cascata de coagulação. Em seguida ocorre a fase inflamatória, em que há a quimiotaxia de células, com a liberação de citocinas e fatores de crescimento. Na fase proliferativa, as células endoteliais se proliferam, ocorrendo a angiogênese. Também há a proliferação dos fibroblastos. Por fim, a fase de maturação e remodelamento é marcada pela deposição de colágeno. Inicialmente ocorre a deposição do colágeno do tipo III, que é gradativamente substituído pelo colágeno do tipo I (Witte e Barbul, 1997). Nos pacientes diabéticos a fase inflamatória e a fase proliferativa estão prejudicadas (Tahergorabi e Khazaei, 2012).

Isto exposto, alterações no processo de angiogênese podem ter efeitos benéficos ou maléficos, dependendo da situação e doenças envolvidas.

1.5. Modelos de avaliação de angiogênese

Ensaios *in vivo* para angiogênese não são muito fáceis de executar, havendo apenas poucos modelos disponíveis. Entretanto, comparativamente são melhores do que ensaios *in vitro*, por causa da natureza complexa da resposta vascular (Patan,

2004). Os modelos mais comumente aplicados são implante de esponjas, *plug matrigel* em linhagens de camundongo sem pelos, angiogênese corneal e saco aéreo dorsal (Almalki *et al.*, 2014).

Para avaliar a angiogênese utilizamos neste trabalho um modelo descrito previamente por Andrade *et al.* (1987). Esse modelo consiste na implantação cirúrgica de uma esponja de poliuretano no tecido subcutâneo do dorso dos animais. Após alguns dias ocorre a formação de vasos nessa matriz implantada. Com a remoção e dissecção das esponjas é possível avaliar morfologicamente essa formação, através da histologia. Também é possível com esse modelo avaliar quantitativamente e qualitativamente a formação de novos vasos, através da dosagem de hemoglobina e de técnicas de histomorfometria. A infiltração de células inflamatórias e formação de fatores inflamatórios e de crescimento também podem ser mensurados, e estes parâmetros são relevantes, pois o crescimento dos vasos é acompanhado pela resposta inflamatória (Ferreira *et al.,* 2004*;* Marques *et al.,*2011).

Paralelamente, a avaliação qualitativa e quantitativa de colágeno permite investigar a resposta fibrogênica. De acordo com os tipos de fibras colágenas presentes é possível avaliar o estágio de maturação em que esta resposta fibrogênica se encontra (Junqueira *et al*., 1979; Oviedo-Socarras *et al.,* 2014).

1.6. Via oxidativa mitocondrial e estresse oxidativo

Estresse oxidativo é a expressão utilizada para descrever os processos deletérios resultantes do desequilíbrio provocado pela excessiva formação de espécies reativas de oxigênio (ROS) e nitrogênio (RNS) e defesas antioxidantes insuficientes. As mitocôndrias são as principais geradoras e também o principal alvo das ROS. Essas organelas possuem uma extensa área de superfície devido às membranas internas, que contêm as enzimas responsáveis pela produção de energia a partir do oxigênio. A transferência de elétrons na cadeia transportadora resulta em uma recíproca transferência de prótons, criando o potencial de membrana mitocondrial. Em parte deste processo as ROS são formadas como produtos da redução incompleta do oxigênio. Cerca de 1% do oxigênio utilizado é convertido em ROS (Turrens, 2003; Murphy, 2009).

As ROS incluem moléculas e radicais livres derivadas do oxigênio molecular. O oxigênio singlete (O_2) é o oxidante resultante da mudança de spin de um dos elétrons não pareados do oxigênio. O ânion superóxido ($O2^{\bullet-}$), por sua vez, é o produto da redução de um elétron do oxigênio. Ele é o precursor de boa parte das ROS (Turrens, 2003). Às vezes, os radicais livres podem iniciar uma reação em cadeia. É o que acontece na peroxidação lipídica. O radical peroxil ($ROO^{\bullet}$), quando formado, remove um átomo de hidrogênio dos ácidos poli-insaturados próximos para continuar o processo, convertendo-se em um peroxilipídeo. O resultado da extensa peroxidação lipídica é a morte celular. Ainda, outras estruturas celulares também são danificadas pelas ROS, incluindo proteínas e o DNA (Salvemini e Cuzzocrea, 2002).

Como a geração de ROS faz parte de vários processos fisiológicos, há defesas celulares antioxidantes para combater seu excesso, assim a remoção das ROS depende da ação das enzimas do sistema antioxidante. A superóxido dismutase (SOD) é a enzima que converte o radical superoxido em peróxido de hidrogênio e oxigênio molecular. A catalase (Cat) converte o peróxido de hidrogênio em oxigênio e água, e a glutationa peroxidase (GPx) converte o peróxido de hidrogênio em água. A GPx precisa da ação de cofatores, como a GSH, para o seu funcionamento adequado. Assim, a ação conjunta dessas três enzimas leva à conversão de espécies reativas em água (Weydert e Cullen, 2010). A ação das enzimas antioxidantes está ilustrada na Figura 1.

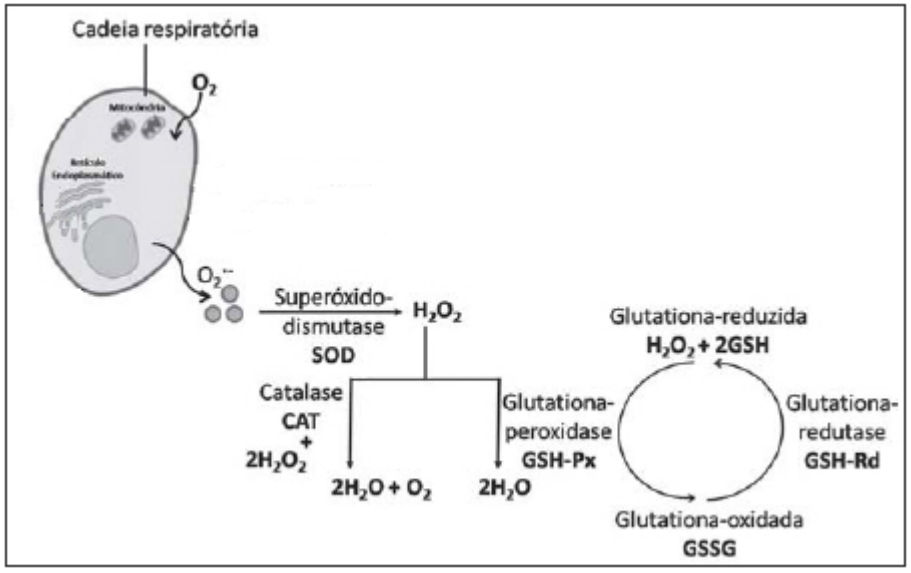

Figura 1: Representação esquemática da ação das enzimas do sistema antioxidante. Fonte: Delbin *et al.*, 2014.

O mecanismo de ação dos agentes diabetogênicos aloxano e estreptozotocina diferem entre si, porém ambos envolvem o dano às células β pancreáticas provocado por espécies reativas de oxigênio (Szkudelski, 2001). Em animais de experimentação, a indução do diabetes leva à redução de enzimas antioxidantes no fígado (Ahmed *et al.*, 2015) e rins (Soto *et al.*, 2010). O estresse oxidativo também está envolvido em complicações do diabetes, como a nefropatia (Faulkner *et al.*, 2015), a hepatopatia (Malekinejad *et al.*, 2012) e a neuropatia diabética (Baluchnejadmojarad *et al.*, 2010).

1.7. Silimarina

As plantas sempre foram uma fonte comum de medicamentos, tanto na forma de principio ativo como na forma de preparações tradicionais, e o reconhecimento do valor clínico e econômico desses produtos tem aumentado consideravelmente. A população muitas vezes utiliza esse recurso por acreditar que os produtos naturais não possuem efeitos colaterais ou por questões financeiras. Assim, é de grande importância que a segurança, a atividade biológica e a toxicidade dos produtos de origem natural sejam avaliadas (Farnsworth *et al.*, 1985; Nicoletti *et al.,* 2010).

A silimarina é um extrato padronizado obtido do fruto da planta *Silybum marianum*. A planta pertence à família Asteraceae, é bienal ou anual de inverno e nativa do sul da Europa e Rússia, e norte da África e Ásia menor, mas também foi introduzida nas Américas, Austrália, China e Europa Central (World Health Organization, 2002). No Brasil, é popularmente conhecida como “cardo de leite” ou “cardo mariano”.

A silimarina é composta principalmente por flavonolignanas, sendo que o isolamento de seus componentes por cromatografia líquida de alta performance (HPLC) revelou que os mais abundantes são silibinina, isosilibinina, silicristina, isosilicristina, silidianina (flavonolignanas) e taxifolina (flavonoide). Estes componentes formam cerca de 68% do extrato, sendo a silibinina a mais abundante, com cerca de 34%. (Pferschy-Wenzig *et al.*, 2014). O termo flavonolignana foi criado em 1968 para nomear uma pequena classe de moléculas híbridas, formadas de flavonóides e lignanas. As primeiras flavonolignanas foram extraídas do *Silybum marianum* (Vue e Chen, 2016). Lignanas são fenóis encontrados no reino vegetal

que possuem atividade antioxidante e antinflamatória. Por essas propriedades são utilizadas na medicina étnica e convencional (Teponno *et al,*. 2016). Flavonóides também são fenóis com propriedades antioxidantes, capazes de remover radicais livres e reduzir a sua formação. Há cerca de 8 mil substâncias flavonoides conhecidas, extraídas de plantas vasculares (Pietta, 2000).

A silimarina é essencialmente indicada para prevenção e tratamento de lesões hepáticas alcoólicas e não alcoólicas, apresentando bons resultados em animais experimentais (Clichici *et al.*, 2016) e em estudos controlados em humanos (Ferenci *et al.*, 1989). Pacientes tratados com silimarina apresentaram redução nos níveis das transaminases séricas, redução de alterações histológicas hepáticas (Salmi e Sarna, 1982) e redução da taxa de mortalidade quando acometidos por cirrose (Benda *et al.*, 1980).

Além de seu uso terapêutico clássico como hepatoprotetor, a silimarina tem sido testada para o tratamento de várias doenças, como diabetes, Alzheimer, Parkinson, sepse, queimaduras, osteoporose, colestase, hipercolesterolemia e neoplasias, com resultados promissores (Milic *et al.*, 2013). Neste contexto, alguns trabalhos já apontam efeitos benéficos da silimarina no tratamento do diabetes. Segundo Soto *et al.,* (2014), o tratamento com a silimarina em animais pancreatectomizados aumentou a expressão gênica da insulina no pâncreas, a neoformação de células β, a insulina sérica e normalizou a glicemia. Sheela *et al.,* (2013) avaliaram o efeito da silimarina em um modelo de diabetes induzida pela estreptozotocina e nicotinamida, no qual os animais tratados tiveram redução da glicemia, volume urinário, albumina urinária, e da creatinina e ácido úrico séricos, demonstrando efeitos nefroprotetores. Em experimentos de cultivo celular, Kim *et al.,* (2014) demonstraram efeitos protetores da silimarina sobre as células β pancreáticas, através da inibição do fator nuclear (NF)-κB e da quinase controlada pela sinalização extracelular (ERK). O tratamento com silimarina em animais e pacientes diabéticos também produziu efeitos antioxidantes (Anestopoulos *et al.*, 2013; Khazim *et al.*, 2013).

Pelo exposto neste e nos itens anteriores, há uma série de complicações resultantes do diabetes. Apesar dos tratamentos disponíveis, com diversos tipos de insulina, secretagogos de insulina, agentes sensibilizadores à insulina e hipoglicemiantes orais, a morbidade e mortalidade no diabetes permanecem altas, sendo assim é necessário explorar novos agentes terapêuticos. Neste contexto, a

silimarina vem ganhando destaque, pois há indícios na literatura de seus efeitos benéficos em animais e pacientes diabéticos. Por outro lado, há evidências de que a silimarina possui efeitos anti-angiogênicos, como por exemplo, no câncer (Singh *et al*., 2008; Vaid *et al*., 2014). Assim, torna-se necessário esclarecer qual o efeito da silimarina sobre a angiogênese em diabéticos, que apresentam déficits no processo cicatricial. Como mencionado anteriormente, a angiogênese é crucial para a cicatrização. Para isso, utilizamos um modelo de angiogênese inflamatória, que consiste no implante de esponja de poliuretano no dorso dos camundongos. Com o passar dos dias ocorre a infiltração de células no implante e a formação de novos vasos, sendo possível mensurar esses e outros eventos após a remoção da esponja. Desta maneira, nossa hipótese é que a silimarina possa modular alguns danos causados pelo diabetes induzido pela STZ, como angiogênese e estresse oxidativo.

2.0. Objetivos

2.1. Objetivo geral

Investigar os efeitos da silimarina em animais diabéticos e normoglicêmicos, dando ênfase aos processos de angiogênese e estresse oxidativo.

2.2. Objetivos específicos

1) Avaliar a angiogênese através de análise histológica do modelo de esponja de poliuretano, com contagem de vasos e mensuração da área vascular, bem como pela determinação de hemoglobina;
2) Avaliar parâmetros inflamatórios no implante da esponja de poliuretano, através da dosagem de enzimas leucocitárias (MPO, presente nos neutrófilos e NAG, presente em linfócitos e monócitos), assim como a dosagem do nitrito, que é uma medida indireta da presença do óxido nítrico;
3) Mensurar o colágeno da esponja de poliuretano, a fim de avaliar o caráter cicatricial no implante, investigando os tipos de colágenos presentes;
4) Avaliar parâmetros de bioquímica plasmática nos animais diabéticos e normoglicêmicos, como creatinina, uréia, aspartato e alanina amino transferase, e amilase;
5) Investigar parâmetros de estresse oxidativo, parâmetros inflamatórios e alterações morfológicas em pâncreas, rins e fígado;
6) Avaliar se todos os parâmetros supracitados diferem em camundongos normoglicêmicos e diabéticos, tratados ou não com a silimarina.

3. Artigo cientifico 1: EFFECTS OF SILYMARIN ON DIABETES MELLITUS COMPLICATIONS: A REVIEW

Artigo submetido à revista *Phytotherapy Research* (Online ISSN: 1099-1573).

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Abstract

Diabetes mellitus (DM) is a common metabolic disorder that is caused by a deficit in the production of (type I) or response to (type II) insulin. DM is characterized by a state of chronic hyperglycemia and such symptoms as weight loss, thirst, polyuria, and blurred vision. These disturbances represent one of the major causes of morbidity and mortality nowadays, despite available treatments, such as insulin, insulin secretagogues, insulin sensitizers, and oral hypoglycemic agents. However, many efforts have been made to discover new drugs for diabetes treatment, including medicinal plant extracts. Silymarin is a powder extract of the seeds from *Silybum marianum*, a plant from the Asteraceae family. The major active ingredients include four isomers: silybin, isosilybin, silychristin, and silydianin. Silymarin is indicated for the treatment of hepatic disorders, such as cirrhosis, chronic hepatitis, and gallstones. Moreover, several studies of other pathologies, including diabetes, sepsis, osteoporosis, arthritis, hypercholesterolemia, cancer, viral infections, and Alzheimer's and Parkinson's disease, have tested the effects of silymarin and reported promising results. This article reviews data from clinical, *in vivo*, and *in vitro* studies on the use of silymarin, with a focus on the complications of diabetes, including nephropathy, neuropathy, healing delays, oxidative stress, hepatotoxicity, and cardiomyopathy.

Keywords: silymarin, silybin, diabetes, healing, hepatopathy, neuropathy.

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Introduction

Diabetes is a metabolic disturbance that is characterized by chronic hyperglycemia, resulting in a deficiency in insulin secretion (type I diabetes) or a deficiency in the response to insulin (type II diabetes). Characteristic symptoms include weight loss, thirst, polyuria, and blurred vision (Alberti and Zimmet, 1999). In 2015, 415 million people were estimated to be living with diabetes, representing one in every 11 adults, and 5.0 million deaths occurred as a consequence of this disease. The number of cases is expected to increase to 642 million in 2040, representing one in every 10 adults, and $673 billion was spent for healthcare for diabetic patients in 2015 (International Diabetes Federation, 2015).

Numerous complications result from diabetes. Despite the available treatments, such as injectable insulin, insulin secretagogues, insulin sensitizers, and oral hypoglycemic agents, the mortality and morbidity associated with diabetes remain high. Moreover, agents that are traditionally used for the treatment of diabetes and diabetes complications can cause severe side effects (Mirhoseini *et al.*, 2013). Therefore, exploring new therapeutic interventions is necessary.

The use of complementary and alternative medicine, including herbal medicine, is increasing, and the action, safety, and efficacy of these products need to be investigated (Mirhoseini *et al*., 2013). Several medicinal plants are used for the treatment of diabetes, but not all of them have been pharmacologically tested (Baharvand-Ahmadi *et al*., 2016). Some plants that are being tested for the treatment of diabetes or diabetes complications have antioxidant properties (Nasri *et al.,* 2015; Nasri and Rafieian-Kopaei, 2014).

In this context, the natural compound silymarin has received increasing interest in recent decades. The aim of this article is to review data from *in vitro*, *in vivo*, and clinical studies on the use of silymarin for the treatment or prevention of diabetes complications, including nephropathy, neuropathy, healing delays, oxidative stress, hepatotoxicity, and cardiomyopathy.

Silymarin

Silymarin is a standardized extract that is obtained from the seeds of *Silybum marianum* (L.) Gaertn (Fig. 1A), a plant from the Asteraceae family (Ottai and Abdel-Moniem, 2006). The extract consists of approximately 65-80% silymarin (i.e., a

flavonolignan complex). Isolation and the complete characterization of the main constituents of silymarin by high-performance liquid chromatography revealed that the most abundant compounds are silybins A and B (also known as silibinins A and B), followed by isosilybins A and B. Three other flavonolignans (i.e., silychristin, isosilychristin, and silydianin) were also isolated, in addition to the flavonoid taxifolin. The structures of compounds (Fig. 1B) were confirmed by two-dimensional nuclear magnetic resonance spectroscopy and circular dichroism spectroscopy (Kim et al., 2003).

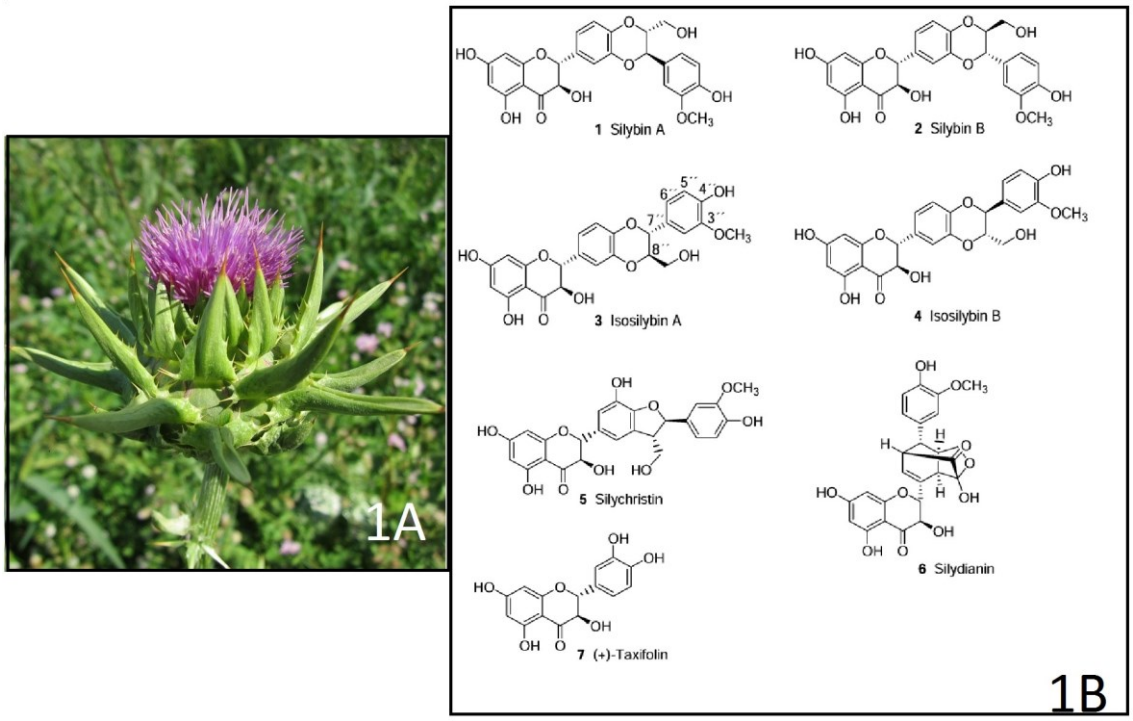

Figure 1. (A) *Silybum marianum* plant (https://pixabay.com, accessed May 2016) and (B) chemical structures of compounds of silymarin (Pferschy-Wenzig *et al,* 2014) [Fig. 1B was reproduced with kind permission of Dr. Atanas G. Atanasov].

The classic indication for silymarin is for the treatment of gallbladder (Kazazis *et al*., 2014) and hepatic diseases, such as cirrhosis, chronic hepatitis (Ferenci *et al.*,1989; Salmi and Sarna, 1982), and non-alcoholic fatty liver disease (NAFLD; Aller *et al*., 2015). The treatment regimen for hepatic cirrhosis is 140 mg three times daily (Ferenci *et al.*, 1989). After oral administration in a single dose, silymarin (Legalon 140™) is well tolerated, even at higher doses (> 700 mg, or more than five capsules). An average of 10% of the administered silybin is found in plasma in an unconjugated form. Four to 6 h after administration, plasma levels are undetectable. Urinary

elimination as total silybin represents only 5% in cirrhotic patients (Weyhenmeyer *et al.,* 1992).

In addition to label indications, many studies have reported the beneficial effects of silymarin for the treatment of several diseases (Fig. 2), including sepsis (Kang *et al*., 2004; Toklu *et al*., 2008), burns (Toklu *et al*., 2007), osteoporosis (Kim *et al.,* 2013; Mohd Fozi *et al*., 2013), arthritis (Gupta *et al*., 2000), hypercholesterolemia (Krecman *et al.,* 1998; Skottova *et al.,* 1998), cancer (Singh and Agarwal, 2008; Bosch-Barrera and Menendez, 2015; Scavo *et al.,* 2015; Yurtcu *et al.,* 2015), viral infections (Lani *et al., 2015*), diabetes (Malekinejad *et al.*, 2012; Soto *et al.*, 2014; Zhang *et al.*, 2014; Ebrahimpour Koujan *et al.*, 2015), Alzheimer's disease (Duan *et al.,* 2015; Kumar *et al.,* 2015), and Parkinson's disease (Haddadi *et al.,* 2014; Lee *et al.,* 2015). Silymarin treatment likewise attenuated the severity of radiotherapy-induced mucositis in a clinical trial (Elyasi *et al.*, 2016). Concerning diabetes, studies have also indicated that silymarin may be used to treat or attenuate specific diabetes complications, as discussed in the following sections and shown in Table 1.

Several articles have reported the findings of *in vitro*, *in vivo*, and clinical studies of silymarin and its constituent compounds and found that its main beneficial action involves antioxidant effects in different organs and diseases. Different mechanistic explanations for the antioxidant effects of silymarin have been described, including (*i*) preventing free radical formation by inhibiting specific reactive oxygen species (ROS)-producing enzymes or improving the integrity of mitochondria in conditions of stress, (*ii*) decreasing inflammatory responses by inhibiting nuclear factor κB (NF-κB)-dependent pathways, and (*iii*) maintaining an optimal redox balance in the cell by activating a range of antioxidant enzymes and non-enzymatic antioxidants, mainly via the activation of nuclear factor-erythroid 2-related factor (Nrf2; Surai, 2015; Tan *et al.*, 2015; Zhao *et al*., 2015; Fallahzadeh *et al.*, 2012; Salamone *et al*., 2012a; Soto *et al.*, 2010; Vengerovskii *et al.,* 2007). Growing evidence indicates that the Nrf2 antioxidant response plays an important role in cellular defense by activating a wide variety of genes that are involved in early antioxidant defense reactions (Surai, 2015), inflammatory processes, apoptotic processes, metabolism, detoxification, and cellular proliferation (Morales-Gonzáles *et al*., 2015). Data have shown that silymarin improves liver regeneration (Morales-Gonzáles *et al*., 2015) and lung injury (Zhao et al., 2015) by mediating the Nrf2 signaling pathway. Nrf2 is a key-regulator of oxidative stress, which is involved in the

pathogenesis of diabetes, and then the therapeutic potential of silymarin for the treatment of diabetes complications has become increasingly evident.

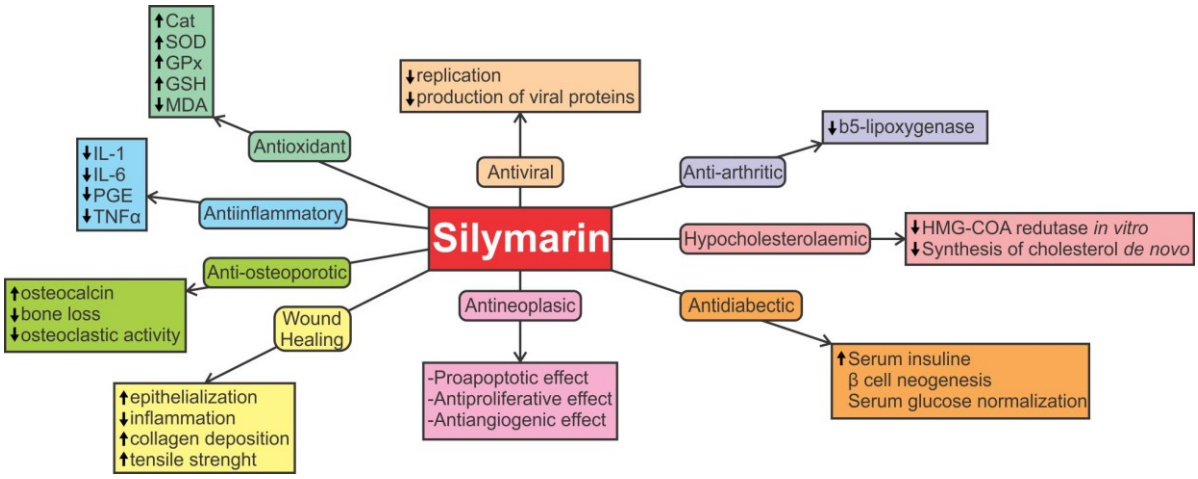

Figure 2. Schematic representation of off-label effects of silymarin reported in the literature.

Table 1. Posological regimen of silymarin or silybin applied experimentally for the treatment of diabetes complications.

Drug	Species	Dose, via and treatment period	Complication	Reference
Silymarin	Rats	100 mg/kg, ip, 2 m	Neuropathy	Baluchnejadmojarad *et al*., 2010
Silybin	Rats	100 mg/kg, vo, 20 d	Neuropathy	Di Cesare Mannelli *et al*., 2012
Silybin	Rats	10 or 30 mg/kg, vo, 22 w	Retinopathy	Zhang *et al*., 2014
Silymarin	Rats	30 mg/kg, t, 5, 50 or 25 d	Healing	Sharifi *et al*., 2012
Silymarin	Rats	6 mg/mL/rat or 12 mg/mL/rat, t, 10, 20 or 30 d	Healing	Oryan *et al, 2012.*
Silymarin	Rats	70 mg/kg, vo, 14 d	Metabolical disorders	Vengerovskii *et al*., 2007
Silymarin	Mice	25, 50 or 100 mg/kg, ip, 3 or 4 w	Cardiomyopahy	Taghiabadi *et al*., 2012
Silymarin	Rats	120 mg/kg, ip,	Cardiomyopahy	Tuorkey *et al*., 2015

		10 d		
Silybin and vitamin E	Rats	47+15 mg/rat, vo, 7, 14, 30 or 60 d	Hepatopathy	Grattagliano *et al.*, 2013
Silymarin	Rats	50mg/kg, vo, 28 d	Hepatopathy	Malekinejad *et al.*, 2012
Silymarin	Gerbil	100 mg/kg, vo, 7 w	Hepatopathy	Bouderba *et al.*, 2014
Silybin-beta-cyclodextrin	Humans	135 mg/d, vo ,6 m	Hepatopathy	Lirussi *et al.*, 2002
Silymarin	Mice	100 mg/kg, vo, 7d	Nephropathy	Tan *et al*., 2015
Silymarin	Rats	60 or 120 mg/Kg, 60 d	Nephropathy	Sheela *et al.*, 2013
Silymarin	Rats	200mg/kg, vo, 9 w	Nephropathy	Soto *et al.,* 2010
Silymarin	Rats	100 mg/kg, vo, 4w	Nephropathy	Vessal *et al.,* 2010
Silymarin	Humans	420 mg/d, vo, 3m	Nephropathy	Fallahzadeh *et al.*, 2012

Legend: vo, oral via; t, topical via; ip, intraperitoneal via; m, months; d, days; w, weeks.

Nephropathy

Diabetic nephropathy involves impairment of the glomerular filtration barrier as a consequence of high blood glucose levels, inflammation, and oxidative stress (Faulkner *et al.*, 2015). High blood pressure also represents an important risk factor for disease onset. Diabetic nephropathy is the primary cause of end-stage renal failure worldwide (Conserva *et al.*, 2016).

Evidence indicates that silymarin may produce beneficial effects in renal diseases. In a model of renal ischemia-reperfusion injury, mice were treated with 100 mg/kg silymarin for 7 days before the induction of ischemia. Compared with the control group, silymarin-treated animals had low levels of serum creatinine and urea. The treatment also attenuated damage in renal tubule cells and the number of apoptotic cells. Renal myeloperoxidase, tumor necrosis factor α (TNF-α), interleukin

1β (IL-1β), and IL-6 levels also decreased. The expression of renal CD68 in silymarin-treated mice was lower, whereas Bcl-2 expression was higher (Tan *et al.*, 2015).

Animal models of diabetes have provided evidence of the beneficial effects of silymarin against diabetic nephropathy. Rats with diabetic nephropathy that was induced by streptozotocin and nicotinamide received 60 and 120 mg/kg silymarin for 60 days. Silymarin-treated animals exhibited reductions of blood glucose, glycosylated hemoglobin, urine albumin and volume, serum creatinine, and uric acid. The histopathological evaluation revealed preservation of the tubular epithelium and a reduction of intertubular hemorrhage, mainly at a higher dose (Sheela *et al.*, 2013). In similar studies, silymarin treatment reduced oxidative stress in renal tissue through the recovery of antioxidant enzymes (Soto *et al.*, 2010; Vessal *et al.*, 2010). Data results from our laboratory corroborated these data, in which we found a reduction of oxidative stress (i.e., decreases in lipoperoxide levels and increases in catalase activity) in homogenates of renal tissue from rats with streptozotocin-induced diabetes that were treated with silymarin (104.1 mg/kg) for 10 days (Stolf, 2016).

A randomized, double-blind, placebo-controlled study was performed in patients with type 2 diabetes with persistent macroalbuminuria. The patients underwent treatment for 6 months with a renin-angiotensin system inhibitor plus an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker at the highest dose that is approved by the US Food and Drug Administration. Among these patients, 60 were divided equally into placebo- and silymarin-treated groups. The silymarin-treated group received three daily doses of 140 mg silymarin for 3 months. Silymarin-treated patients presented a better urinary albumin-creatinine ratio and lower levels of urinary TNF-α and urinary and serum malondialdehyde (Fallahzadeh *et al.*, 2012).

Neuropathy

Approximately 50% of diabetic patients develop neuropathy. This condition can be either symptomatic or asymptomatic. Symptoms can include numbness, prickling, pain, and allodynia. The mechanisms of diabetic neuropathy involve ischemia, impaired growth factor support, and oxidative stress. Severe cases can

lead to toe, foot, and leg amputation (Gordois *et al*., 2003; Baluchnejadmojarad *et al*., 2010).

Some studies have reported the potential beneficial effects of silymarin in *in vitro* and *in vivo* models of neuropathy. Di Cesare Mannelli *et al*. (2013) evaluated the effects of treatment with silybin and α-tocopherol in the neuronal-derived SH-SY5Y cell line and primary cultures of rat cortical astrocytes that were challenged with oxaliplatin, an antineoplasic agent that can induce neurotoxicity. Such toxicity is caused by oxidative damage in nervous system tissues, leading to neuropathic pain. The treatment protected the cells against the generation of superoxide anions, malondialdehyde, carbonylated proteins, and DNA oxidation. The treatment did not suppress the apoptotic effect of oxaliplatin when tested in neoplastic cells. A similar study was performed in rats. Oxaliplatin was administered intraperitoneally daily for 21 days at a dose of 2.4 mg/kg, leading to neuropathic pain and increases in oxidative stress parameters in the sciatic nerve, spinal cord, and plasma. Treatment with 100 mg/kg silybin and α-tocopherol reduced pain and improved motor coordination (Di Cesare Mannelli *et al*., 2012).

In rats with streptozotocin-induced diabetes, daily treatment with 100 mg/kg silymarin for 2 months reduced the deficit in sciatic motor nerve conduction velocity and reduced hyperalgesia. The activity of superoxide dismutase was restored, and malondialdehyde levels decreased (Baluchnejadmojarad *et al*., 2010). The authors concluded that silymarin had a potential beneficial effect in the treatment and prevention of diabetic neuropathy. However, these findings have not yet been confirmed in clinical trials.

Retinopathy

Retinopathy is one of the most common complications associated with diabetes and one of the major causes of adult blindness (International Diabetes Federation, 2015). The mechanisms that are involved in this condition include damaged retinal vasculature, neurodegeneration, and inflammation (Jonsson *et al*., 2016; Vindeirinho *et al*., 2016). Ocular imaging techniques are very important for diagnosis and the evaluation of treatment efficacy (Tan *et al*., 2016).

Lin *et al*. (2013) evaluated the effects of silybin in age-related macular degeneration *in vitro* and *in vivo.* Retinal pigmented epithelial cells were subjected to hypoxic conditions. The treatment increased the expression of proline hydroxylase-2 and inhibited hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor, molecules that are related to angiogenesis. In rats, silybin prevented retinal edema and neovascularization. In another experiment with Sprague-Dawley rats, diabetes was induced by streptozotocin and a high-fat diet. Silybin was administered for 22 weeks (Table 1). This treatment prevented the obliteration of retinal capillaries and reduced leukostasis and retinal intercellular adhesion molecule-1 (ICAM-1) in experimental diabetes (Zhang *et al*., 2014). The authors concluded that silybin prevented vascular retinal damage, which at least partially occurred as a consequence of a reduction of ICAM molecules and leukostasis. We found no clinical trials on silymarin for the treatment of retinopathy.

Impaired healing

Neovascularization is essential to wound healing (King *et al*., 2014). Because of this, another consequence of microvascular deficiency in diabetic patients is impaired healing. Reports have indicated that silymarin and related compounds can improve healing. An *in vitro* study evaluated the efficacy of silybin against the toxicity of sulfur mustard, a vesicant agent that can cause serious skin injuries. HaCat cells (i.e., an aneuploid immortal keratinocyte cell line from adult human skin) were treated with silybin-bis-succinate, a water-soluble prodrug of silybin. The treatment reduced cytotoxicity, apoptosis, and IL-6 and IL-8 production, with a reduction of inflammation. These results may suggest the potential use of silybin for the treatment of skin injuries (Balszuweit *et al*., 2013). Gadad *et al.* (2013) developed sterile lyophilized wafers that were impregnated with silymarin and investigated their *in vitro* actions. They used a model of cell migration and microvascular endothelial cells from the adult dermis (HMVECad). In an environment with high glucose concentrations that simulated cellular conditions associated with diabetes, the use of the silymarin-containing wafers improved endothelial cell migration. The results indicated that silymarin at least partially prevented the reduction of angiogenesis in diabetes, improved endothelial cell migration, and consequently promoted better healing in these patients.

The topical application of 30 mg/kg silymarin in Wistar rats reduced inflammation and increased epithelialization in excision wounds, with no differences in the percentage of wound contraction, collagen deposition, or hydroxyproline levels (Sharifi *et al*., 2012). However, another study with Wistar rats reported differences in collagen and hydroxyproline levels. Oryan *et al.* (2012) investigated the effects of topical application of silymarin on incisional wounds. Treatment with 6 or 12 mg/ml in rats for 10, 20, or 30 days increased tissue healing, collagen and glycosaminoglycan deposition, and tensile strength. Differences were observed between the 6 and 12 mg/mg doses. The lower dose decreased the number of lymphocytes and increased the number of fibrocytes. The higher dose increased the number of lymphocytes and macrophages and increased the number of fibrocytes. The topical application of 10% and 20% silybin accelerated the time of healing and increased the levels of stromelysine 1 (i.e., an enzyme involved in the remodeling process), *N*-acetyl glucosamine, *N*-acetyl galactosamine, and hydroxyproline (i.e., components of the extracellular matrix) with 10, 20, or 30 days of treatment. These effects occurred in a dose- and time-dependent manner. The results indicated an improvement in the remodeling phase and augmentation of the index of collagen and glycosaminoglycan content that was induced by silybin in rats (Tabandeh *et al.,* 2013). We found no clinical trials concerning would healing in patients who are treated with silymarin or its constituent compounds.

Metabolic disorders

Diabetes involves disturbances in the metabolism of carbohydrates, fat, and protein, resulting from alterations in insulin secretion or action. In severe forms, ketoacidosis may occur, leading to stupor, coma, and death. Mitochondrial mutations are also related to diabetes (Alberti and Zimmet, 1999). Evidence shows that silymarin can reverse metabolic damage and improve mitochondrial respiratory activity. Streptozotocin-induced diabetes in Wistar rats was treated intragastrically with 70 mg/kg silymarin for 14 days. The treated animals exhibited lower levels of blood glucose and cholesterol, a reduction of the lipoperoxidation index, and normalized oxidative phosphorylation disturbances in liver mitochondria (Vengerovskii *et al.,* 2007). Similarly, Detaille *et al.* (2008) evaluated the activity of silymarin in isolated hepatocytes. After isolation, the cells were perfused with Krebs-

bicarbonate-calcium in the presence or absence of silybin. This compound decreased hepatic glycolysis by inhibiting pyruvate kinase and reducing dihydroxyacetone phosphorylation. Silybin also reduced the levels of ROS that were produced within the electron transport chain. Reactive oxygen species production was fully abrogated by 100 μM silybin.

Liver gluconeogenesis and glycogenolysis were evaluated in a perfusion system, in which silybin treatment reduced glucose-6-phosphate hydrolysis, leading to the inhibition of gluconeogenesis and glycogenolysis. These effects were attributed to inhibition of the enzyme glucose-6-phosphatase in rat liver microsomes (Vengerovskii *et al.,* 2007).

Ligeret *et al.* (2008) evaluated hepatic alterations after cold preservation (i.e., a necessary condition for organ transplants). The assays involved liver perfusion after 24 h with silybin added to the cold preservation solution. Compared with the control group, silybin restored bile secretion and glutathione (GSH) production, reduced lipoperoxidation (LPO) and superoxide production, and increased the respiratory control ratio. The results suggested that silybin may be useful for improving the outcome of liver transplantation (Ligeret *et al.,* 2008). These data are relevant because some patients develop diabetes after liver transplantation. The preoperative risks for developing this condition include advanced age, alcoholic hepatitis, ascites, hepatic coma, and esophageal varices (Liu *et al.,* 2016).

Although only a few clinical studies of silymarin have been conducted, the results have reflected the findings of laboratory studies (Costa Pereira *et al*., 2016), including with regard to glycemia and lipid profiles. A clinical trial that included patients with type 2 diabetes (*n* = 25) who received silymarin (200 mg/day) for 4 months reported significant decreases in fasting glycemia, total cholesterol, low-density lipoprotein, and triglycerides compared with placebo and compared with the respective indices at the beginning of the study. The authors concluded that silymarin treatment has a beneficial effect on improving the glycemic profile (Huseini *et al*., 2006).

Hepatopathy

Other complications of diabetes include liver diseases that are mainly caused by insulin resistance and oxidative stress (Bourdeba *et al.,* 2014). Two hepatic

complications are largely present in patients with diabetes: (*i*) concomitant autoimmune hepatitis (Matsumoto *et al*., 2016) and *(ii)* NAFLD (Forlani *et al*., 2016). A study of 118 diabetic patients found a prevalence of 24.5% of concomitant autoimmune hepatitis in diabetic patients (Matsumoto *et al*., 2016). NAFLD is a public health concern, the major treatment for which remains lifestyle changes, including weight reduction, the prevention of weight gain, a healthy diet, and physical activity (Zelber-Sagi *et al*., 2016).

Oxidative stress is involved in the pathogenesis of NAFLD and non-alcoholic steatohepatitis (NASH), and treatment with antioxidant substances, including silymarin and silibinin, can contribute to the control of these imbalances in lipid metabolism (Salomone *et al.,* 2016; Salamone *et al*., 2012a). Treatment with silibinin (20 mg/kg) reduced the activation of NF-κB in a model of obese mice with NASH and counteracted the progression of liver injury through antiinflammatory actions (Salamone *et al*., 2012a). These data have translational importance because patients the type 2 diabetes also commonly have NAFLD, with a very high rate of NASH (Tilg *et al*., 2016). In another model of liver steatosis, Wistar rats were treated with a complex that contained silymarin and vitamin E. The treated animals presented less hepatic lipid infiltration, lower serum transaminase levels, and improvements in parameters of nitrosative and oxidative stress, including reductions of malondialdehyde and thiobarbituric acid, nitrosothiols, nitrotyrosine, and proinflammatory keratins in the liver and blood. Mitochondrial oxidative phosphorylation complexes were evaluated in the liver, heart, and skeletal muscles. The treatment exerted a protective effect on respiratory chain proteins, which were more expressive in the liver (Grattagliano *et al.,* 2013). Notably, however, silymarin (47 mg) was combined with vitamin E (15 mg) in the study by Grattagliano *et al*. (2013), thus hindering definitive conclusions concerning the antioxidant effects of silymarin *per se*. Cytochrome P450 (CYP) 3A2 and oxidative stress were also evaluated in the liver in rats with streptozotocin-induced diabetes. Treatment with 50 mg/kg silymarin for 28 days abrogated oxidative stress, reflected by reductions of malondialdehyde and nitric oxide levels and an increase in glutathione peroxidase and total thiol molecules. In diabetic animals, CYP3A2 was upregulated, and silymarin treatment restored these levels to normal (Malekinejad *et al.*, 2012).

The sand rat (*Psammomys obesus*) is a rodent species of the Gerbillidae class. In the natural environment, its diet is based on vegetables. When these

animals receive a high-fat diet, they develop diabetes, obesity, and metabolic syndrome. For this reason, this species is frequently used as an animal model of human diabetes (Berdja *et al.,* 2016; Bolton *et al.,* 2012; Scherzer *et al.,* 2011). Bourdeba *et al.* (2014) induced diabetes in *P. obesus* with a high-calorie diet for 14 weeks. Diabetic animals presented hepatic steatosis, hepatic and plasma oxidative stress, hyperinsulinemia, hyperglycemia, and dyslipidemia. Daily treatment with 100 mg/kg silybin beginning on week 7 reduced oxidative stress, hepatic steatosis, triglycerides, and insulin resistance. In mice with streptozotocin-induced diabetes, silymarin treatment restored the activity of hepatic catalase (Stolf, 2016).

Silybin-β-cyclodextrin (IβI/S) is a formulation of silybin with improved solubility and absorption. A double-blind, randomized study of IβI/S *vs.* placebo was performed in patients with diabetes and chronic liver disease who received 6 months of treatment. The treated group presented reductions of blood glucose, triglycerides, and malondialdehyde. The conclusion of this study was that silymarin may be useful in conditions with augmented mitochondrial ROS formation because of its antioxidant properties (Lirussi *et al.,* 2002). Thus, regarding hepatic benefits, silymarin acts through its antioxidant and antiinflammatory properties and stimulates liver regeneration (Bahmani *et al*., 2015; Vargas-Mendoza *et al*., 2014).

Cardiomyopathy

Metabolic alterations in diabetic patients lead to functional and structural changes in the myocardium. Diabetic cardiomyopathy is associated with high rates of mortality (Hayat *et al.,* 2004). This form of cardiomyopathy is related to insulin resistance, hyperinsulinemia, and alterations in mitochondria and the endoplasmic reticulum and occurs independently of hypertension and coronary artery disease (Jia *et al.,* 2016).

In an *in vitro* model of hypoxia/reoxygenation, neonatal rat cardiomyocytes were treated with 2,3-dehydrosilybin, a minor component of silymarin. Treated cells had a lower index of cell death and lower activity of lactate dehydrogenase. Treated cells also exhibited decreases in ROS, reactive nitrogen species, hydrogen peroxide, and the formation of protein carbonyls (i.e., a marker of protein oxidative modification). Furthermore, the treatment regulated the activity and phosphorylation of protein kinase Cε, a component of cell survival and mitogenesis (Gabrielova *et al.,*

2015). The treatment of H9c2 embryonic rat heart cells with silybin reduced the hypertrophic response that was induced by phenylephrine by inhibiting oxidative stress (Anestopoulos *et al.,* 2013).

Acrolein is a highly reactive aldehyde that is able to induce apoptosis, oxidative stress, and inflammation, thus leading to neurodegenerative and cardiac diseases. A protective effect was demonstrated in mice that were treated with silymarin. Lower malondialdehyde levels and the restoration of GSH, superoxide dismutase (SOD), and catalase levels were observed in the heart in treated animals, indicating a reduction of oxidative stress. Serum cardiac troponin and creatine kinase-MB, indicators of cardiac lesions, were also reduced. The Bax/Bcl-2 ratio, cytosolic cytochrome *c* content, and cleaved caspase-3 were also reduced, indicating protection against apoptosis (Taghiabadi *et al.,* 2012). Another study that treated db/db mice with 20 mg/kg silybin, i.p., daily for 4 weeks found that silybin exerted antioxidant and antiinflammatory actions by lowering the levels of isoprostanes, 8-deoxyguanosine, nitrites/nitrates, and TNF-α in the heart and liver. Silybin treatment also restored GSH levels in both tissues (Salamone *et al.*, 2012b). These authors concluded that silybin improved both myocardial and hepatic injury.

Silymarin afforded cardiac protection against hyperglycemia-induced apoptosis in cardiomyocytes. In rats with alloxan-induced diabetes that was treated with 120 mg/kg silymarin, immunohistochemistry of the heart showed lower immunoreactivity of caspase 3, a pro-apoptotic protein, and augmented Bcl-2, an anti-apoptotic protein, in treated animals. The ratio of DNA cardiac fragmentation was also reduced in treated animals, indicating protection against apoptosis. The treatment also reduced the plasma levels of cholesterol, triglycerides, glycemia, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), and the AST/ALT ratio. Plasma insulin levels were restored after treatment, reflecting the restoration of pancreatic cells (Tuorkey *et al.,* 2015). We found no clinical trials that evaluated the effects of silymarin in cardiomyopathy.

Final considerations

The studies reviewed herein provide evidence that treatment with silymarin or its constituent compounds are capable of attenuating common diabetic complications in several organs. The data were obtained from *in vitro* investigations, animal

models, and clinical studies. However, data are still lacking concerning the safety of silymarin and its constituent compounds in diabetic patients, mainly with prolonged treatment. Cheng *et al.* (2014) warned about this problem because their study showed that silymarin treatment led to insulin resistance in rats. A recent meta-analysis evaluated five randomized controlled trials that included 270 diabetic patients who were treated with silymarin. Silymarin reduced fasting blood glucose but had no effect on lipid profiles. Few data are available on the effects of silymarin on diabetic complications in randomized controlled trials (Voroneanu *et al.,* 2016) or its pharmacokinetics profile. Regarding drug interactions, silymarin may interact with metronidazole (Izzo *et al.,* 2016) and ribavirin (Liao et al., 2016), lowering the plasma concentrations of both drugs and thus compromising its pharmacokinetics and therapeutic efficacy. Silymarin appears to be a promising drug for controlling diabetes complications, but further investigations of its pharmacokinetics, safety, and possible adverse effects in this particular patient population are necessary and should be encouraged.

Conflict of interest

The authors declare no conflict of interest.

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4. Artigo científico 2: EFFECTS OF SILYMARIN ON ANGIOGENESIS AND OXIDATIVE STRESS ON NORMOGLYCEMIC AND DIABETIC MICE

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Abstract

Background: Silymarin is the extract obtained from *Silybum marianum,* used to treat and prevent liver diseases. However, it has been tested to treat other diseases, including diabetes. Previous studies showed that silymarin can have a beneficial effect in glycemic control. In this study we evaluated the effect of acute treatment of silymarin in angiogenesis, in normoglycemic or diabetic animals, and also investigated its effects in kidney, liver and pancreas oxidative stress and inflammation.

Experimental approach: Male Swiss mice, six weeks aged, were used in the experiments. Diabetes was induced by administration of 80 mg/kg of streptozotocin (STZ) intraperitonially. Animals with glycemia above 250 mg/dL were used in the experiments, as well as normoglycemic mice. A polyether-polyurethane sponge was surgically implanted on back of mice. Animals were divided in groups, diabetic or normoglycemic, treated with oral silymarin or water for 10 days, and latter anaesthetized for collecting the sponges, blood and organs. Myeloperoxidase, N-acetylglucosaminidase, collagen and hemoglobin were measured in sponges, and oxidative stress and inflammatory parameters were evaluated in organs. Liver mitochondrial enzymatic activity, histological analysis of sponges and organs, and plasmatic biochemistry were also performed.

Results: The diabetes leads to impairment of antioxidant defenses demonstrate by reduction of pancreatic superoxide dismutase, hepatic and renal catalase and augment of pancreatic lipoperoxidation. Inflammatory reaction in diabetic mice was observed by the augment of pancreatic tumor necrosis factor (TNF)-α and infiltration of inflammatory cells in islets. The number of vessels was reduced in implants of diabetic mice. Treatment with silymarin was capable to attenuate this damage, restoring antioxidant enzymes and reducing pancreatic TNF-α and inflammatory infiltration. However, the treatment did not restore angiogenesis.

Conclusion: The STZ-induced diabetes produces alterations in several tissues. Treatment with silymarin reduced oxidative stress and inflammation in this model. Silymarin seems to be a promising drug for controlling diabetes complications.

Key words: Diabetes, silymarin, streptozotocin, angiogenesis, oxidative stress, inflammation.

1. INTRODUCTION

Diabetes is a metabolic disturbance disease, characterized by chronic hyperglycemia (1, 2), associated with high risk of death and reduced life expectancy (3). The number of adults with diabetes more than doubled in the last three decades (4). Its complications are triggered by several disturbances in homeostasis, including vascular complications, which are associated with impaired angiogenesis (5) and poor wound healing (6), leading sometimes to limb amputations (7).

Angiogenesis is the process responsible for vessels sprouts formation and stabilization. Its regulation is complex and involves several growth factors. Alterations in these process leads to malignant, ischemic and inflammatory disorders. Augmented angiogenesis is related with several diseases, including cancer, asthma and obesity. In other hand, reduced angiogenesis also triggers to illness, like osteoporosis, Alzheimer disease and hair loss (8).

In diabetes, complications can be developed by reductions or by augment in angiogenesis. Diabetic complications related with disturbances in angiogenesis include retinopathy, nephropathy, neuropathy, malignancy, cardiovascular diseases and impaired wound healing. In diabetic retinopathy, abnormal blood vessels were formed in retina and optic disc. In diabetic nephropathy occurs glomerular hypertrophy, with increase in length and number of capillaries. In wound healing, the retard is a consequence of a deficit in inflammation and proliferation stages (9).

It has been shown that chronic hyperglycemia leads to reactive oxygen species (ROS) production, as well as the impairment of angiogenesis is also involved with mitochondrial overproduction of ROS (2). More recently, evidences showed that fluctuation of blood glucose leads to microvascular damage by oxidative stress (10). Thus, therapy with antioxidants substances, including silymarin, may attenuate diabetes complications (11).

Silymarin is the extract obtained of *Silybum marianum* fruits*,* known for its hepatoprotective effects (12). Several data suggest a potential application for treating diabetes complications (13-16). Besides, it was reported that silymarin possess antioxidant (17) and healing (18) properties, and that is capable to influence angiogenesis process (19). However, the influence of silymarin in diabetic conditions, characterized by angiogenesis deficit and oxidative stress, was not yet well established. In this context, the aim of this study was to evaluate the effects of

silymarin in angiogenesis, oxidative stress and inflammation in diabetic and normoglycemic mice.

2. MATERIAL AND METHODS

2.1. Animals. All the experimental protocols were approved by the Ethical Committee for Animal Use (CEUA) of Biological Science Sector of Federal University of Paraná (certificate number 876). Six weeks old male Swiss mice (*Mus musculus*), housed at 22 ± 2ºC and maintained in a 12 - 12 light:dark cycle, were utilized in the experiments. The animals had access to water and standard laboratory chow *ad libitum* during the treatment.

2.2. Diabetes induction. After 12 hours of starvation, mice received an intraperitoneal injection of streptozotocin (STZ, 80 mg/kg) or vehicle (citrate buffer 10mM, pH 4.5) (19). Glycemia was measured 72 hours and 7 days after induction with a glucometer (Accu-Chek® Active - Roche Diagnostics, Germany) by tail puncture. The animals that received streptozotocin and had blood glucose higher than 250 mg/dL were considered diabetic.

2.3. Sponge surgical implant. Polyether–polyurethane sponges (Vitafoam Ltd., Manchester, UK) were immersed in ethanol 70% overnight and boiled in distilled water before implantation. Mice were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg). The dorsal hair was shaved and the skin wiped with 70% ethanol. An incision of 1 cm was made to implantation of the sponge on subcutaneous tissue. The skin incision was sutured with mononylon 5-0.

2.4. Animal treatments. The animals were separated in 6 different groups, as described in Table 1. Mice were treated with silymarin (Pharma Nostra®, Rio de Janeiro, Brazil) or water, via gavage, once a day, during 10 consecutive days. The doses were obtained by alometric extrapolation (20) from the doses indicated for the treatment of liver diseases in humans. A 10-times higher dose was also tested, as the safety factor. Measurement of glycemia and body weight was performed during the treatment.

Table 1: Experimental mice groups and treatments applied in the experiments.

Group number	n*	Group name	STZ Injection	Oral Gavage
1	11	Normoglycemic/ Water	No	Water
2	11	Normoglycemic/ Silymarin	No	Silymarin alometric dose (10.41 mg/kg)
3	11	Normoglycemic/ Silymarin 10	No	Silymarin 10 times dose (104.1 mg/kg)
4	8	Diabetic/ Water	80 mg/kg	Water
5	10	Diabetic/ Silymarin	80 mg/kg	Silymarin alometric dose (10.41 mg/kg)
6	9	Diabetic/ Silymarin 10	80 mg/kg	Silymarin 10 times dose (104.1 mg/kg)

*The different 'n' amongst the groups comes from the animal death in diabetic groups; STZ = streptozotocin.

2.5. Sample collection. Twenty-four hours after the end of the treatment, the animals were weighed and anesthetized as described previously in section 2.3. The sponges were collected, dissected, weighed and homogenized as described in the item 2.6 or stored in 4% buffered formalin for histological analysis. The blood was draw from abdominal cava vein with heparinized syringes. The plasma was separated by centrifugation and stored at -70ºC for further analysis. The liver, kidney and pancreas were rapidly harvested, weighed, frozen in liquid nitrogen and stored at -80ºC for oxidative stress, mitochondrial and TNFα measurement; or kept at 4% buffered formalin for histological analysis. The organs and the mice masses were used to calculate the organs index (relation between the organ mass and body mass multiplied by 100). After the sample collection, the animals were euthanized by deeper anesthesia. The experimental design is resumed in Figure 1.

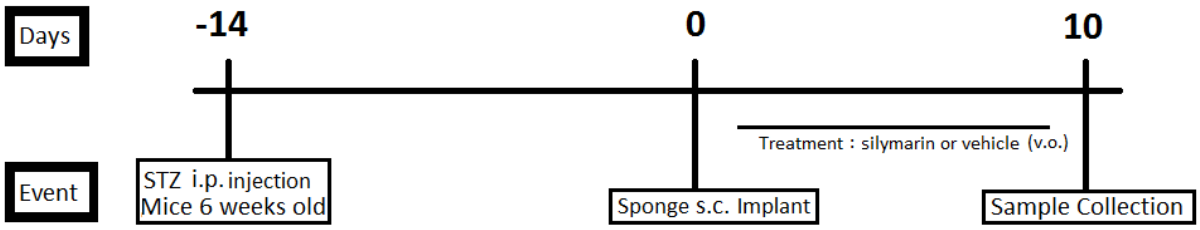


Figure 1. Schematic representation of the experimental design applied to normoglycemic and diabetic mice. Legend: STZ, streptozotocin; v.o., oral via; s.c., subcutaneous; i.p., intraperitoneal.

2.6. Measurements in sponges. For the hemoglobin dosage, implants were homogenized in Drabikin reagent (Labtest®, São Paulo, Brazil), centrifuged and filtered through a milipore filter. The hemoglobin concentration was determined spectrophotometrically (22). For the collagen, nitric oxide (NO), myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) measurements, implants were homogenized in NaCl solution 0.9% containing 0.1 Triton X-100 (Promega®, Madison, USA). The collagen determination was performed by the Sirius red reagent-based assay (23). For the myeloperoxidase the main reagents utilized were tetramethylbenzidine (Sigma-Aldrich®, St Louis, USA) and H_2O_2 (24) and for the NAG determination, p-nitrophenyl-N-acetyl-beta-D-glucosaminide (Sigma-Aldrich®, St Louis, USA) (25), both following established methods (19, 20). To evaluate nitric oxide release, the nitrite levels was measured as an indirect indication of the NO amount in each sample (26). The homogenate was incubated with Griess reagent (0.1% N-1- naphthylediamine, 1% sulfanilamide in 5% H_3PO_4). All reactions and spectrophotometric determinations were performed like described for Castro *et al* (27).

2.7. Hepatic, renal and pancreatic oxidative stress. To evaluate oxidative stress in tissues usually affected in diabetes, we measured the antioxidant enzyme activities, lipid peroxidation and reduced glutathione. The tissue samples were homogenized in phosphate-buffered solution (PBS, pH 6.5) and centrifuged at 10000 *g* at 4ºC, for 20 minutes. The supernatant was used for measuring catalase (Cat) (28), superoxide-dismutase (SOD) (29) and glutathione-S-transferase (GST) activities (30). The tissues were homogenized with methanol to measure the lipid peroxidation (LPO) rate (31) and with trichloroacetic acid to measure reduced glutathione (GSH) level

(32). The results were expressed in relation of the protein amount in each homogenate. The protein concentration was determined by the Bradford method (33).

2.8. Hepatic, renal, pancreatic and sponges histology. The tissue sections were fixed in 4% buffered formalin at room temperature. After fixed, the samples were dehydrated in a graded series of ethanol before paraffin embedding. Thereafter, sections of 5 μm were processed for histology and the tissues were stained with hematoxylin and eosin (HE) or picrosirius red (only for sponges collagen qualification). Later on, the slides were analyzed by optic microscopy, and polarized lens are used for collagen differentiation.

2.9. Morphometric analysis of vessels and collagen in sponges. Images of sponge slides were captured with an Olympus DP72 camera attached to an Olympus BX51 microscope. The pictures were transferred to image analyzer software (Image J - National Institutes of Health, USA). For vessels quantifications, images of 15 random fields per group stained with HE were captured (x200 magnification, 713.79 x 533.79 μm per field). The vessels of each field were summed. The vascular area was considered by the sum of vessels area divided of the number of vessels in each field. For the collagen analyses, images of sponges sections stained with picrosirius red were analyzed with polarized microscopy, as described for Junqueira *et al* (1979) (34). Images of 5 fields were captured (x40 magnification, 3566.44 x 2671.81 μm per field). The values were expressed as mean ± SEM.

2.10. Plasma biochemistry. Plasmatic concentrations of creatinine, urea, aspartate aminotransferase (AST) and amylase were analyzed by an automated system, following the manufacturer's instructions (Kovalent, Reagelabor ™, São Paulo-SP, Brazil).

2.11. Mitochondrial enzymatic activity in liver. The mitochondrial fraction was obtained from frozen livers by differential centrifugation as described by Voss *et al* (35), and then the protein concentrations in mitochondria homogenate were

determined (36). Disrupted mitochondria obtained by a freeze-thawing treatment were used to determine polarographically the activity of reduced nicotinamide adenine dinucleotide (NADH) oxidase and succinate oxidase, according to Singer (37). The oxygen uptake in mitochondrial fraction was monitorated by high resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). The measurements were made in two chamber (2 mL each one) at 37°C under agitation. The results are expressed as oxygen flow per mg protein [pmols/(s*mg protein)] as media ± SEM. For the treated mice, these experiments were performed only in those who received the highest dose of silymarin.

2.12. Pancreatic and hepatic TNFα measurement. The tumor necrosis factor (TNF)-α concentration in homogenate tissues was measured by immunoassay kits (R&D Systems, USA), according to the manufacturer protocol.

2.13. Statistical analysis. The results were expressed as mean ± SEM and were statistically analyzed by one-way ANOVA and Newman-Keuls as the post-hoc test Comparisons between only two groups were performed by paired T test. The glycemic curve was analyzed by two-way ANOVA and Bonferroni as post-hoc test, with ‘treatment’ and ‘time’ as the variables. Significance level adopted was $p<0.05$ in all analyses. The GraphPad Prism™ 5.0 program was used for both statistical analysis and figures design.

3. RESULTS

3.1. Glycemia and body mass

All diabetic mice, independent of the treatment, presented higher levels of blood glucose during the experiment course when compared with animals who did not receive streptozotocin (Figure 2A). The measurements were performed in fed status. Treatment with higher dose of silymarin was capable to prevent marked elevation of glycemia, reducing its variation (-19.71%) in comparison to diabetic mice treated with water (Figure 2B). However, even in this treated group the glycemia was kept elevated along the 10 days of treatment. Both variables, treatment and diabetes, did not influence the body mass of mice (data not shown).

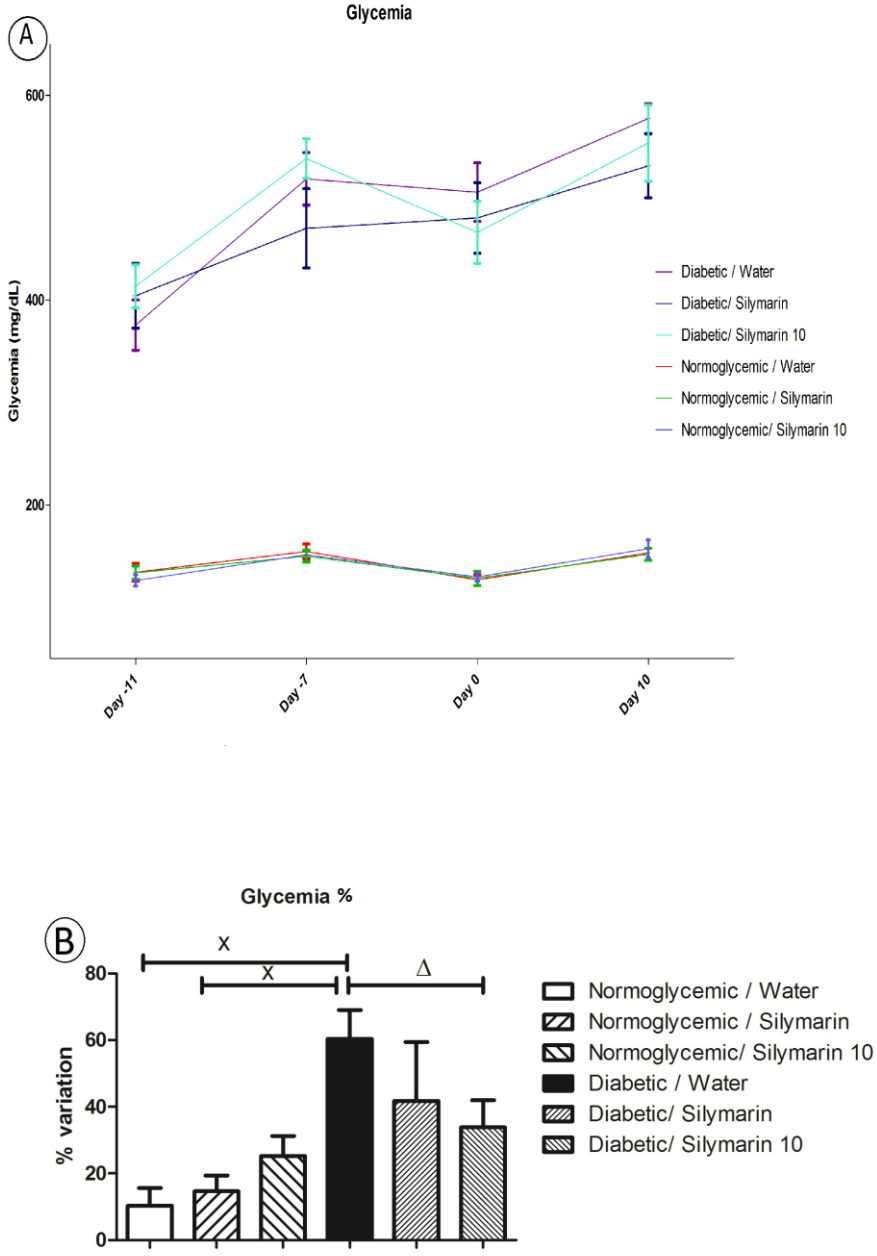


Figure 2. Glycemia of STZ-induced diabetic mice, treated with silymarin or vehicle during 10 days. **(A)** Glycemic curve. **(B)** Percentage of variation before (day -11) and after the treatment (day 10). Data are presented as mean ± SEM and compared by (A) two-way ANOVA and Bonferroni; or (B) one way ANOVA and Newman-Keuls, and paired T test for comparing diabetic/water and diabetic/silymarin 10 groups. Symbols: ^{x} comparison between normoglycemic and diabetic groups; ^{xxx} $p<0.001$; $^{\Delta}$ comparison with T test.

3.2. Sponge analysis

No differences among groups were observed in sponge dosages of hemoglobin, collagen, nitric oxide, myeloperoxidase and N-acetylglucosaminidase (Figure 3).

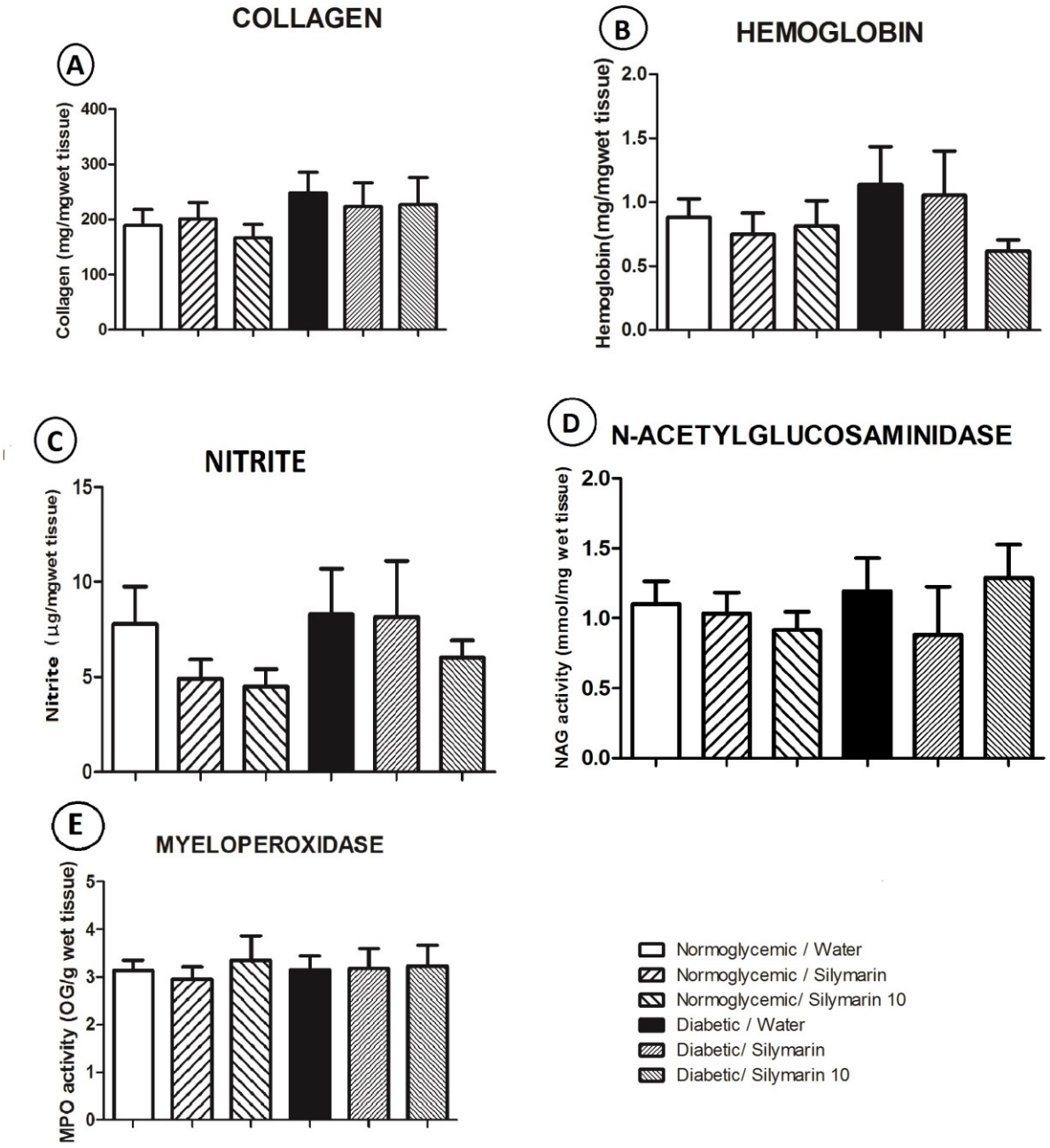


Figure 3. Dosages in sponge implants removed from normoglycemic or diabetic mice after 10 days of implantation and treatment with silymarin or vehicle. (A) collagen, (B) hemoglobin, (C) nitrite, (D) N-acetylglucosaminidase and (E) myeloperoxidase. Data are presented as mean ± SEM and compared by one-way ANOVA and Newman-Keuls.

3.3. Morphometric analysis of vessels

Diabetes leads to reduction of number of vessels in implanted sponges when compared with normoglycemic/water group. However, no differences were observed in vascular area measured in slices of sponges stained with HE (Figure 4). Silymarin treatment did not change both vascular parameters.

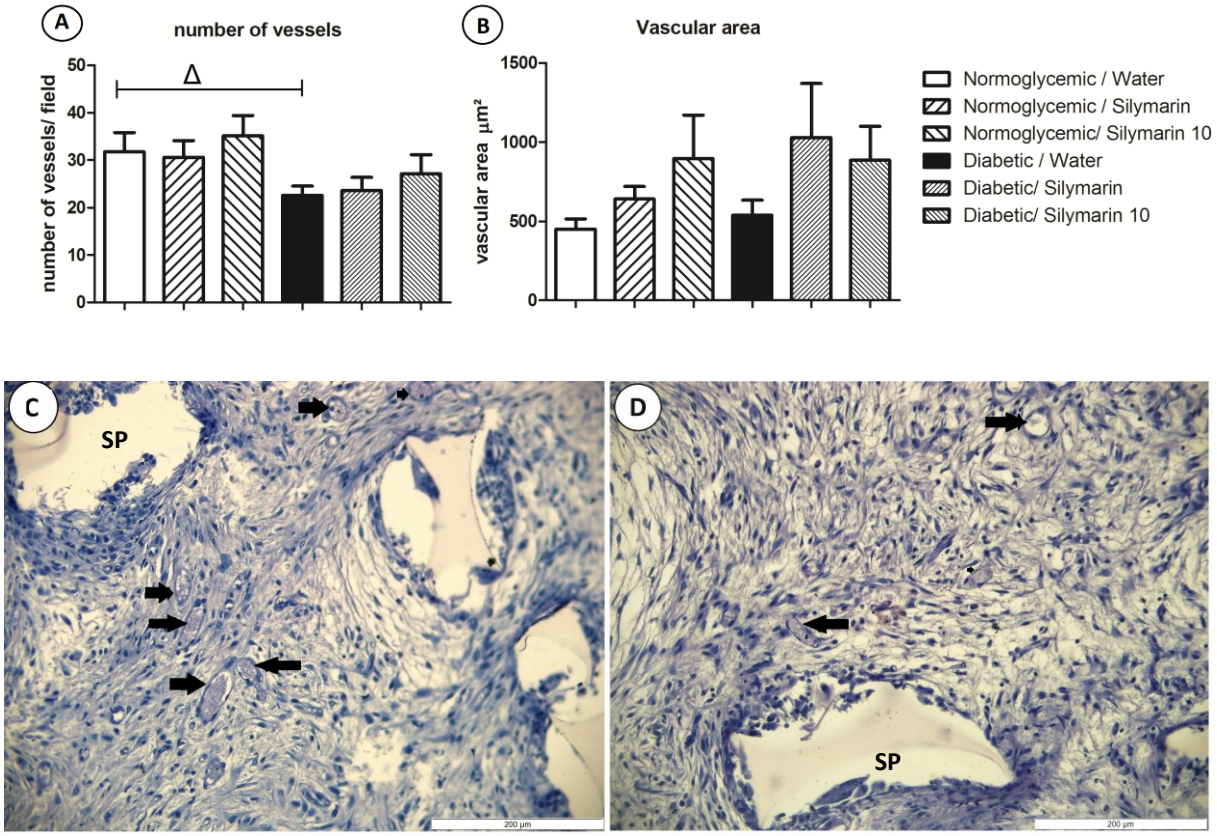


Figure 4. Morphometric analysis of vessels in sponges stained with HE after 10 days of implantation and treatment with silymarin or vehicle. **(A)** Number of vessels. **(B)** Vascular area (sum of vessels area/ number of vessels in each field). Representative images of sponges in HE staining **(C)** normoglycemic/ water **(D)** diabetic/ water (x200 magnification). Data are presented as mean ± SEM and analyzed by one way ANOVA and Newman-Keuls for comparing all groups, or paired T test for comparing normoglycemic/water group and diabetic/water group. $p<0.05$. Legend: $^{\Delta}$ comparison with T test; Black arrows indicate blood vessels; SP: sponge pore.

3.4. Collagen analysis with polarized microscopy

No differences among groups were observed in collagen types measurement, referring to type I, type III and total collagen (Figure 5).

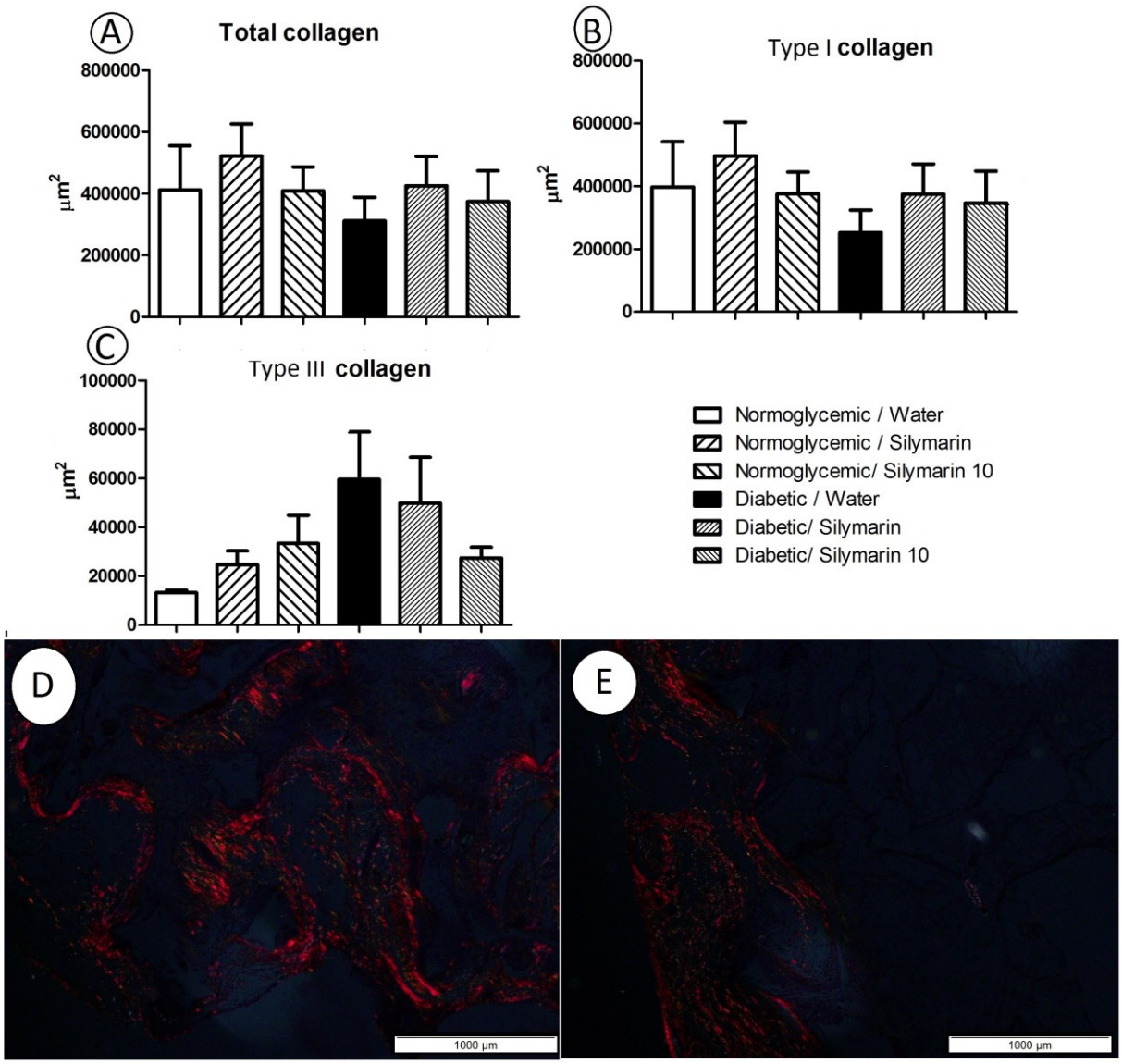


Figure 5. Morphometric analysis of vessels in sponges stained with picrosirius red after 10 days of implant and treatment with silymarin or vehicle. Images were captured with polarized microscopy. **(A)** total collagen, **(B)** type I collagen, **(C)** type III collagen, and **(D)** and **(E)** are representative images of normoglycemic/ water and diabetic/ water groups (x40 magnification), respectively.

3.5. Pancreatic parameters

Diabetes leads to reduction in activity of pancreatic SOD and augment of LPO levels. In LPO assay, differences were observed between the normoglycemic/water and diabetic silymarin groups as well. Diabetes also raised the levels of TNF-α in pancreas and leads to infiltration of inflammatory cells in pancreatic islets and reduction of size and number of islets, observed in optic microscopy with HE. Decrease of serum amylase also was observed in diabetic mice. All these results are presented in Figure 6.

Treatment with both doses of silymarin was capable to restore the SOD activity and abrogated the inflammatory infiltrate observed in optical microscopy. The alometric dose of silymarin was also capable to reverse the increase of TNF-α (Figure 6). No differences among groups were observed in GST and GSH assays (data not shown).

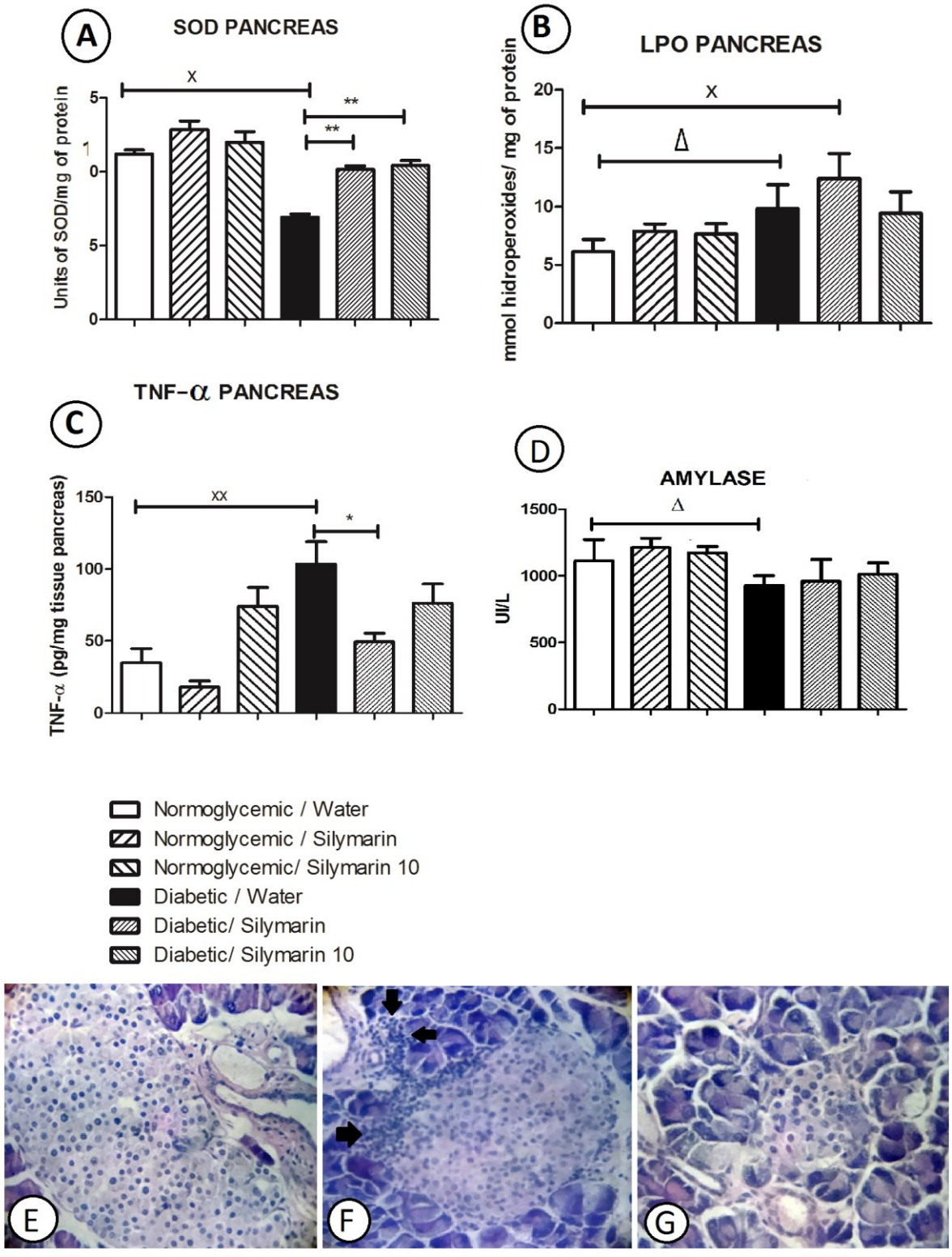


Figure 6. Parameters of oxidative stress, inflammation, pancreatic function and histologycal analysis from pancreas collected from normoglycemic and diabetic mice after 10 days of treatment with silymarin or vehicle. **(A)** pancreatic SOD activity, **(B)** LPO and **(C)** TNF-α levels, **(D)** plasmatic amylase, **(E)**, **(F)** and **(G)** are representative slides of pancreas in HE stain (x400 magnification). **(E)**, **(F)** and **(G)** indicate

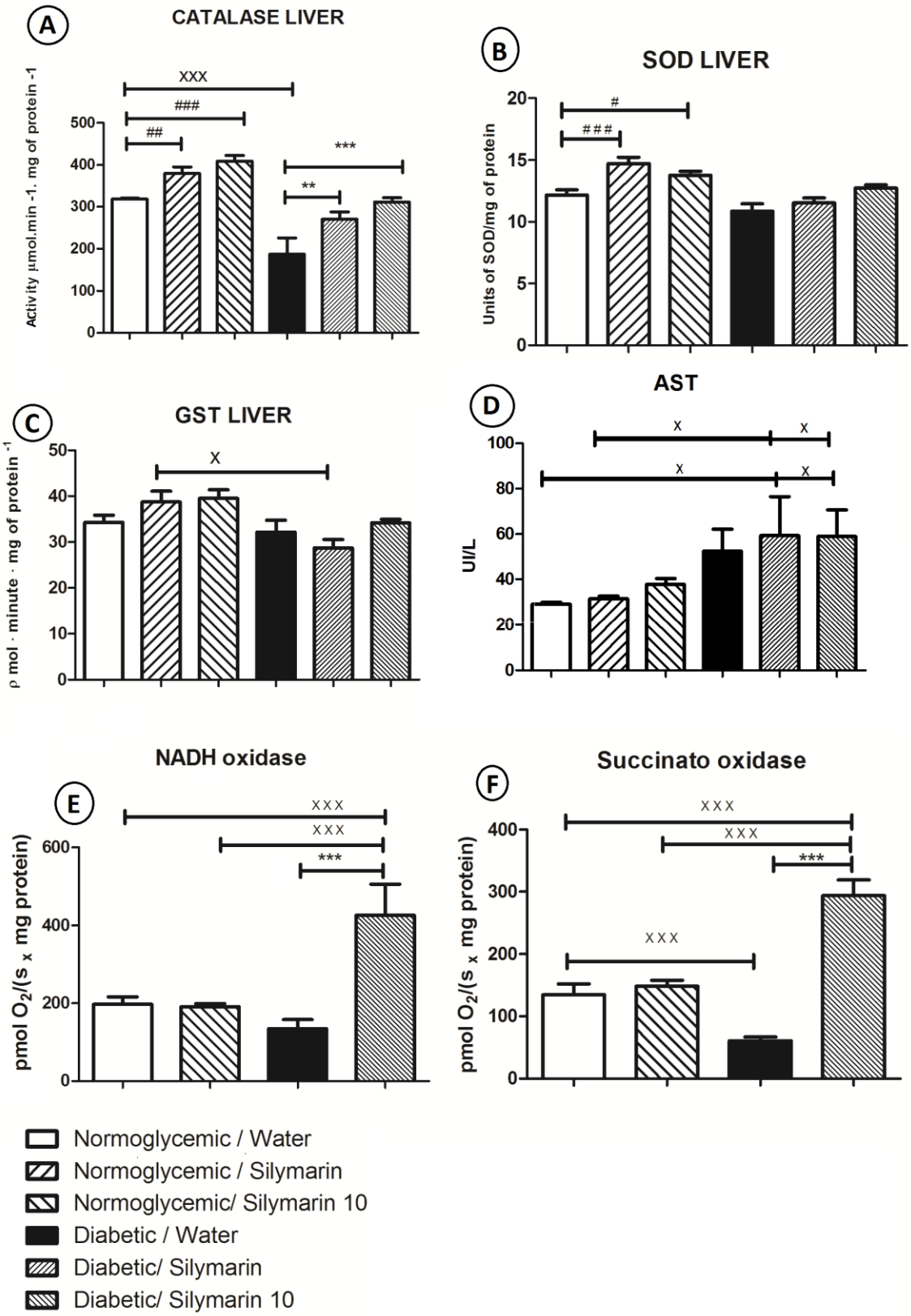
normoglycemic/water, diabetic/water and diabetic/ silymarin 10 groups, respectively. Arrows indicate inflammatory infiltration. Data are presented as mean ± SEM and compared by one way ANOVA and Newman-Keuls and paired T test for comparing normoglycemic/water group and diabetic/water group. Symbols: $^{\Delta}$ comparison with T test; ^{x} comparison between normoglycemic and diabetic groups; * comparison among diabetic groups; # comparison among normoglycemic groups; * and ^{x} $p<0.05$; ** and ^{xx} $p<0.01$.

3.6. Hepatic parameters

The diabetes caused depletion in hepatic catalase activity. Differences in GST activity were observed only between normoglycemic/silymarin group and diabetic/silymarin group. The plasmatic AST levels were higher in diabetic treated groups.

The treatment with silymarin improved the catalase activity in diabetic and normoglycemic groups, while the hepatic SOD activity was improved only in normoglycemic treated groups (Figure 7A,B). No differences among groups were observed in GSH, LPO nor TNF–α assays (data not shown).

The activity of hepatic mitochondrial enzymes was measured and an expressive increase in oxygen flux through NADH oxidase and succinate oxidase was observed in diabetic/silymarin 10 group when compared to diabetic/water group. Curiously, the oxygen flux in this group was ≅ 2-fold higher also when compared with normoglycemic groups (Figure 7E,F).


A
CATALASE LIVER
Activity µmol.min -1. mg of protein -1
xxx
###
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B
SOD LIVER
Units of SOD/mg of protein
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NADH oxidase
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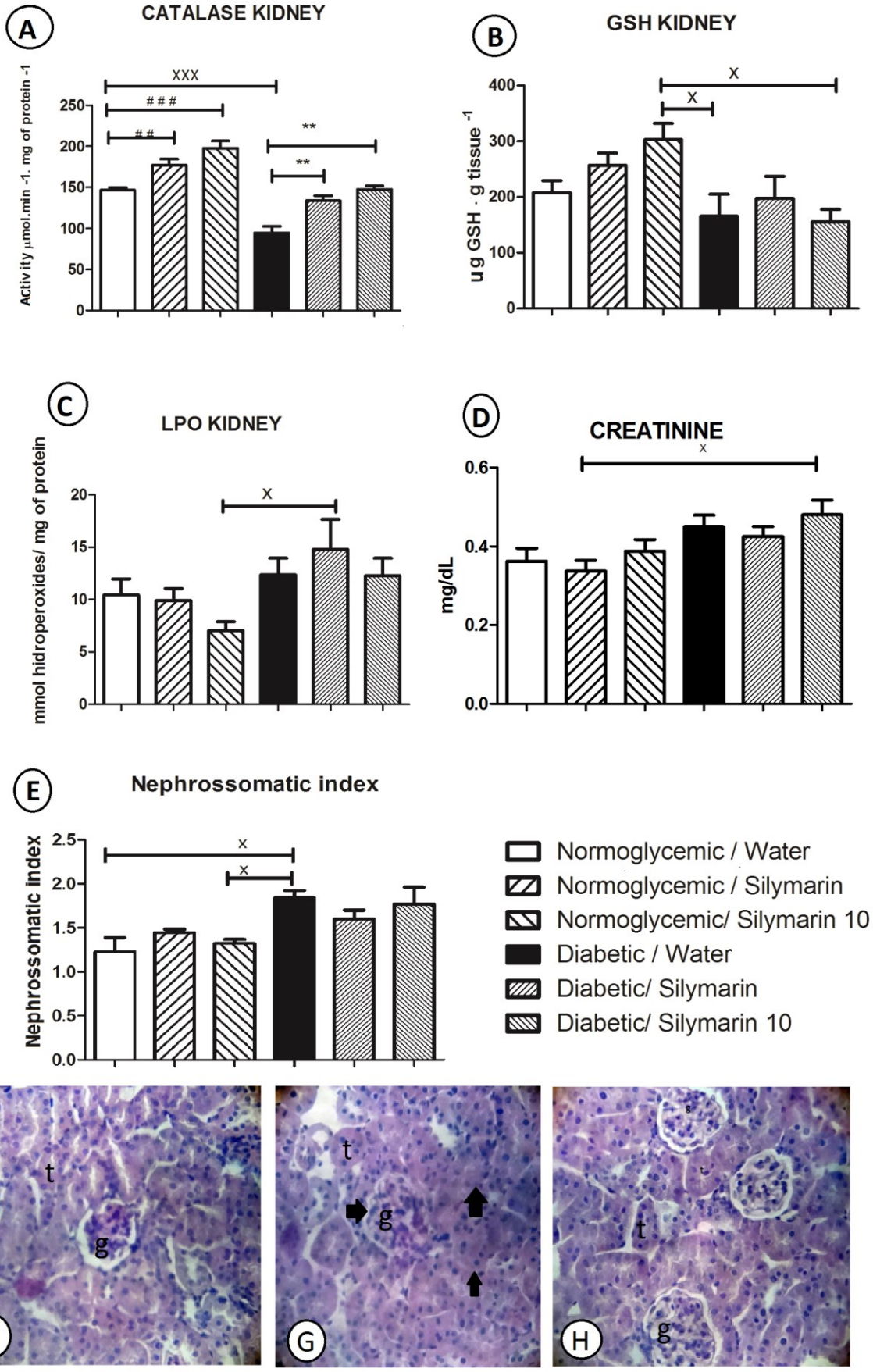
Normoglycemic / Water
Normoglycemic / Silymarin
Normoglycemic/ Silymarin 10
Diabetic / Water
Diabetic/ Silymarin
Diabetic/ Silymarin 10

Figure 7. Oxidative stress, mitochondrial and plasmatic parameters that indicate liver function of normoglycemic and diabetic mice, after 10 days of treatment with silymarin or vehicle. **(A)** Cat, **(B)** SOD, **(C)** GST, **(D)** AST, **(E)** NADH oxidase and **(F)** Succinato oxidase. Data are presented as mean ± SEM and compared by one way ANOVA and Newman-Keuls. Symbols: ^{x} comparison between normoglycemic and diabetic groups; * comparison among diabetic groups; $^{\#}$ comparison among normoglicemic groups; ^{x} and $^{\#}$ $p<0.05$; ** and $^{\#\#}$ $p<0.01$; ***, ^{xxx,} and $^{\#\#\#}$ $p<0.001$.

3.7. Renal parameters

The diabetes reduced the catalase activity in kidneys. The nephrossomatic index was higher in diabetic mice, as well as the cell tumefaction, observed in HE kidney histology of those mice (Figure 8A,E,G). Treatment with silymarin restored and improved the activity of catalase in diabetic and normoglycemic groups, respectively. The GSH level was improved by the higher dose of silymarin in normoglycemic animals, but not in the diabetic ones (Figure 8B). Treatment with silymarin also reduced the cell tumefaction evidenced in renal histology of diabetic mice (Figure 8H). No differences amongst groups were observed in renal SOD and GST, and plasmatic urea assays (data not shown).


A
CATALASE KIDNEY
Activity µmol.min -1. mg of protein -1
250
200
150
100
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XXX
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GSH KIDNEY
ug GSH . g tissue -1
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mmol hidroperoxides/ mg of protein
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Figure 8. Parameters of oxidative stress, plasmatic biochemistry nephrossomatic index and histologycal analysis in kidneys collected from normoglycemic and diabetic mice after 10 days of treatment with silymarin or vehicle. **(A)** Cat, **(B)** GSH, **(C)** LPO, **(D)** creatinine, **(E)** nephrossomatic index, **(F)**, **(G) and (H)** representative slides of kidneys in HE stain (x400 magnification). **(F)**, **(G)** and **(H)** indicate normoglycemic/water, diabetic/water and diabetic/ silymarin 10 groups, respectively. Arrows indicate reduction in Bowman's space and spaces among tubules; (t) and (g) indicate tubules and glomerulus, respectively. Data are presented as mean ± SEM and compared by one way ANOVA and Newman-Keuls. Symbols: ^{x} comparison between normoglycemic and diabetic groups; * comparison among diabetic groups; # comparison among normoglicemic groups; ^{x} $p<0.05$; ** and $^{\#\#}$ $p<0.01$; ^{XXX,} and $^{\#\#\#}$ $p<0.001$.

4. DISCUSSION

In this work we evaluate for the first time the response of silymarin treatment in normoglicemic and diabetic mice utilizing a model of inflammatory angiogenesis. Our data showed that STZ-induced diabetes leads to hyperglycemia, inflammatory reaction and impairment of oxidative defenses in several organs, as well as impaired angiogenesis. The treatment with silymarin was capable to attenuate some of these damages. For inducing diabetes in mice, we utilized the model described for Rakieten *et al* in 1963 (38). A single dose of 80 mg/kg STZ was enough to produce hyperglycemia after 72 hours in more than 85% of mice who receive STZ, reaching the average of 550.5 ± 90.71 mg/dL. Some authors used higher (39) or repeated doses of STZ to induce diabetes on mice. However, in a previous experience of our group, 150 mg/kg of streptozotocin in mice with the same age (6 weeks old) led to extremely high mortality index (unpublished data).

To assess the influence of diabetes in the angiogenesis, vascularization and healing response we used the model of subcutaneous implanted sponges. In the implants, the hemoglobin, vascular area and number of vessels were measured. Inflammatory parameters and collagen were also evaluated, because the synthetic matrix implant leads to a non-specific inflammation in the host, analog to healing (40-42). The picrosirius red staining associated with polarized microscopy allowed to observe the types of collagen present in tissues (34), thus we could evaluate the stage of healing. In the early stages of healing, type III collagen fibers are formed.

These fibers are thin and incompact. Gradually, activity and number of fibroblasts increased, and these fibers are replaced by fibers of type I collagen, thick and dense (Witte and Barbul, 1997). In the present study, we did not find differences in collagen types among groups (Figure 5). Diabetes reduced the number of vessels in sponges (Figure 4A). This result corroborate with Thomson *et al* (2010) (43) and Oviedo-Socarrás *et al* (2014) (44). The first one evaluated the angiogenesis in implants placed subcutaneously in baboons after diabetes induction, while the second evaluated angiogenesis in diabetic rats, in implants placed in peritoneal cavity. We did not observe differences in other parameters analyzed in sponges (Figure 3), and the treatment with silymarin also did not improve the angiogenesis (Figure 4) or collagen deposition (Figure 5). Maybe a longer time of treatment was necessary to observe differences in vessels of mice. The use of young mice is necessary to the application of the sponge model, since angiogenesis is largely influenced by age (45, 46). However this necessity created a limitation in the present study, because the maximum time we could treat the mice in order to keep the ideal age for the sponge experiment was 10 days.

The STZ-induced diabetes leads to alterations in several parameters and organs. The diabetic model was characterized by relevant impairment of oxidative defenses, evidenced by depletion of Cat activity in liver (Figure 7A) and kidney (Figure 8A), GST activity in liver (Figure 7C), SOD activity in pancreas (Figure 6A), and GSH level in kidney (Figure 8B). Additionally, an elevation of LPO levels in pancreas (Figure 6B), was observed. Our data corroborate the alterations in oxidative stress of diabetic animals reported previously. Sharma *et al* (2015) reported an increase of LPO and a reduction in GSH, SOD, Cat, gluthatione-peroxydase (GPx), and GST in pancreas of diabetic rats (47). In accordance, Ahmed *et al* (2015) reported depletion in hepatic Cat, SOD, GSH, and GPx in diabetic rats (48). In kidney, Zhang *et al* (2015) reported decreased activity of SOD, Cat and GPx, and augment of malondialdehyde (MDA)(49). In the present study, the treatment with silymarin restored the oxidative defenses in diabetic mice in all organs analyzed. The improvement was evidenced by the restoration of SOD activity in pancreas and Cat activities in liver and kidney. These findings corroborates with Bouderba *et al* (2014) (50). In the present study, silymarin also improved the hepatic Cat activity of normoglycemic mice, as previously reported (17). Our data confirm the antioxidant effects of silymarin, already known in the literature (50,51).

Since mitochondria are closely related with oxidative stress by producing significant amount of ROS, we measured the oxygen mitochondria flux in liver (Figure 7E,F). The succinate oxidase measurement showed a reduction in the oxygen flux in diabetic/ water group. Satav and Katyare (2004) (52), reported that the activity of succinate oxidase in diabetic rats is variable, according to the time after the diabetic induction. One week after the STZ administration in rats, the oxygen flux in liver mitochondria through succinate oxidase is higher when comparing with control rats; however the result is reverse after one month of induction. In the present study, in diabetic/silymarin 10 group occurred an augment in oxygen flux through NADH oxidase and succinate oxidase when compared to diabetic/water group. Colturato *et al* (2011) observed an augment of oxygen flux in presence of succinate in consequence of silibinin infusion, in a concentration-dependent manner (53). Curiously, the oxygen flux in diabetic/silymarin 10 group was higher than the normoglycemic groups. Since the enzyme AST is present in mitochondria of hepatocytes, its elevation in plasma of diabetic/silymarin mice (Figure 7D), may be a consequence of damages in these organelle in liver.

On pancreas, the diabetes induction leads to augment of TNF-α (Figure 6C), corroborating the data of Fathy *et al* (54). This find is compatible with the infiltration of inflammatory cells in islets, observed in histology, while the size and number of islets are reduced in all STZ-induced diabetic groups. The plasmatic amylase, produced by pancreas and responsible for hydrolyzing the dietary starch, was reduced in diabetic mice (Figure 6D), corroborating data already reported (55). The treatment with silymarin in alometric dose reversed the augment of concentrations of TNF-α (Figure 6C). Similarly, the treatment was capable to reduce inflammatory cells infiltration in pancreatic islets, but in this case, the higher dose was more effective (Figure 6D,F,G). The inflammatory cells infiltration in pancreatic islets is one of the responsible factors for the β-cells destruction in type I diabetes (56). Thus, the silymarin treatment showed benefits upon pancreatic tissue of diabetic mice.

The STZ-induced diabetes also leads to kidney alterations. We observed an augment of the nephrossomatic index (Figure 8E), which may be caused by the cell tumefaction, evidenced by reduction in Bowman's space and in space among tubules, observed in kidney histology (Figure 8F,G,H). Silymarin treatment was capable to attenuate this tumefaction. Despite these findings, plasmatic urea levels were not significantly increased. These results can be related with the short evolution

time of the disease in our model, because diabetic nephropathy is a chronic, and not an acute complication of diabetes. It is characterized by glomerular hypertrophy, proteinuria, decreased glomerular filtration, and renal fibrosis resulting in the loss of renal function (57).

To evaluate the effects of silymarin treatment in diabetes, we administrated a dose of 10.41 mg/kg. This was calculated based on the hepatoprotective dose for humans (210 mg), by alometric extrapolation according to Pachaly *et al* (20). We also used a 10-times higher dose (104.1 mg/kg), as a safety factor, in order to evaluate possible toxic effects. In several studies with silymarin, similar doses are used to treat diabetes complications (100-120 mg/kg) (58-60). The glycemia reduction in rats with resembling dose of silymarin (120 mg/kg) was already reported (60). In the present study, higher dose of silymarin prevented the augment of glycemia (Figure 2B), but did not turn mice to normoglycemic condition (2A). In some parameters analyzed in this work, it was not find an evident dose-response for silymarin. However, it should be mention that apparently the higher dose was more effective in diabetic mice, without induces toxicity.

5. CONCLUSIONS

Diabetes leads to inflammatory reaction and impairment of oxidative defenses and angiogenesis. Treatment with silymarin was capable to attenuate, at least in part, these damages. Besides, silymarin is considered a safety drug, with a low incidence of side effects. In view of the side effects presented by drugs commonly utilized as adjuvant in diabetes treatment, silymarin can be a candidate drug for treating diabetes complications. More studies covering the effects of silymarin in diabetes, in combination with other therapies and for longer periods, and clinical trials should be stimulated.

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5. Considerações Finais

Frente aos dados apresentados e aos resultados obtidos neste trabalho, conclui-se que:

- Neste trabalho, nós avaliamos a ação da silimarina em camundongos normoglicêmicos e diabéticos. Pela primeira vez foi utilizado um modelo de angiogênese inflamatória para esta finalidade.

- O diabetes é uma doença grave caracterizada por várias complicações, como vasculopatias, nefropatias, hepatopatias e neuropatias. O estresse oxidativo faz parte da patogênese de várias destas complicações, nas quais a silimarina mostrou-se efetiva terapeuticamente.

- O diabetes induzido por STZ prejudicou as defesas antioxidantes em vários órgãos, alterou a angiogênese, a respiração mitocondrial hepática e induziu resposta inflamatória no tecido pancreático.

- O tratamento com silimarina na dose alométrica e na dose 10 vezes maior foi capaz de restabelecer algumas defesas antioxidantes e atenuar a resposta inflamatória.

- A silimarina não alterou diretamente a formação de vasos nas esponjas, o que pode ter ocorrido devido ao curto tempo de tratamento, determinado neste estudo pela necessidade de utilizar animais jovens, com 6 a 7 semanas de vida, que são mais indicados para estudos relacionados com a angiogênese.

- A silimarina apresentou resultados promissores em complicações relacionadas ao diabetes. Assim, novos estudos abordando outros protocolos de tratamento e diferentes modelos de angiogênese devem ser continuados.

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