EDSON DA SILVA

EFEITOS DO DIABETES EXPERIMENTAL SOBRE A MORFOLOGIA E FUNÇÃO DO VENTRICULO ESQUERDO DE RATOS WISTAR PÚBERES: IMPACTO DO TREINAMENTO DE NATAÇÃO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 08 de novembro de 2013.

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Luciana Duarte Novais Silva Ita de Oliveira Silva

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Sirlene Souza Rodrigues Sartori Silvia Almeida Cardoso

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Izabel Regina dos Santos Costa Maldonado
(Orientadora)

O saber a gente aprende com os mestres e os livros.
A sabedoria se aprende é com a vida e com os humildes.

Cora Coralina

Dedico à minha esposa Marileila,
e aos que me dedicaram suas vidas:
meus pais, Luíza e Gedi (presente na memória),
e aos meus irmãos Noêmia, Luís e Eduardo.

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SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

AMPK – Proteína quinase ativada por AMP

ANOVA – Análise de variância

ATP – Adenosina trifosfato

ATPase – Enzima que catalisa a hidrólise do ATP

BG – Blood glucose

bmp – Beats per minute

BW – Body weight

Ca^{2+} – Íon cálcio

$CaCl_2$ – Calcium chloride

CaMKII – Calcium/calmodulin-dependent protein kinase II

CE – Controle exercitado

CEUA – Ethics Committee on Animal Experimentation

CRP – C-reactive protein

CS – Controle sedentário

DCM – Diabetic cardiomyophaty

DE – Diabético exercitado

dL – Decilitro

DM – Diabetes mellitus

DS – Diabético sedentário

EC – Exercise control

ECG – Electrocardiogram

ECM – Extracellular matrix

ED – Exercise diabetic

EGTA – Ethylene glycol-bis (ß-aminoethyl ether)-N, N, N', N'-tetraacetic acid

ELISA – Enzyme linked immunosorbent assay

eNOs – Endothelial nitric oxide synthase

g – Gramas

GLUT 1 – Transportador de glicose 1

GLUT 4 – Transportador de glicose 4

Hepes – N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

HF – Heart failure

HMW – High molecular weight

HR – Heart rate

Hz – Hertz

ICAM-1 – Intercellular adhesion molecule-1

IL – Interleukin

IUR – Uniform random section

KCl – Cloreto de potássio

Kg – Quilograma

L – Litro

LMW – Low molecular weight

LV – Left ventricle

M – Molar

mg – Miligrama

$MgCl_2$ – Cloreto de magnésio

min – Minuto

mL – Mililitro

mM – Milimolar

MMW – Middle molecular weight

ms – Milissegundo

NaCl – Cloreto de sódio

NCX – Na/Ca Exchange

ng – Nanogramas

NO – Nitric oxide

ºC – Grau Celsius

PAS – Periodic acid-Schiff

pH – Potencial hidrogeniônico

PLB – Phospholamban

PN– Programa de natação

PPARγ – Receptor ativado por proliferadores de peroxissoma gama

RyR2 – Canais receptores de rianodina

S1P – Esfingosina-1 fosfato

SC – Sendentary control

SD – Sedentary diabetic

SEM – Standard error of the mean

SERCA2 – Sarcoplasmic reticulum Ca^{2+} ATPase

SphK1 – Esfingosina quinase 1

SR – Sarcoplasmic reticulum

STZ – Estreptozotocina

T1DM – Type 1 diabetes mellitus

T2DM – Type 2 diabetes mellitus

TNF-α – Fator de necrose tumoral alfa

VCAM-1 – Vascular cell adhesion molecule-1

VE – Ventrículo esquerdo

vs – Versus

VW – Weight gain

WG – Ventricular weight

% r.c.l – Percentage of resting cell length

µg – Microgramas

µm – Micrômetro

µM – Micromolar

µL – Microlitro

RESUMO

SILVA, Edson da, D.S., Universidade Federal de Viçosa, Novembro de 2013. **Efeitos do diabetes experimental sobre a morfologia e função do ventrículo esquerdo de ratos Wistar púberes: impacto do treinamento de natação.** Orientadora: Izabel Regina dos Santos Costa Maldonado. Coorientadores: Antônio José Natali e Leandro Licursi de Oliveira.

A cardiomiopatia diabética está associada não só com o remodelamento cardíaco e disfunção miocárdica, mas também com a ocorrência de inflamação de baixo grau e redução dos níveis de adiponectina cardíaca, resultando em significativa morbidade e mortalidade em pacientes com Diabetes mellitus tipo 1 (DM1). Por outro lado, o exercício físico é uma estratégia importante para o tratamento do diabetes, que pode reduzir a inflamação, atenuar o remodelamento cardíaco adverso e a disfunção contrátil. O objetivo deste estudo foi investigar a influência do treinamento de natação de baixa intensidade no remodelamento estrutural, nos níveis de citocinas cardíacas, na inflamação e na disfunção contrátil de cardiomiócitos do ventrículo esquerdo (VE) de ratos púberes com diabetes experimental não tratado. Ratos Wistar machos, com trinta dias de idade, foram divididos em quatro grupos (n = 19 por grupo): controle sedentário (CS), controle exercitado (CE), diabético sedentário (DS) e diabético exercitado (DE). O diabetes foi induzido por estreptozotocina (STZ, 60 mg kg^{-1} de peso corporal). Animais dos grupos CE e DE foram submetidos a um treinamento de natação (5 dias/semana , 90 min/dia, com carga de 5 % do peso corporal) durante 8 semanas. Após a eutanásia, o VE foi removido para análises molecular, morfológica e mecânica de cardiomiócitos. Secções do VE foram coradas com Periodic acid-Schiff (PAS), Sirius Red, reticulina de Gomori, tricrômico de Gomori e azul de toluidina/borato de sódio 1%. O VE de animais diabéticos apresentou aumento de colágeno intersticial e fibras reticulares na matriz extracelular, acúmulo de glicogênio, desorganização da histoarquitetura do miocárdio, infiltrado inflamatório e necrose. A densidade capilar foi significativamente menor nos animais diabéticos. Cardiomiócitos do VE dos animais diabéticos apresentaram o tempo para o pico de contração e o tempo para 50% de relaxamento mais longos do que os animais do grupo controle, mas não houve diferença na amplitude de contração. Os níveis cardíacos de IL-10, óxido nítrico, adiponectina total e adiponectina HMW foram significativamente menores nos

animais diabéticos. O exercício físico atenuou o nível de TNF-α e os parâmetros histopatológicos avaliados, e aumentou a densidade capilar no VE de ratos diabéticos. Em conclusão, o remodelamento estrutural cardíaco induzido pelo DM1 experimental coexiste com níveis reduzidos de adiponectina total e HMW, inflamação crônica e disfunção de contratilidade dos cardiomiócitos. Mais importante ainda, o treinamento de natação de baixa intensidade atenuou parte destas alterações patológicas, indicando o papel benéfico do exercício regular no DM1 não tratado.

ABSTRACT

SILVA, Edson da, D.S., Universidade Federal de Viçosa, November, 2013. **Effects of experimental diabetes on the morphology and function of the left ventricle of pubescent rats: impact of swimming training**. Adviser: Izabel Regina dos Santos Costa Maldonado. Co-advisers: Antônio José Natali e Leandro Licursi de Oliveira.

Diabetic cardiomyopathy is associated not only with cardiac remodeling and myocardial dysfunction but also with the occurrence of low-grade inflammation and reduced cardiac adiponectin resulting in significant morbidity and mortality in patients with type 1 diabetes mellitus (T1DM). On the other hand, physical exercise is an important strategy for the management of diabetes which can reduce inflammation and attenuate adverse cardiac remodeling and contractile dysfunction. The aim of this study was to investigate the influence of low-intensity swimming training on the structural remodeling, cardiac cytokines, inflammation, and cardiomyocyte contractile dysfunction of the left ventricle (LV) in pubescent rats with unmanaged experimental diabetes. Thirty-day-old male Wistar rats were divided into four groups (n = 19, per group): sedentary control (SC), exercised control (EC), sedentary diabetic (SD), and exercised diabetic (ED). Diabetes was induced by streptozotocin (STZ, 60 mg kg^{-1} body weight). Animals from EC and ED groups were submitted to a swimming training (5 days/week, 90 min/day, load of 5% body weight) for 8 weeks. After euthanasia LV was removed for molecular, morphological, and cardiomyocyte mechanical analysis. Sections of LV were stained with Periodic acid-Schiff (PAS), Sirius Red, Gomori's reticulin, Gomori's trichrome, and toluidine blue/sodium borate 1%. The LV of diabetic animals presented increased interstitial collagen and reticular fibers on the extracellular matrix, accumulation of glycogen, myocardial histoarchitectural disorganization, inflammatory infiltrate and necrosis. The capillary density was significantly lower in diabetic animals. Left ventricular cardiomyocytes from diabetic animals exhibited more prolonged time to the peak of contraction and the time to half relaxation than those from control animals, but no difference in cell shortening was observed. The cardiac levels of interleukin 10, nitric oxide, total and HMW adiponectin were significantly decreased in diabetic animals. Exercise training attenuated the level of TNF-α and the histopathological parameters assessed, and increased the capillary density in the LV of diabetic rats. In conclusion, the cardiac structural remodeling

induced by experimental T1DM coexists with reduced levels of total and HMW adiponectin, chronic inflammation and cardiomyocyte contractility dysfunction. More important, low-intensity swimming training attenuated part of these pathological changes which indicate the beneficial role for regular exercise in untreated T1DM.

INTRODUÇÃO GERAL

O Diabetes mellitus (DM) é uma desordem metabólica caracterizada pelo aumento da glicemia resultante de defeitos na secreção de insulina, ação da insulina, ou em ambos (AMERICAN DIABETES ASSOCIATION, 2013); com alta prevalência em adultos e adolescentes (MA, CHAN, 2009). O número de crianças, adolescentes e jovens adultos com DM aumenta a um ritmo alarmante (STEHNO-BITTEL, 2012; TRUONG et al., 2012). As doenças cardiovasculares representam a principal complicação do diabetes e podem ter início na infância (NADEAU et al., 2011) e influenciar de forma significativa a morbidade e a mortalidade na fase adulta (RAJESH et al., 2012; GIANNINI et al., 2011; GUO et al., 2007; LIBBY et al., 2005) .

A maioria dos casos de diabetes divide-se em duas categorias etiopatogenéticas: Diabetes Mellitus tipo 1 (DM1) e tipo 2 (DM2) (AMERICAN DIABETES ASSOCIATION, 2013). No DM1 a causa é uma deficiência absoluta da secreção de insulina, enquanto no DM2 a causa é uma combinação da resistência à ação da insulina e uma resposta secretora inadequada de insulina (AMERICAN DIABETES ASSOCIATION, 2013).

O DM1 é uma doença que ocorre principalmente por deficiência de insulina, a partir da destruição das células beta pancreáticas (AMERICAN DIABETES ASSOCIATION, 2013) e as doenças cardiovasculares podem estar associadas à resistência à insulina nesta forma de diabetes (PEREIRA et al., 2012; NADEAU et al., 2010; KILPATRICK et al., 2007; DEFRONZO et al., 1982). Além disto, o DM1 tem sido relacionado à inflamação de baixo grau e às complicações micro e macrovasculares em adultos (LLAURADÓ et al., 2012; GONZALEZ-CLEMENTE et al., 2007) e crianças (MANGGE et al., 2004). A inflamação sistêmica de baixo grau pode ser definida como a elevação, em duas a quatro vezes, das concentrações de citocinas que desencadeiam ações pró e anti-inflamatórias (BRUUNSGAARD, 2005).

As vias que levam à disfunção cardíaca induzida por DM e suas alterações histopatológicas ainda não estão totalmente esclarecidas (NADEAU et al., 2010; SALEM et al., 2009; NEMOTO et al., 2006), em parte, devido à natureza complexa e multifatorial do diabetes (LAW et al., 2012). Possivelmente, o papel mais importante tem sido atribuído à hiperglicemia persistente (LI et al., 2012), condição a

qual aumenta os riscos para o desenvolvimento de insuficiência cardíaca de duas a cinco vezes (BELL, 1995).

Pessoas com diabetes podem desenvolver uma disfunção cardíaca denominada cardiomiopatia diabética (LAW et al., 2012). Esta é caracterizada por dilatação e hipertrofia do miocárdio, acompanhada por disfunção sistólica e/ou diastólica do ventrículo esquerdo (VE) e a sua presença é independente da coexistência de doença cardíaca isquêmica ou hipertensão (HAYAT et al., 2004). Apesar de a cardiomiopatia diabética ser uma condição subclínica crônica (VOULGARI et al., 2010), eventualmente leva à redução da elasticidade da matriz extracelular e diminuição da função contrátil do coração (FALCÃO-PIRES, LEITE-MOREIRA, 2012; RAJESH et al., 2012; ARAGNO et al., 2008; SEARLS et al., 2004). A associação entre o desenvolvimento da cardiomiopatia diabética e os elevados níveis de glicose têm efeitos significativos sobre a expressão, organização e modificação de componentes da matriz extracelular no coração (LAW et al., 2012).

A matriz extracelular cardíaca é uma rede dinâmica bem definida, constituída de proteínas estruturais, proteoglicanos, fatores de crescimento, citocinas e enzimas, essenciais para a organização e estrutura do tecido (LAW et al., 2012). É composta por colágeno (CAULFIELD, BORG, 1979), sendo colágeno fibrilar dos tipos I e III localizados no interstício do miocárdio, e colágeno não fibrilar dos tipos IV e VI e as glicoproteínas fibronectina e laminina, predominantes na membrana basal dos cardiomiócitos (BROWER et al., 2006; BISHOP, LAURENT, 1995; EGHBALI, WEBER, 1990). Elevações no estresse do miocárdio iniciam um remodelamento estrutural do coração, na tentativa de normalizar o estresse imposto. Este processo compreende a hipertrofia de cardiomiócitos com mudanças na quantidade e no fenótipo do colágeno, ocorrência de ligações cruzadas (*cross-linking*) entre moléculas de colágeno e remodelamento progressivo de componentes musculares, vasculares e da matriz extracelular do coração (BROWER et al., 2006). O aumento das concentrações intersticiais de colágeno e/ou de ligações cruzadas resulta em rigidez do miocárdio e disfunção diastólica do ventrículo esquerdo (BROWER et al., 2006).

Adicionalmente, no DM a inflamação de baixo grau pode estar relacionada ao aumento dos níveis sanguíneos de proteínas pró-inflamatórias, tais como fator de necrose tumoral alfa (TNF-α), interleucina-6 (IL-6), proteína C-reactiva (PCR) e à redução dos níveis de proteínas anti-inflamatórias (LLAURADÓ et al., 2012; RYBA

et al., 2011). Um estado inflamatório crônico ocasiona alterações histopatológicas com lesão tecidual, disfunção endotelial e remodelamento cardíaco, os quais resultam em deterioração da contratilidade miocárdica e da função ventricular esquerda (LLAURADÓ ET AL., 2012; DUNCAN ET AL., 2007). A redução do transiente de Ca^{2+} (DUNCAN et al., 2007) e a redução da sensibilidade dos miofilamentos contrácteis ao Ca^{2+} são mecanismos possivelmente envolvidos neste processo (GOLDHABER et al., 1996).

Estudos confirmam que a adiponectina desempenha um papel importante no metabolismo de glicose e de lipídios (PEREIRA et al., 2012; GAREKANI et a.l, 2011; NUMAO et al., 2008), além de seu impacto relevante na patogênese do diabetes, da resistência à insulina e da lesão vascular (FENGER, 2013; GU et al., 2012). A adiponectina é uma proteína plasmática, secretada principalmente pelos adipócitos (BOBBERT et al., 2011; SUN, CHEN, 2010) e possui propriedades antidiabéticas, anti-inflamatórias, antiapoptóticas, antiaterogênicas (FORSBLOM et al., 2011; BOBBERT et al., 2011), imunomoduladoras e cardioprotetoras (JENKE et al., 2013; LETH et al., 2008). Dentre outras células, os cardiomiócitos são capazes de sintetizar adiponectina (PIÑEIRO et al., 2005; MAIA-FERNANDES et al., 2008), a qual tem função autócrina potencializando seu efeito cardioprotetor (HUI et al., 2012). As vias de sinalização dos efeitos cardioprotetores da adiponectina são mediadas principalmente pelo aumento de proteína quinase ativada por adenosina monofosfato (AMPK), receptor ativado por proliferadores de peroxissoma gama (PPARγ), esfingosina-1 fosfato (S1P) e esfingosina quinase 1 (SphK1) (HUI et al., 2012).

Existem três principais isoformas de adiponectina: adiponectina de baixo peso molecular (*low molecular weight*, LMW), médio peso molecular (*middle molecular weight*, MMW) e alto peso molecular (*high-molecular weight*, HMW) (HICKMAN, WHITEHEAD, 2012). Acredita-se que a adiponectina HMW é a isoforma mais ativa nos tecidos periféricos (GOTO et al., 2013; MAIA-FERNANDES et al., 2008). Há evidências de que a adiponectina protege o coração de lesões isquêmicas, cardiomiopatias e disfunção sistólica (GOLDSTEIN, SCALIA, MA, 2009). Além disto, a adiponectina é capaz de inibir a hipertrofia patológica dos cardiomiócitos e a fibrose do miocárdio (MAIA-FERANDES et al., 2008; HAN et al, 2007), reduzir o stress oxidativo e nitrativo (WANG et al., 2013; GOLDSTEIN, SCALIA, MA, 2009), inibir a expressão de TNF-α e de IL-6, e aumentar a expressão

de IL-10 no coração (LO, MITSNEFES, 2012; GOLDSTEIN, SCALIA, MA, 2009; MAIA-FERANDES et al., 2008; HAN et al., 2007).

Por outro lado, o exercício físico regular é parte importante no manejo do DM1, devido aos efeitos benéficos à saúde, especialmente a prevenção de doenças cardiovasculares (GALASSET, RIDDELL, 2013). Assim, o treinamento físico tem sido recomendado como estratégia não farmacológica útil para reduzir a resistência à insulina, aumentar o metabolismo da glicose e de lipídios (KHAN, 2013), atenuar as alterações morfológicas e a disfunção contrátil em animais e humanos com cardiomiopatia diabética no DM1 (SILVA et al., 2013; CHIMEN et al, 2012; LOGANATHAN et al., 2012; RAJESH et al, 2012; SILVA et al., 2011; BIDASEE et al, 2008; SEARLS et al., 2004; DE ANGELIS et al., 2000). De modo geral, melhorias na função contrátil do miocárdio são observadas em seres humanos e animais, *in vivo*, em corações isolados e preparações multicelulares, submetidos a diferentes modelos de exercício (DI BELLO et al., 1996).

Além disto, a atividade física regular está associada à longevidade e à menor incidência de complicações decorrentes do diabetes (YARDLEY et al., 2012). Enquanto o treinamento físico de alta intensidade pode promover remodelamento cardíaco patológico em ratos (BENITO et al., 2011), o exercício aeróbico de baixa intensidade melhora a aptidão física e a força, reduz fatores de risco cardiovascular, melhora o bem-estar, reduz a necessidade de insulina e melhora a função autonômica cardíaca (CHIMEN et al, 2012; YARDLEY et al., 2012; DE ANGELIS et al., 2000). A literatura comprova que o exercício aeróbico de baixa a moderada intensidade é capaz de induzir adaptações cardiovasculares benéficas (por exemplo, melhora a função cardíaca, a estrutura do miocárdio, reduz a pressão arterial, e aumenta a sensibilidade baro e quimiorreflexa, o ritmo cardíaco intrínseco e o débito cardíaco), em ratos com diabetes induzido por estreptozotocina, um modelo que imita muito o DM1 humano (JORGE et al., 2012; LOGANATHAN et al., 2012; MOSTARDA et al., 2009; LOGANATHAN et al., 2007; DE ANGELIS et al., 2000). No entanto, os mecanismos subjacentes à histopatologia do DM1 e sua relação com o exercício físico, o remodelamento cardíaco e a disfunção cardíaca não são claros. Diante disto, torna-se importante conhecer os ajustes estruturais e morfológicos do miocárdio após o treinamento físico.

A regulação da glicose ou do metabolismo lipídico por adiponectina, em resposta ao treinamento físico, têm sido investigada (GARENAKI et al., 2011;

ANDO et al., 2009) principalmente com análises dos níveis circulantes de adiponectina total e HMW (ANDO et al., 2009). Até o momento, poucos estudos examinaram a relação de fatores que influenciam os níveis de adiponectina em crianças e adolescentes com DM1, e os resultados destes estudos são inconsistentes (KARAMIFAR et al, 2013; MENON, 2012; HABEEB et al., 2011; MESSAAOUI, 2012; HUERTA, 2006). Estudos recentes exibem redução na expressão de adiponectina cardíaca em ratos com diabetes induzido por estreptozotocina (PEI et al, 2013), porém, a relação entre adiponectina cardíaca em ratos púberes com DM1 e os efeitos de um programa de treinamento de natação nunca foi investigada.

Embora os relatos evidenciem de remodelamento cardíaco em humanos e animais adultos, dados semelhantes em ratos púberes com DM1 são escassos (SILVA et al., 2013; LAW et al., 2012; BROWER et al., 2006). Crianças e adolescentes com diabetes têm muitos dos benefícios que os adultos adquirem com a prática de exercícios, por isto devem praticar exercício físico com segurança, tanto para a saúde como para o lazer (ROBERTSON et al., 2009). Uma vez que os ratos são considerados um modelo animal válido para a compreensão do DM e são constantemente utilizados para este fim, acreditamos que ratos jovens podem servir como modelos para orientar o desenvolvimento de novas estratégias de prevenção e tratamento do DM.

Deste modo, o presente estudo foi realizado para verificar a influência do treinamento de natação de baixa intensidade sobre a estrutura dos cardiomiócitos, o remodelamento estrutural do miocárdio, níveis de citocinas e de óxido nítrico, e a densidade capilar do miocárdio, assim como a disfunção contrátil de cardiomiócitos do VE de ratos púberes com diabetes experimental não tratado com insulina.

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Artigo 1

Ventricular remodeling in growing rats with experimental diabetes: the impact of swimming training

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Abstract

Diabetic cardiomyopathy is associated with cardiac muscle remodeling, resulting in myocardial dysfunction, whereas exercise training (ET) is a useful nonpharmacological strategy for the therapy of cardiac diseases. This study tested the effects of low intensity swimming training on the structural remodeling of the left ventricle (LV) in growing rats with unmanaged experimental diabetes. Thirty day old male Wistar rats were divided into four groups (n = 5/group): sedentary control (SC), exercised control (EC), sedentary diabetic (SD), and exercised diabetic (ED). Swimming training rats exercised 5 days/week, 90 min/day, with a load of 5% BW during 8 weeks. Sections of LV were stained with Periodic acid Schiff, Sirius Red, and Gomori's reticulin. Seven days and 8 weeks after streptozotocin (STZ) induction (60 mg kg^{-1} BW), blood glucose (BG) in the diabetic groups (SD = 581.40 ± 40.48; ED = 558.00 ± 48.89) was greater ($p < 0.05$) than in their controls (SC = 88.80 ± 21.70; EC = 85.60 ± 11.55). Swimming-training reduced BG by 23 mg dL^{-1} in the diabetics ($p > 0.05$). The LV of diabetic rats had increased interstitial collagen and reticular fibers on the extracellular matrix and presented glycogen accumulation. More importantly, all these adverse tissue changes induced by STZ were attenuated by ET. Together, these findings support the idea of a beneficial role of exercise in the LV remodeling in rats with unmanaged type 1 diabetes mellitus.

Introduction

Diabetes mellitus (DM) and its long-term complications are considered one of the major health problems worldwide [33]. The number of children, adolescents, and young adults with DM continue to rise at a dramatic rate [54,55]. In patients with diabetes, cardiovascular complications represent the chief cause of morbidity and mortality in adults [46], children, and adolescents [20].

The pathways leading to DM-induced cardiac dysfunction and its histopathological changes are yet to be fully clarified [42,44,48]. However, this could be in part due to the complex and multifactorial nature of diabetes [31]. Probably the most important role has been attributed to persistent hyperglycemia [33]. This condition increases the risks for developing heart failure (HF) from two- to five fold [8].

Individuals with diabetes can develop cardiac dysfunction, termed diabetic cardiomyopathy (DCM) [31]. Diabetic cardiomyopathy is characterized by myocardial dilatation and hypertrophy, accompanied by systolic and diastolic left ventricle (LV) dysfunction, and its presence is independent of the coexistence of ischemic heart disease or hypertension [25]. Although DCM may be subclinical for a long time [58], eventually, it leads to decreased myocardial elasticity and impaired heart contractile function [3,18,46,49]. The link between elevated glucose levels and the development of DCM has significant effects on the expression, organization, and modification of extracellular matrix (ECM) components in the heart [31].

The cardiac ECM is a dynamic well-defined network consisting of structural proteins, proteoglycans, growth factors/cytokines, and enzymes that provide essential cues and scaffolding for tissue organization and structure [31]. It is composed of collagen [14], with fibrillar collagen types I and III localized within the myocardial interstitium and with nonfibrillar collagen types IV and VI, and the glycoproteins fibronectin and laminin predominating in the cardiomyocyte basement membrane [11,17]. Elevations in myocardial stress initiate structural remodeling of the heart in an attempt to normalize the imposed stress. This process comprises cardiomyocyte hypertrophy and changes in the amount of collagen, collagen phenotyping, and collagen cross-linking with progressive remodeling of the muscular, vascular, and ECM components of the heart [12]. Increased interstitial collagen concentrations

and/or cross-linking results in a stiffer myocardium and ventricular diastolic dysfunction [12].

Exercise training is of particular interest in both the prevention and treatment of DCM in type 1 DM [35]. Thus, exercise training has been suggested to be a useful nonpharmacological strategy to attenuate morphological changes and contractile dysfunction in both animals and human with DM [10,15,16,49]. Furthermore, regular physical activity is associated with greater longevity and lower frequency and severity of diabetes complications [59]. While long-term intensive exercise training is suggested to promote adverse remodeling (i.e., fibrosis) in a rat model [9], low-intensity aerobic exercises improves physical fitness and strength, reduces cardiovascular risk factors, improves well-being, reduces insulin requirements, and improves cardiac autonomic function [15,16,59]. Indeed, low- to moderate-intensity aerobic exercise has been shown to induce beneficial cardiovascular adaptations (e.g., improves cardiac function, myocardial structure; reduces blood pressure; increases baro- and chemoreflex sensitivity, intrinsic heart rate, and cardiac output) in STZ-induced diabetic rats, a model that imitates many of the human type 1 DM [16,29,34,35,41]. Nevertheless, the mechanisms underlying the histopathology of type 1 DM and their relationship to physical exercise, myocardial interstitial fibrosis, and cardiac functional impairment are unclear. Although reports show evidence of cardiac remodeling in adult humans and animals, similar data in growing rats with type 1 DM are lacking [12,31]. Children and adolescents with diabetes should have many of the same health and leisure benefits as adults and should be allowed to practice physical exercise with safety [47]. Since rats are considered a valid animal model for understanding the DM and are consistently used for this purpose, we believe that young rats could serve as models for guide the development of novel strategies for prevention and management of DM. Therefore, the present study was undertaken to verify the effects of low-intensity swimming training on the structural remodeling of the LV of growing rats with unmanaged experimental diabetes.

Materials and Methods

Animals and experimental groups

Male Wistar rats weighing 87.40 ± 13.03 g, 30 days old, were obtained from the animal facility at the Federal University of Viçosa, Brazil, and were randomly

divided into four groups (n = 5, per group): sedentary control (SC), exercised control (EC), sedentary diabetic (SD), and exercised diabetic (ED). Rats were maintained on 12-h dark/light cycle at 22°C, housed in groups of five, and fed standard commercial rodent chow and water *ad libitum*. All procedures followed the Guidelines for Ethical Care of Experimental Animals and were approved by the Ethics Committee on Animal Experimentation (CEUA) from the Federal University of Viçosa (protocol number 46/2011).

Diabetes induction

Severe diabetes was induced in the animals by intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) dissolved in 0.1 M citrate buffer solution (0.1 M, pH 4.5) at the dose of 60 mg kg^{-1} body weight (BW) [28]. Equivalent volume (1 mL kg^{-1}) of vehicle was injected into the rats assigned to the control groups. Animals were fasted overnight for 12 h prior to STZ administration. Water and food were available immediately after dosing. Development of diabetes was determined by observing hyperglycemia (> 300 mg dL^{-1}) [51] as measured by an Accu-Chek Advantage glucometer (Boehringer Mannheim Corporation, Indianapolis, IN). Body weights and blood glucose levels were recorded once a week throughout the study. All animals were euthanatized 8 weeks after diabetes induction by intraperitoneal injection of sodium pentobarbital (120 mg kg^{-1}).

Exercise protocol

After seven days of diabetes induction and confirmation of consistent hyperglycemia, animals from the exercised groups (ED and EC) were submitted to a swimming training program (adapted from Gomes et al. [22]) for 8 weeks. Rats were placed in water tank with 65 cm high by 75 cm in diameter, filled with warm water (28°C to 30°C) at a depth of 45 cm and forced to swim. Animals were then dried and returned to the home cage. Training intensity varied by changing a load that was placed around the animal's chest from 0% to 5% of its BW. Briefly, in the first week, animals exercised in the water for 10 to 50 min, with no load, while duration was increased by 10 min each/day. In the second week, animals remained exercising with no load and with the duration incremented by 10 min/day until a maximum of 90 min of continuous swimming. From the fourth week, animals began swimming with a load until the end of the training program (8 weeks). The load was progressively

increased by 1% of the animal's BW from the fourth week on, such that at the eighth week, animals swam with a total load of 5% of their BW. During the swimming sessions, animals from the sedentary group (SD and SC) were placed in a polypropylene box containing warm water (28°C to 30°C) with a depth of 10 cm.

Electrocardiogram

Animals were anesthetized in an induction chamber with 2% isoflurane and 100% oxygen at a constant flow of 1 L/min. Once unconscious, they were placed on a platform in dorsal recumbency, with the four limbs fixed. Isoflurane was maintained at a concentration sufficient for restrain (0.5% to 1.0%), and animals were able to maintain spontaneous respiration during the electrocardiogram (ECG). A trichotomy of approximately 1 cm^2 was performed in the forelimbs and left hindlimb for electrode insertion. Derivation II of the ECG was recorded using the data acquisition system PowerLab® (AD Instruments, São Paulo, Brazil), and data analysis was done with the program LabChart Pro® (ADInstruments LabChart 7, São Paulo, Brazil).

Electrocardiograms were performed at the end of the experiments by an experienced fellow blind to the study groups and treatments. Resting heart rate (HR) was derived from the ECG, following established guidelines [26].

Histological processing and histochemistry

Following euthanasia, the heart was excised, washed with saline solution, and fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2–7.4, for 24 h. The LV was dissected and weighed separately. Left ventricle fragments were obtained through the *orientator* method to define isotropic and uniform random sections (IUR) required in the stereological study [38]. These fragments were dehydrated in ethanol, cleared in xylol, and embedded in paraffin. Blocks were cut into 5 μm sections and mounted on histological slides. The LV sections were stained by Periodic acid-Schiff (PAS) for glycogen, Sirius Red (for total collagen), and Gomori's reticulin (for silver impregnation of reticular fibers, which mainly consist of collagen type III) to evaluate the series of histopathological changes in the diabetic myocardium. Digital images were captured using a light microscope (mod. Primo Star, Carl Zeiss AG, Oberkochen, Germany) connected to a digital camera (AxioCam ERc5s, Carl Zeiss AG).

Analysis of myocardial collagen content

To quantify interstitial fibrosis, LV was evaluated in histological sections 5 µm thick stained with Sirius Red dye (Sirius Red F3B, Mobay Chemical Co., Union, NJ, USA), which marks collagen fibers types I and III for further observation under a polarizing microscope [30]. The distribution of total collagen content was analyzed using the image analysis software Image Pro-Plus 4.5® (Media Cybernetics, Silver Spring, MD, USA) based on the birefringence properties of the collagen fibrils under polarized light. In this analysis, 12 microscopic fields from five rats per group were investigated (magnification ×200) randomly. A 208-point grid was superimposed on each image, and the total of 2,496 points was assigned for each rat. The area was determined by adding up these points, then dividing the result by the total points for the cross-section. Results were expressed as mean ± SD. Samples were observed under an Olympus BX53 microscope equipped with a DP-73 digital camera and imaging system (CellSens, Olympus Corporation, Tokyo, Japan).

Periodic acid-Schiff and Gomori's reticulin staining

A qualitative analysis based on the intensity of the staining reaction with the ventricular muscle was performed using PAS stain and Gomori's reticulin stain. In this analysis, 12 microscopic fields from five rats per group were investigated (magnification ×1,000) randomly. Tissue sections of LV were stained with PAS for detection of polysaccharides [53], and amylase-treated sections served as negative controls. Other sections of LV were stained by Gomori's reticulin. The level of polysaccharides/collagen staining received a score that varied from 1 cross (+), representing weak reaction for polysaccharides/collagen fibers, to 4 crosses (++++), representing the strongest staining for polysaccharides/collagen (adapted from Spillman et al. [53]).

Statistical analysis

Results were expressed as mean ± SD. The Kolmogorov-Smirnov test for normality was initially performed. Statistical differences were evaluated by two-way ANOVA or unpaired *t*-test when appropriate, and the post hoc Tukey test was applied for multiple comparisons. The analysis was performed using the Sigma Stat software, version 3.0, and statistical significance was defined as $p \leq 0.05$.

Results

The animals of the groups SD and ED developed the expected hyperglycemia of severe diabetes compared with control groups. Initially, on the baseline, there were no differences in blood glucose levels between diabetic (SD *vs*. ED) and control rats (SC *vs*. EC) (Table 1). Blood glucose increased throughout the experiment in both SD and ED rats. Seven days after the application of STZ and at the end of the study, the glycemic levels for the diabetic groups (SD and ED) were significantly greater compared with their controls (Table 1). Swimming training reduced BG level by 23 mg/dL in the diabetic groups, but this did not reach statistical difference.

The analyses of the baseline body weights were not different among the four groups (Table 1). Diabetic rats had lower BW gain compared with normal rats (SC and EC) ($p < 0.05$). Similarly, the LV weighted significantly less in the diabetic rats (SD and ED).

Table 1. Biometrical and functional parameters

Parameters	**SC (*n* = 5)**	**EC (*n* = 5)**	**SD (*n* = 5)**	**ED (*n* = 5)**
Baseline BW, g	90.40 ± 3.29	93.60 ± 17.64	78.00 ± 17.78	87.60 ± 6.43
Final BW, g	353.60 ± 63.01	345.60 ± 63.56	174.80 ± 50.49 *	175.00 ± 43.89 †
WG, g	263.20 ± 63.03	252.00 ± 76.80	96.80 ± 40.68 *	87.40 ± 43.07 †
VW, mg	364.20 ± 40.90	354.60 ± 104.84	231.60 ± 43.28*	198.00 ± 47.94†
VW/BW, mg/g	1.03 ± 0.27	1.03 ± 0.34	1.32 ± 0.15*	1.13 ± 0.30 †
Baseline BG, mg/dL	66.20 ± 9.04	65.40 ± 5.50	73.40 ± 12.10	73.80 ± 11.13
BG Post-STZ mg/dL	72.40 ± 6.02	63.40 ± 9.02	418.80 ± 98.98 *	480.60 ± 68.34 †
Final BG, mg/dL	88.80 ± 21.70	85.60 ± 11.55	581.40± 40.48 *	558.00± 48.89 †
Resting HR, bmp	352.58 ± 17.07	300.50 ± 47.60*	256.6± 12.83 *	266.34± 31.29

Data are presented as mean ± SD; *n*, number of animals; SC, sedentary controls; EC, exercised controls; SD, sedentary diabetics; ED, exercised diabetics; BG, blood glucose; BW, body weight; VW, ventricular weight; WG, weight gain; HR, heart rate. *, different from SC; †, different from EC; ($p < 0.05$).

As expected, STZ-induced diabetes resulted in decreased resting HR in SD animals compared with SC (Table 1). Resting HR did not differ between ED and EC rats. The swimming training program was capable of reducing the resting HR for the EC group when baseline and end-study comparisons were made.

Diabetes was associated with interstitial fibrosis by the accumulation of myocardial collagen (Fig. 2 and 3). Collagen content was significantly greater in SD rats compared with SC (7.41 ± 2.08% *vs*. 3.04±0.26%, respectively; $p < 0.05$) and in ED compared with EC rats (4.29 ± 0.36 *vs*. 2.82±0.38, respectively; $p < 0.047$) (Fig. 1 and 2). Interestingly, the exercise training reduced collagen accumulation in the LV of diabetic animals (SD *vs*. ED, $p < 0.05$). However, this was not true in the nondiabetic animals (Fig. 1, 2 and 3).

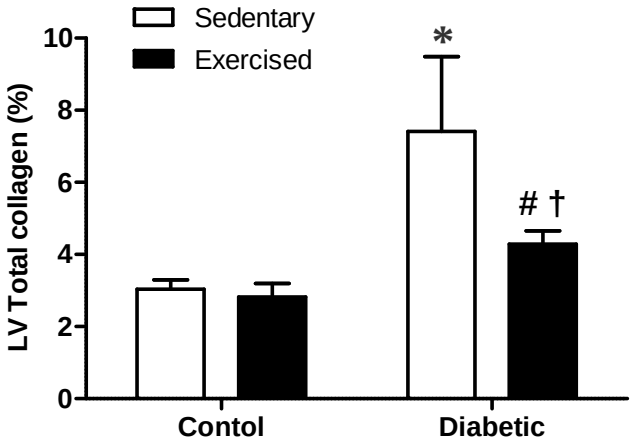

Figure 1. Exercise prevented the accumulation of myocardial collagen. Left ventricular total collagen content expressed as mean ± SD in all four experimental groups. Statistics: *, significant difference from sedentary control (SC); †, significant difference from exercised control (EC); #, significant difference from sedentary diabetic (SD). LV: left ventricle; magnification, ×200.

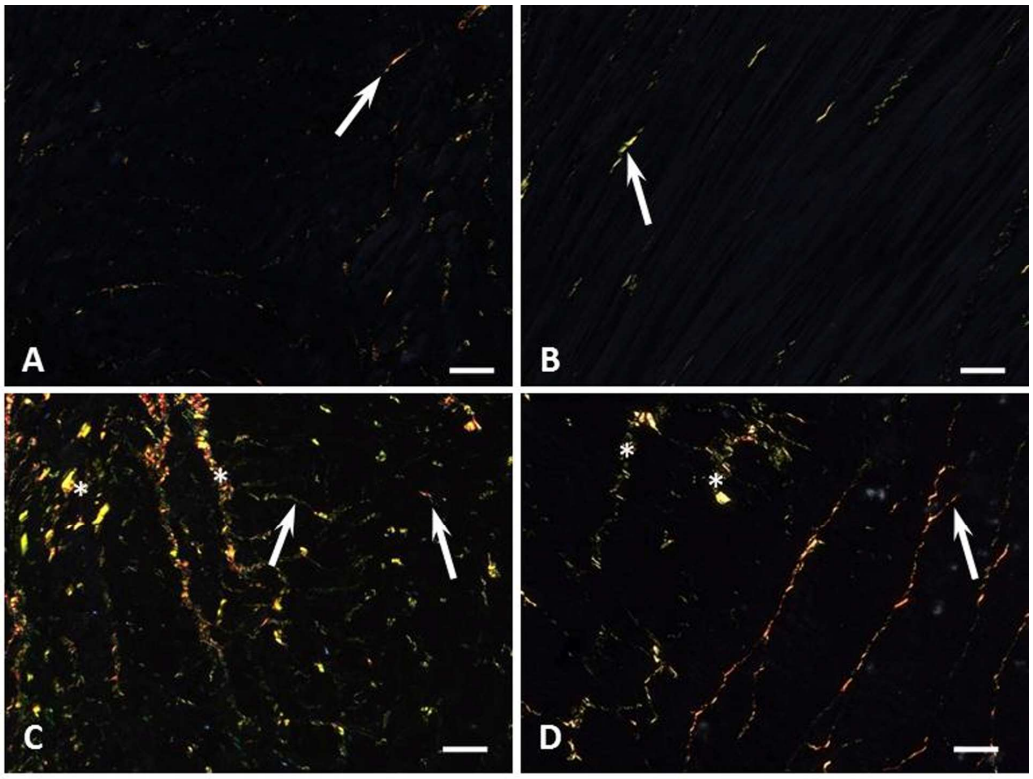

Figure 2. Representative image of the myocardium stained with Sirius Red, observed under a polarizing microscope. (A) Sedentary control, (B) exercised control, (C) sedentary diabetic, and (D) exercised diabetic groups. Arrows = collagen fibers. Arrows pinpoint areas filled with collagen. Observe the increase of collagen fibers in panel C and its less collagen formation in panel D for those submitted to 8 weeks of swimming training. Asterisks = interstitial fibrosis; magnification, ×200; bar: 15 µm.

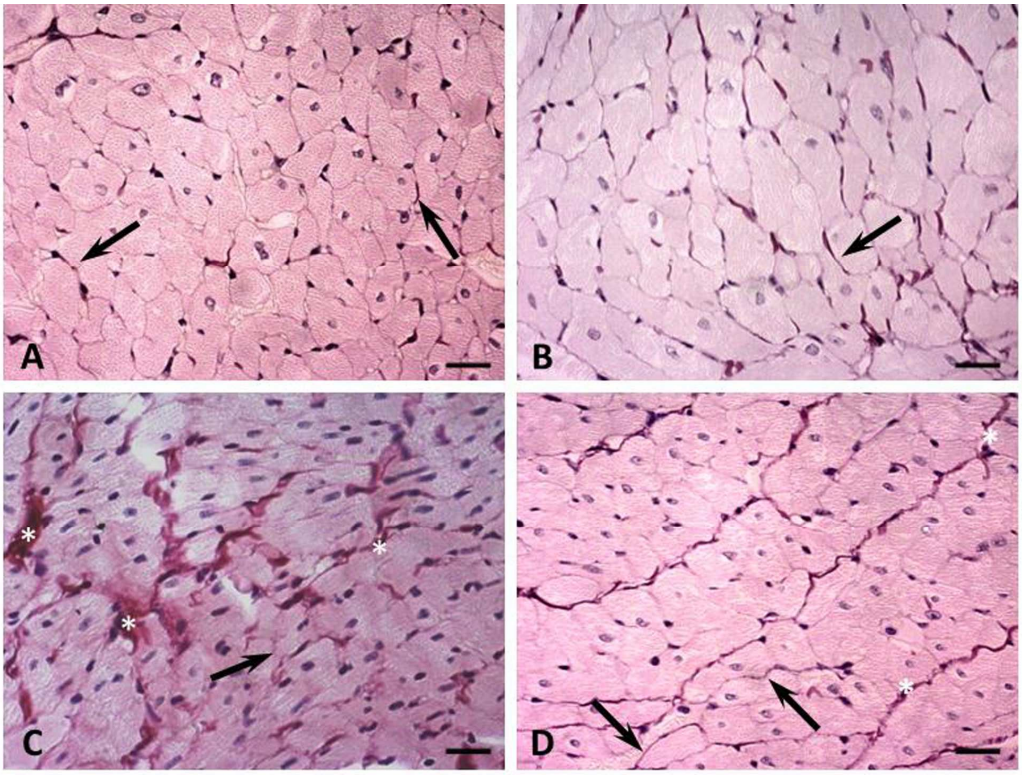

Figure 3. Diabetes-induced cardiac fibrosis. The red color of Sirius Red staining under light microscopy indicates total collagen deposition. (A) Sedentary control, (B) exercised control, (C) sedentary diabetic, and (D) exercised diabetic groups. Arrows = collagen fibers. Observe the increased amount of collagen fibers in panel C and its reduction (near normality) in panel D. Asterisks = interstitial fibrosis; magnification, ×400; bar: 30 µm.

Qualitative analysis of the myocardium using PAS and Gomori's reticulin staining is summarized in Table 2. The sarcoplasm and endomysium of the LV of diabetic sedentary rats had a more prominent staining for polysaccharides than the exercised diabetic (Fig. 4). Animals from the ED group showed a PAS-positive reaction partially reduced in sarcoplasms and in endomysium compared with the EC group. Moreover, the accumulation of PAS-positive material was not observed in amylase-treated sections (negative controls: Fig. 4E and F).

Table 2. Qualitative analysis of the LV of rats by Periodic acid-Schiff (PAS) and Gomori's reticulin histochemical techniques

Histochemistry techniques	**SC (*n* = 5)**	**EC (*n* = 5)**	**SD (*n* = 5)**	**ED (*n* = 5)**
PAS-sarcoplasm	+	+	++++	+++
PAS-endomysium	+	+	++++	++
Gomori's reticulin	+	+	+++	++

PAS staining and Gomori's reticulin staining score: (+) weak reaction with the staining technique, (++) moderate reaction with the staining technique, (+++) strong reaction with the staining technique, (++++) intense reaction with the staining technique. (A) Sedentary control, (B) exercised control, (C) sedentary diabetic, and (D) exercised diabetic groups.

The silver impregnation of reticular fibers by Gomori's reticulin technique demonstrated the fine structure of myocardial collagen, observed as delicate meshworks of fine fibrils stained black by the silver impregnation. This technique demonstrated that there were differences among the contents of reticular fibers in diabetic animals (Fig. 5C and D) compared with nondiabetic (Fig. 5A and B). Animals from the SD group (Fig. 5C) had greater silver impregnation reaction compared with animals from the SC group (Fig. 5A). Similarly, the ED animals (Fig. 5D) presented a more intense reaction than those in the EC group. When comparing the diabetic groups, it appears that exercise training was capable of reducing the occurrence of reticular fibers in these animals.

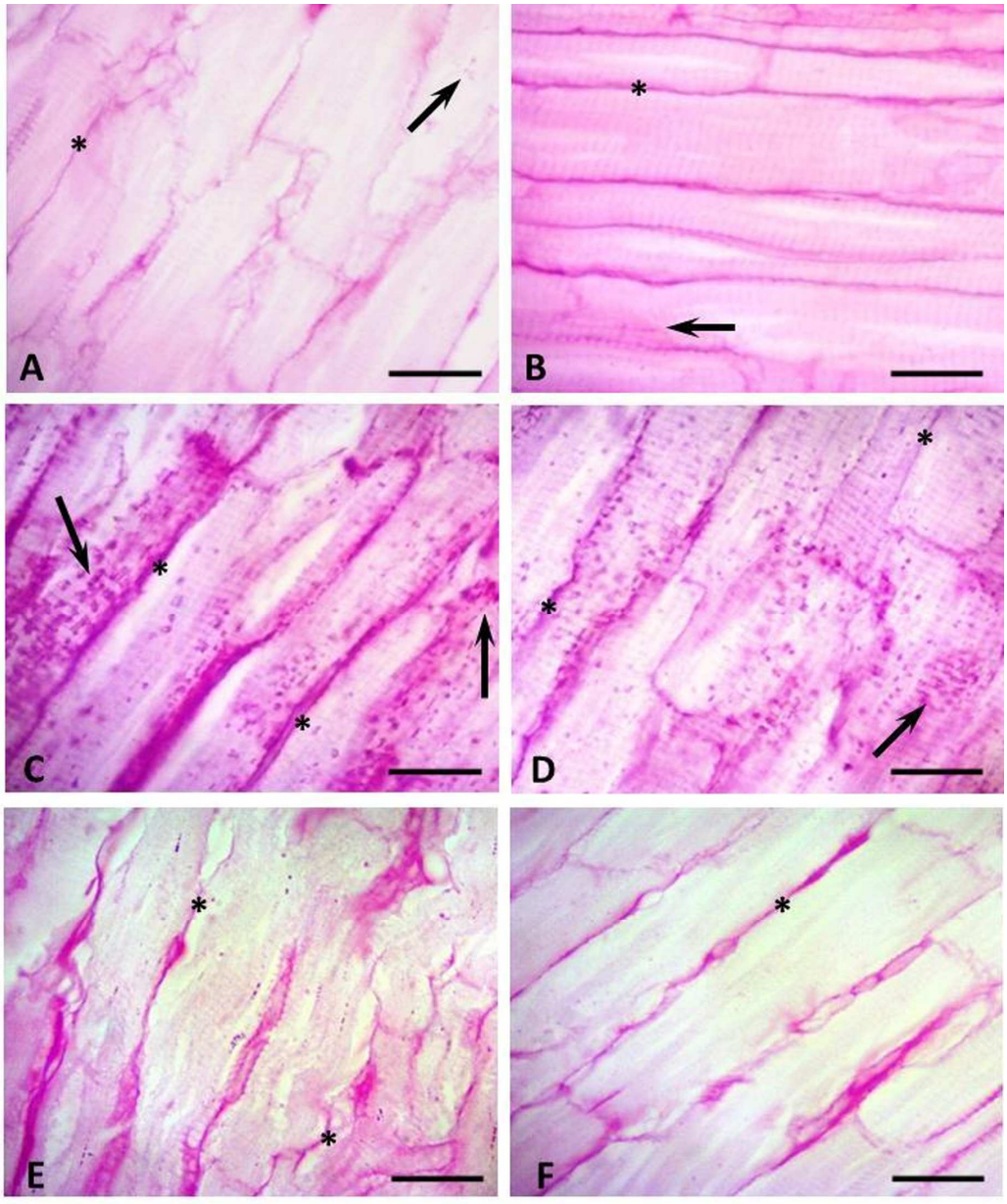

Figure 4. Periodic acid-Schiff (PAS) staining for polysaccharides. Qualitative analysis shows glycogen accumulation and PAS-positive staining in cardiomyocytes after 8 weeks of exercise training. (A) Sedentary control, (B) exercised control, (C) sedentary diabetic, and (D) exercised diabetic groups. In the groups in panel E (sedentary diabetic) and panel F (exercised diabetic), note the absence of glycogen in the sarcoplasm in amylase-treated sections (negative controls). Arrows indicate intracellular glycogen, and asterisks indicate the PAS-positive endomysium. Magnification, ×1,000; bar: 20 μm.

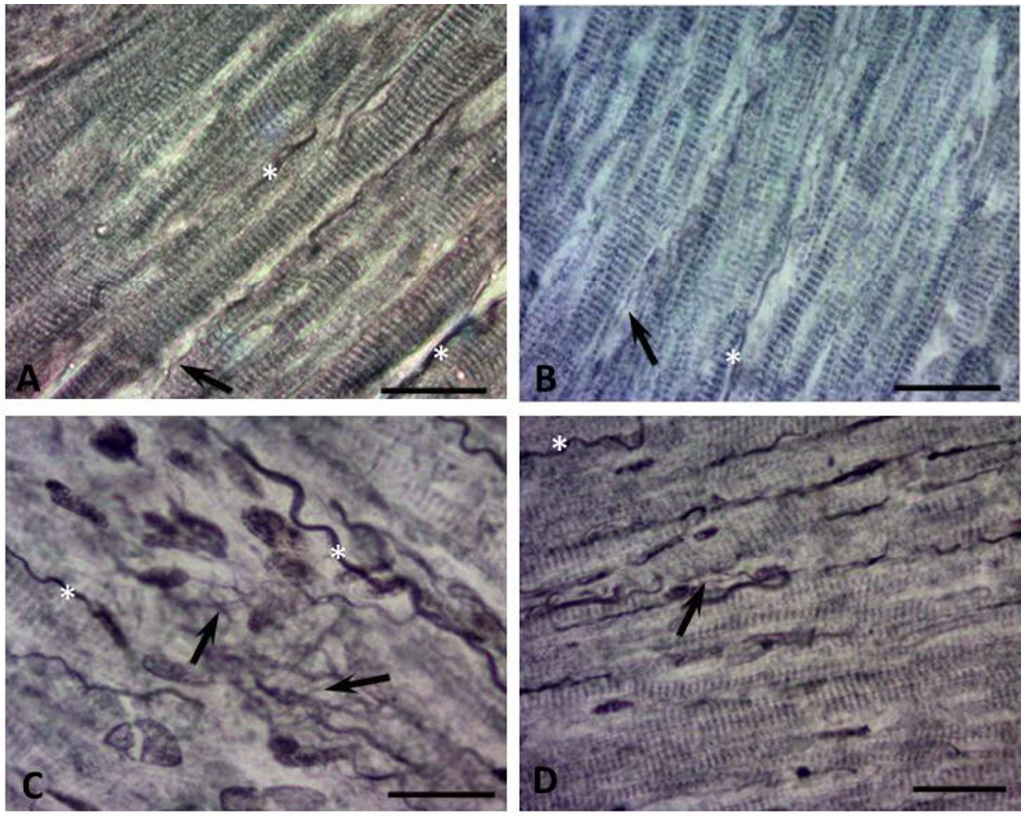

Figure 5. Silver-impregnated section of myocardium subjected to the Gomori's reticulin technique. (A) Sedentary control, (B) exercised control, (C) sedentary diabetic, and (D) exercised diabetic groups. Arrows = reticular fibers consisting mainly of collagen type III. Observe the increase of reticular fibers in panel C and its reduction in panel D. Note in panel C the reticular fibers associated with cardiomyocyte degeneration and possibly a process of recovery from injury. Asterisks = thick collagen fibers; magnification, ×1,000; bar: 20 µm.

Discussion

The present study evaluated the effects of low-intensity swimming training on the structural remodeling of the LV in growing rats with untreated experimental diabetes. Our data showed that the STZ dosage used induced DCM as indicated by morphological changes such as increased interstitial collagen, increased fine reticular fiber, and the accumulation of glycogen in the LV's extracellular collagen matrix. Interestingly, part of the adverse cardiac remodeling was attenuated in response to

chronic exercise, as observed by reduced collagen deposition and the accumulation of glycogen.

We found increased LV collagen deposition on STZ-induced diabetic rats, which is in agreement with previous studies [2,4,13,32,33]. Our results showed that the degree of total collagen deposition in SD rats was significantly more intense, and consequently, fibrosis was more evident compared with normal rats. These data substantiates a histomorphological remodeling process, which is able to endanger the heart function. In fact, collagen deposition leads to a stiffer myocardium, and consequently, diastolic dysfunction is observed [12]. Even though this was not directly evaluated in this study, based on collagen findings, it is possible that diabetic animals have developed diastolic dysfunction as observed by Loganathan et al. [35].

Additionally, the degree of interstitial fibrosis in ED rats was reduced compared with the SD group. Cardiac fibrosis is characterized by the proliferation of cardiac fibroblasts and the excessive accumulation of matrix proteins, mainly collagen types I and III, in the extracellular space [31,56,60]. This occurs due to imbalanced ECM metabolism, characterized by increased collagen synthesis and decreased collagen degradation [56]. Moreover, glycated proteins can undergo a series of chemical rearrangements to form complex compounds and cross-links known as advanced glycation end products (AGEs) [40]. The accumulation of AGE in collagen was associated with reduced collagen turnover, increased cross-linking of collagen, and stiffness of arteries and myocardium [40]. Furthermore, collagen glycation increases the formation and migration of myofibroblasts in the heart, a critical event during fibrosis development in diabetes [60].

Our study demonstrated LV fibrosis in growing diabetic rats, and similar changes were demonstrated by others either in young and adult rodents [31] or in humans [60]. Interstitial and perivascular fibrosis has been described in the myocardium of animals and patients with DM [27]. Myocardial fibrosis is the major hypothesis for the pathogenesis of DCM [2,6]. Thus, after prolonged hyperglycemia, the ECM of the cardiac interstitium can be profoundly affected in diabetes [31,60], which may manifest as increased cross-linking of collagen and alteration of its functional properties [60]. Currently, the understanding of the effects of type 1 DM on the morphological characteristics in the myocardium of pediatric populations is vague, although there are evidences that short-term diabetes in the otherwise-healthy

child and adolescent can produce myocardial dysfunction that may be a threat to the development of severe cardiomyopathy at adulthood [7].

Our qualitative analysis of PAS staining demonstrated a greater tissue distribution of polysaccharides in the LV myocardium of diabetics compared with control animals. The accumulation of PAS-positive material was also observed in the sarcoplasm of cardiomyocyte of the SD rats, which is in concordance with previous studies [13,36,43].

The effects of diabetes on myocardial glycogen metabolism are directly related to decreased glucose transport, which might result in increased myocardial glycogen [43]. However, the excess of glycogen brings about cardiac structural and physiological impairments, including changes in pH, ionic imbalances, and stimulations of pathways leading to hypertrophic signaling [45]. It is known that energy metabolism is rapidly shifted in the diabetic metabolism, resulting in augmented fatty acid and decreased glucose consumption [21]. The switch of cardiac energy substrate utilization from carbohydrate to lipids increases intracellular glycogen, probably through increased glycogen synthesis or impaired glycogenolysis or a combination of both [24]. Therefore, the greatest intensity in the PAS-positive reaction observed in the LV of sedentary diabetic rats in the present study may be related to glycogen stored and spared in the myocardium [13]. In addition, a higher reaction found in the endomysium by PAS staining may be related to morphological changes involved in the mechanisms of type III and type IV collagen deposition, both positive to PAS technique [13,19].

Although exercise seemed to improve accumulation of glycogen in our trained animals, diabetes played its role. This was demonstrated by a slightly greater reaction of PAS-positive material in the sarcoplasm of the exercised diabetic rats when compared with the nondiabetic animals. These results highlight the benefits that the exercise training exerted on these animals by partially recovering the polysaccharides tissue distribution. According to Castellar et al. [13], this recovery might be attributed to an improved metabolic status provided by physical exercise, which apparently reduces the necessity for glycogen production.

In this study, histochemistry by Gomori's reticulin staining showed reticular fibers surrounding the cardiomyocytes, including the endomysium with a more intense stain reaction in the diabetics groups. Our histological and histochemical analyses confirm the remodeling of the interstitial matrix demonstrated by increased

total collagen deposition in the ECM of diabetic animals reported previously [31]. This adaptation was reduced by exercise training (ED animals) and was unchanged in nondiabetic animals (SC and EC). However, the reduction of reticular fiber in ED animals is partially different from previous reports [13]. Castellar and colleagues [13] found only a slight increased stain reaction to silver impregnation in sedentary diabetic rats compared with exercised diabetic and control animals. These controversial results could be attributed to differences in exercise training protocols or even to age differences among these studies. In this study, the duration of the training sessions was 30 min longer, and the animals were 40 days younger than those of Castellar's protocol [13]. A higher reaction of the endomysium by Gomori's reticulin staining in our study also may be involved in the mechanisms of type III collagen deposition, corroborating with our data of PAS staining [13]. Though the interactions of ECM components with silver may not be specific, they can nevertheless provide important insights into the mechanisms of the chemical reactions involved in ECM remodeling [57]. However, the precise mechanism for the protection associated with decreased reticular fiber by exercise in our study is unknown.

Fasting plasma glucose at rest was not affected by the swimming program in the current study. These results are consistent with other reports [34,35,50]. It is likely that there has been an improvement in glucose uptake in ED animals. Glucose uptake into cardiomyocyte occurs through GLUT1 and GLUT4 transporters [1]. Studies have demonstrated that AMP-activated protein kinase (AMPK) promotes GLUT4 redistribution to the sarcolemmal membrane, improving glucose capitation [1]. Moreover, it is possible that there has been an increase in glucagon secretion in ED animals, and its counterregulatory action has helped maintain hyperglycemia [50]. Thus, a possible improvement of glucose uptake may have been able to prevent the morphological changes demonstrated in diabetic animals. However, studies have demonstrated that exercise was able to improve glucose metabolism in diabetic rats with the reduction of blood glucose levels [5,23]. In agreement with Chimen et al. [15], there is poor evidence for a beneficial effect of physical activity on glycemic control. Nevertheless, there are evidences to recommend physical activity in the management of type 1 DM [15], although the duration and intensity of exercise, especially for the young, needs further clarification.

In the current study, we found that diabetes in rats resulted in decreased HR at rest. It was surprising that we found no statistical differences in the HR between exercised and sedentary diabetic animals. Bradycardia has been reported previously in adult rats [16,52] and adolescent [37] with type 1 DM. Thus, the decreased HR observed in our study might be associated with autonomic dysfunction. Bradycardia is a very early indication of DCM [52]. Furthermore, cardiovascular autonomic neuropathy in diabetes commonly leads to abnormalities in HR control and vascular dynamics [39]. Additionally, in rats, degenerative changes in autonomic neurons can be observed from 3 days to several weeks after STZ injection [16].

Conversely, studies have reported that exercise training prevents cardiac autonomic nervous dysfunction in diabetics [15,59] and reverses bradycardia [16]. Diabetic rats were exercised at a low intensity during 7 weeks, and there was a significant decrease in resting and post-stress test HR [52]. However, further investigation is needed to clarify the causes of the lower heart rate in young rats with type 1 DM.

Conclusion

We concluded that low-intensity swimming training attenuated total collagen deposition on the ECM and the accumulation of glycogen in the LV of growing rats with untreated severe experimental diabetes. Our results support the idea that this type of regular physical activity plays a beneficial role in the adverse remodeling of the myocardium in rats with type 1 DM. Further studies focusing on the metabolic disorders associated with diabetes in the youth are required.

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Artigo 2

Swimming training attenuates the morphological reorganization of the myocardium and local inflammation in the left ventricle of growing rats with untreated experimental diabetes

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Abstract

Diabetic cardiomyopathy is associated not only with cardiac remodeling and myocardial dysfunction but also with the occurrence of low-grade inflammation and reduced cardiac adiponectin resulting in significant morbidity and mortality in patients with type 1 diabetes mellitus (T1DM). On the other hand, physical exercise is an important strategy for the management of diabetes which can reduce inflammation and attenuate adverse cardiac remodeling and contractile dysfunction. The aim of this study was to investigate the influence of low-intensity swimming training in cardiac cytokines, inflammation, structural remodeling, and cardiomyocyte contractile dysfunction in growing rats with untreated experimental T1DM. Thirty-day-old male Wistar rats were divided into four groups (n = 14, per group): sedentary control (SC), exercised control (EC), sedentary diabetic (SD), and exercised diabetic (ED). Diabetes was induced by streptozotocin (STZ, 60 mg kg^{-1} body weight). Animals from EC and ED groups were submitted to a swimming training (5 days/week, 90 min/day, load of 5% body weight) for 8 weeks. After euthanasia, the left ventricle (LV) was removed for molecular, morphological, and cardiomyocyte mechanical analysis. Diabetic animals presented cardiac remodeling with myocardial histoarchitectural disorganization, fibrosis, inflammatory infiltrate and necrosis. The capillary density was significantly lower in diabetic animals. Left ventricular cardiomyocytes from diabetic animals exhibited more prolonged time to the peak of contraction and the time to half relaxation than those from control animals, but no difference in cell shortening was observed. The cardiac levels of interleukin 10, nitric oxide, total and HMW adiponectin were significantly decreased in diabetic animals. Exercise training reduced the level of TNF-α, increased capillary density and attenuated the histopathological parameters assessed in the LV of diabetic rats. In conclusion, the adverse cardiac structural remodeling induced by experimental T1DM coexists with reduced levels of total and HMW adiponectin, chronic inflammation and cardiomyocyte contractility dysfunction. More important, low-intensity swimming training attenuated part of these pathological changes which indicate the beneficial role for regular exercise untreated T1DM.

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, 2013) with high prevalence in both adults and adolescent populations (Ma and Chan, 2009). Cardiovascular disease (CVD) is one of the major complications of diabetes that commence in childhood (Nadeau et al., 2011) and greatly impacts mortality and morbidity (Guo et al., 2007; Rajesh et al., 2012).

Type 1 DM (T1DM) is primarily a disease of insulin deficiency from pancreatic beta-cell destruction (American Diabetes Association, 2013) and CVD may be linked to insulin resistance in this form of diabetes (DeFronzo et al., 1982; Pereira et al., 2012). Moreover, DM has been related to low-grade inflammation and micro- and macrovascular complications in adults (Gonzalez-Clemente et al., 2007; Llauradó et al., 2012) and children with T1DM (Mangge et al., 2004).

Low-grade inflammation has been associated with an increase pro-inflammatory circulating proteins, such tumor necrosis factor-alpha (TNF-α), interleukin (IL-6), C-reactive protein (CPR) and with a reduction in the anti-inflammatory proteins such as IL-10 (Ryba et al., 2011; Llauradó et al., 2012). A chronic inflammation status leads to histopathological changes with tissue injury, endothelial dysfunction, and cardiac remodeling resulting in deterioration of myocardial contractility and left ventricular function (Llauradó et al., 2012). The suggested mechanisms are through the reduction of Ca^{2+} transient (Duncan et al., 2007) and reduction of Ca^{2+} sensitivity of the contractile myofilaments (Goldhaber et al., 1996).

Adiponectin also majorly impacts the pathogenesis of insulin resistance, diabetes and vascular injury (Gu and Li, 2012; Fenger, 2013), and plays an important role in glucose and lipid metabolism (Numao et al., 2008; Garekani et al., 2011; Pereira et al., 2012). Adiponectin is a plasma protein mainly secreted by adipocytes (Bobbert et al., 2011) with anti-diabetic, anti-inflammatory, antiatherogenic properties (Garekani et al., 2011; Forsblom et al., 2011). Moreover, cardiomyocytes are also capable of synthesizing adiponectin (Piñeiro et al., 2005; Maia-Fernandes et al., 2008). Studies suggest that, adiponectin, in particular, the high-molecular weight (HMW) adiponectin isoform (Leth et al., 2008; Numao et al., 2008), is a potent immunomodulatory and cardioprotective molecule (Essick et al., 2011; Jenke et al.,

2013). In fact, this protein protects the heart from ischemic injury, cardiomyopathy and systolic dysfunction (Goldstein et al., 2009), inhibits pathological cardiomyocyte hypertrophy and myocardial fibrosis (Han et al., 2007; Maia-Ferandes et al., 2008), reduces oxidative and nitrative stress (Goldstein et al., 2009; Wang et al., 2013), reduces TNF-α and IL-6, and increases expression of IL-10 in the heart (Han et al., 2007; Maia-Ferandes et al., 2008; Goldstein et al., 2009).

Adiponectin improves cardiomyocyte contractile function in db/db diabetic obese possibly by alleviating endoplasmic reticulum stress (Dong and Ren, 2009). In addition, in diabetes, endothelial nitric oxide synthase (eNOS) protein expression is progressively reduced in myocardium and nitric oxide (NO) content is decreased (Wang et al., 2011; Wang et al., 2013, Nagareddy et al., 2005).

Regular physical exercise is an important strategy for the management of T1DM, due the beneficial health effects, especially cardiovascular disease prevention (Galassetti and Riddell, 2013). Aerobic exercise training can reduce inflammation and cardiovascular risks (Rosa et al., 2010; Galassetti and Riddell, 2013), reduces insulin resistance, improves glucose and lipid metabolism (Khan, 2013), attenuates morphological changes (Silva et al., 2013) and contractile dysfunction in both animals and human with DM (Bidasee et al., 2008; Chimen et al., 2012; Rajesh et al., 2012).

The regulation of glucose or lipid metabolism by adiponectin through exercise training have been investigated (Ando et al., 2009; Garenaki et al., 2011), however, inconsistent findings have emerged, mainly on total circulating and HMW adiponectin levels (Ando et al., 2009).

So far, few studies have examined the relationship of factors influencing adiponectin levels in children and adolescents with type 1 DM, and the results of those studies are very inconsistent (Huerta, 2006; Habeeb et al., 2012; Menon, 2012; Karamifar et al., 2013). In addition, recent studies showed reduction in the cardiac adiponectin expression in STZ-induced diabetic rats (Pei et al., 2013). However, the relationship between cardiac adiponectin in growing rats with T1DM and the effects of a low-intensity swimming training has not been investigated yet.

Therefore, we hypothesized that low-intensity swimming training can reduce the effects of STZ-induced diabetes on the cardiac histopathology, cytokines, NO, capillary density as well as on the cardiomyocyte contractile dysfunction.

Materials and Methods

Animals and experimental groups

Male Wistar rats weighing 90.0 ± 5.0 g, 30 days old, were obtained from the animal facility at the Federal University of Viçosa, Brazil, and were randomly divided into four groups (n = 14, per group): sedentary control (SC), exercised control (EC), sedentary diabetic (SD), and exercised diabetic (ED). Rats were maintained on 12-h dark/light cycle at 22°C, humidity at 60–70%, housed in groups of five, and fed standard commercial rodent chow and water *ad libitum*. All procedures followed the Guidelines for Ethical Care of Experimental Animals and were approved by the Ethics Committee on Animal Experimentation (CEUA) from the Federal University of Viçosa (protocol number 46/2011).

Diabetes induction

Severe diabetes was induced in the animals by intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO) dissolved in 0.1 M citrate buffer solution (0.1 M, pH 4.5) at the dose of 60 mg kg^{-1} body weight (BW) (Howarth et al., 2010). Equivalent volume (1 mL kg^{-1}) of vehicle was injected into the rats assigned to the control groups. Animals were fasted overnight for 12 h prior to STZ administration. Water and food were available immediately after dosing. Diabetes was determined seven days after STZ injection. Glycemia (> 300 mg/dL) (Siu et al., 2006) was dosed using the Accu-Chek Advantage glucometer (Boehringer Mannheim Corporation, Indianapolis, IN) after a fasting period of 12-hours overnight. Body weights and blood glucose levels were recorded once a week throughout the study. All animals were euthanatized 8 weeks after diabetes induction by intraperitoneal injection of sodium pentobarbital (120 mg kg^{-1}).

Exercise protocol

After seven days of diabetes induction and confirmation of consistent hyperglycemia, animals from the exercised groups (ED and EC) were submitted to a swimming training program (adapted from Gomes et al., 2006) for 8 weeks. Rats were placed in water tank measuring 65 cm in height and 75 cm in diameter, filled with warm water (28°C to 30°C) at a depth of 45 cm and forced to swim. Animals were then dried and returned to the home cage. Training intensity varied by changing

a load that was placed around the animal's chest from 0% to 5% of its BW. Briefly, in the first week, animals exercised in the water for 10 to 50 min, with no load, while duration was increased by 10 min each/day. In the second week, animals remained exercising with no load and with the duration incremented by 10 min/day until a maximum of 90 min of continuous swimming. From the fourth week, animals began swimming with a load until the end of the training program (8 weeks). The load was progressively increased by 1% of the animal's BW from the fourth week on, such that at the eighth week, animals swam with a total load of 5% of their BW. During the swimming sessions, animals from the sedentary group (SD and SC) were placed in a polypropylene box containing warm water (28°C to 30°C) with a depth of 10 cm.

Electrocardiogram

Four animals per group randomly selected were anesthetized in an induction chamber flushed with 2% isoflurane and 100% oxygen at a constant flow of 1 L min^{-1}. Once unconscious, they were placed on a platform in dorsal recumbency, with the four limbs fixed. Isoflurane was maintained at a concentration sufficient for restrain (0.5% to 1.0%), and animals were able to maintain spontaneous breathing during the electrocardiogram (ECG). A trichotomy of approximately 1 cm^2 was performed in the forelimbs and left hindlimb for electrode insertion. Derivation II of the ECG was recorded using the data acquisition system PowerLab® (AD Instruments, São Paulo, Brazil), and data analysis was done with the program LabChart Pro® (ADInstruments LabChart 7, São Paulo, Brazil).

Electrocardiograms were performed at the end of the experiments by an experienced fellow blind to the study groups and treatments. Resting heart rate (HR) was derived from the ECG, following established guidelines (Heffernan and Jae, 2007).

Histological processing and histochemistry

Following euthanasia, the heart of five animals per group randomly selected was excised, washed with saline solution, dissected and weighed separately. The LV was dissected and weighed separately. Half of the LV tissue was rapidly frozen in liquid nitrogen and stored at -80° C for subsequent assay, and the other half of the LV was fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer,

pH 7.2–7.4, for 24 h. Left ventricle fragments were obtained through the *orientator* method to define isotropic and uniform random sections (IUR) required in the stereological study (Mandarim-de-Lacerda et al., 2003). These fragments were dehydrated in ethanol, cleared in xylol, and embedded in paraffin or glycolmethacrylate resin (Historesin® – Leica). Blocks were cut into 5 μm or 2 μm sections and mounted on histological slides. The LV sections were stained by Sirius red (for total collagen), Gomori's trichrome (for microcirculation), and toluidine blue/sodium borate 1% to evaluate the series of histopathological changes in the diabetic myocardium. Digital images were captured using a light microscope (mod. Primo Star, Carl Zeiss AG, Oberkochen, Germany) connected to a digital camera (AxioCam ERc5s, Carl Zeiss AG).

Analysis of myocardial microcirculation

To quantify intramyocardial capillaries, LV was evaluated in histological sections 5 μm thick stained with Gomori's trichrome. The distribution of capillary density was analyzed using the image analysis software Image Pro-Plus 4.5® (Media Cybernetics, Silver Spring, MD, USA). In this analysis, 12 microscopic fields from five rats per group were randomly investigated (magnification ×400). A 208-point grid was superimposed on each image, and the total of 2,496 points were assigned for each rat. The area was determined by adding up these points, then dividing the result by the total points for the cross-section. Results were expressed as mean ± SEM.

Sirius red and toluidine blue/sodium borate staining

Pathological analysis of the left ventricle was performed using toluidine blue/sodium borate 1% and Sirius red stain. In this analysis, 12 microscopic fields from five rats per group were randomly investigated (magnification ×400). Tissue sections of LV were stained with Sirius red for detection of collagen accumulation, perivascular fibrosis and interstitial fibrosis, and toluidine blue for general pathological alterations.

ELISA-based cytokine detection assay

The levels of Interleukin 10 (IL-10), Tumor Necrosis Factor alpha (TNF-α), adiponectin and High molecular weight (HMW) adiponectin were measured from the

LV homogenate by ELISA. Cytokines IL-10, TNF-α and adiponectin were assayed using Uscn life science Inc. (Wuhan in China) kits and high molecular weight (HMW) adiponectin from BioVendor Co. (Heidelberg, German). The ELISA procedure was performed according to the manufacturer's protocol. The cytokine concentrations were determined with reference to a standard curve for serial two-fold dilutions of the rat recombinant cytokines.

NO Production

NO production was quantified by the standard Griess reaction (Sambrook and Russell, 2001). Briefly, 50 µl of supernatants from the left ventricle homogenates were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine dihydrochloride, and 2.5% phosphoric acid) at room temperature, for 10 minutes. The absorbance was measured at 550 nm in a microplate scanning spectrophotometer (Power Wave X, Bio-Tek Instruments, Inc., Winooski, VT). The conversion of absorbance into micromolar concentrations of NO was deduced from a standard curve using a known concentration of sodium nitrite.

Cardiomyocyte isolation

Nine animals from each group were used to cardiomyocyte contractile function measurement. Two days after the last training session, the rats were weighed and euthanized by cervical dislocation under resting conditions, and their hearts were quickly removed. Left ventricular myocytes were enzymatically isolated as previously described (Natali et al., 2002). Briefly, the hearts were mounted on a Langendorff system and perfused for ~5 min with a modified Hepes–Tyrode solution of the following composition (in mM): 130 NaCl, 1.43 $MgCl_2$, 5.4 KCl, 0.75 $CaCl_2$, 5.0 Hepes, 10.0 glucose, 20.0 taurine, and 10.0 creatine, pH 7.3 at 37 °C. The perfusion solution was changed for the calcium-free solution with EGTA (0.1 mM) for 6 min. Afterwards, the hearts were perfused for 15–20 min with a solution containing 1 mg mL^{-1} collagenase type II (Worthington, USA). The digested heart was then removed from the cannula, and the ventricles were removed and weighed. The left ventricle was separated, weighed, and cut into small pieces. The left ventricle fragments were placed into small conical flasks with collagenase-containing solution supplemented with 1% bovine serum albumin. The cells were dispersed by agitating the flasks at 37 °C for periods of 5 min. Then, single cells were separated

from the non-dispersed tissue by filtration. The resulting cell suspension was centrifuged and resuspended in Hepes–Tyrode solution. Non-dispersed tissue was subjected to further enzyme treatment. The isolated cells were stored at 5 °C until use. Only calcium-tolerant, quiescent, rod-shaped cardiomyocytes showing clear cross-striations were studied. The isolated cardiomyocytes were used within 2–3 h of isolation.

Measurements of cell contractility

Cell contractility was evaluated as previously described (Roman-Campos et al., 2009). Briefly, isolated cells were placed in a chamber with a glass coverslip base mounted on the stage of an inverted microscope (Nikon Eclipse-TS100, USA). The chamber was perfused with Hepes–Tyrode solution at 37 °C. Steady-state 1 Hz contractions were elicited via platinum bath electrodes (Myopacer, Field Stimulator, Ionoptix, USA) with 5 ms duration voltage pulses and an intensity of 20 V. Cells were visualized on a PC monitor with a NTSC camera (Myocam, Ionoptix, USA) in partial scanning mode. This image was used to measure cell shortening (our index of contractility) in response to electrical stimulation using a video motion edge detector (IonWizard, Ionoptix, USA). The cell image was sampled at 240 Hz. Cell shortening was calculated from the output of the edge detector using an IonWizard A/D converter (Ionoptix, Milton, MA, USA). Cell shortening (expressed as a percentage of resting cell length), time to peak shortening and time to half relaxation were calculated.

Statistical analysis

Results were expressed as mean ± SEM. The Kolmogorov-Smirnov test for normality was initially performed. Statistical significance differences were evaluated by ANOVA one way on Ranks followed by the Dunn's test. Two-way ANOVA or unpaired *t*-test was used when appropriate, and the post hoc Tukey test was applied for multiple comparisons. The analysis was performed using the Sigma Stat software, version 3.0, and statistical significance was defined as $p \leq 0.05$.

Results

The animals from SD and ED groups presented the expected hyperglycemia found in severe diabetes. At the baseline, there were no differences in blood glucose

(BG) levels between diabetic (SD *vs*. ED) and control rats (SC *vs*. EC) (Table 1). Blood glucose increased throughout the experiment in both SD and ED rats. Seven days after the application of STZ and at the end of the study, the glycemic levels for the diabetic groups (SD and ED) were significantly greater (Table 1). Swimming training reduced the BG level by 34 mg dL^{-1} in the diabetic groups, however this was not statistically different ($p = 0.16$).

Mean body weight at baseline was similar across all groups (Table 1). Diabetic rats had lower BW gain compared with normal rats (SC and EC). Similarly, the LV weighted significantly less in the diabetic rats (SD and ED). Streptozotocin induced diabetes increased the index of LV hypertrophy (VW/BW ratio) observed in SD *vs*. SC and ED vs. EC rats (Table 1). The index of hypertrophy did not differ between ED and SD rats (Table 1).

Table 1. Biometrical and functional parameters

Parameters	**SC**	**EC**	**SD**	**ED**
Baseline BW, g	90.80 ± 3.37	89.40 ± 5.01	86.20 ± 5.67	91.20 ± 6.87
Final BW, g	320.60 ± 26.05	334.60 ± 13.57	164.60 ± 5.89*	170.60 ± 5.18†
WG, g	229.80 ± 25.42	245.20 ± 13.17	75.20 ± 7.69*	79.40 ± 4.76†
VW, mg	339.20 ± 17.45	379.20 ± 19.09	211.80 ± 6.67*	222.40 ± 4.04†
VW/BW, mg g^{-1}	1.06 ± 0.30	1.13 ± 0.63	1.29 ± 0.51*	1.30 ± 0.35†
Baseline BG, mg dL^{-1}	73.20 ± 5.35	68.20 ± 5.47	74.20 ± 4.33	73.60 ± 8.54
BG Post-STZ, mg dL^{-1}	72.60 ± 3.31	65.40 ± 2.48	429.20 ± 33.65*	461.18 ± 11.07†
Final BG, mg dL^{-1}	98.20 ± 7.93	89.80 ± 2.71	567.80± 19.78*	533.00 ± 6.25†
Resting HR, bmp	356.40 ± 7.39	300.50 ± 20.44*	262.98 ± 7.39*	274.35± 20.53

Data are presented as mean ± SEM (Resting HR, four animals per group, other parameters n = 14 animals per group); SC, sedentary controls; EC, exercised controls; SD, sedentary diabetics; ED, exercised diabetics; BG, blood glucose; BW, body weight; VW, ventricular weight; WG, weight gain; HR, heart rate. *, different from SC; †, different from EC; ($p < 0.05$).

Diabetes caused a decreased resting HR in SD *vs*. SC rats ($p < 0.05$). However, resting HR did not differ between animals that exercised (Table 1). The swimming training program reduced the resting HR for the EC group when compared to SC measurements.

As shown in Fig. 1, total adiponectin tissue levels were significantly lower in the diabetic rats (SD 3.13 ± 0.13 and ED 3.41 ± 0.38) compared with controls (SC 4.92 ± 0.25 and EC 5.64 ± 0.26). Similarly, the HMW adiponectin level was significantly lower in the SD *vs*. SC (1.78 ± 0.01 and 3.01 ± 0.02, respectively) and ED *vs*. EC (2.19 ± 0.01 and 3.41 ± 0.02, respectively) rats. Swimming training program promoted a non-significant increasing trend in the levels of HMW adiponectin in the EC ($p = 0.06$) and ED ($p = 0.07$) animals.

Tissue levels of IL-10 were significantly lower in the diabetic rats (SD 0.21 ± 0.01 and ED 0.41 ± 0.15) compared with controls (SC 0.78 ± 0.06 and EC 1.43 ± 0.32). Diabetes increased TNF-α levels in the LV of diabetic rats (SD and ED) compared with control rats (SC and EC, respectively). Exercise promoted a non-significant increasing trend in the IL-10 levels in both group, control ($p = 0.08$) or diabetic animals ($p = 0.09$) (Figure 1C), and reduced TNF-α levels in the diabetic group (Fig. 1d).

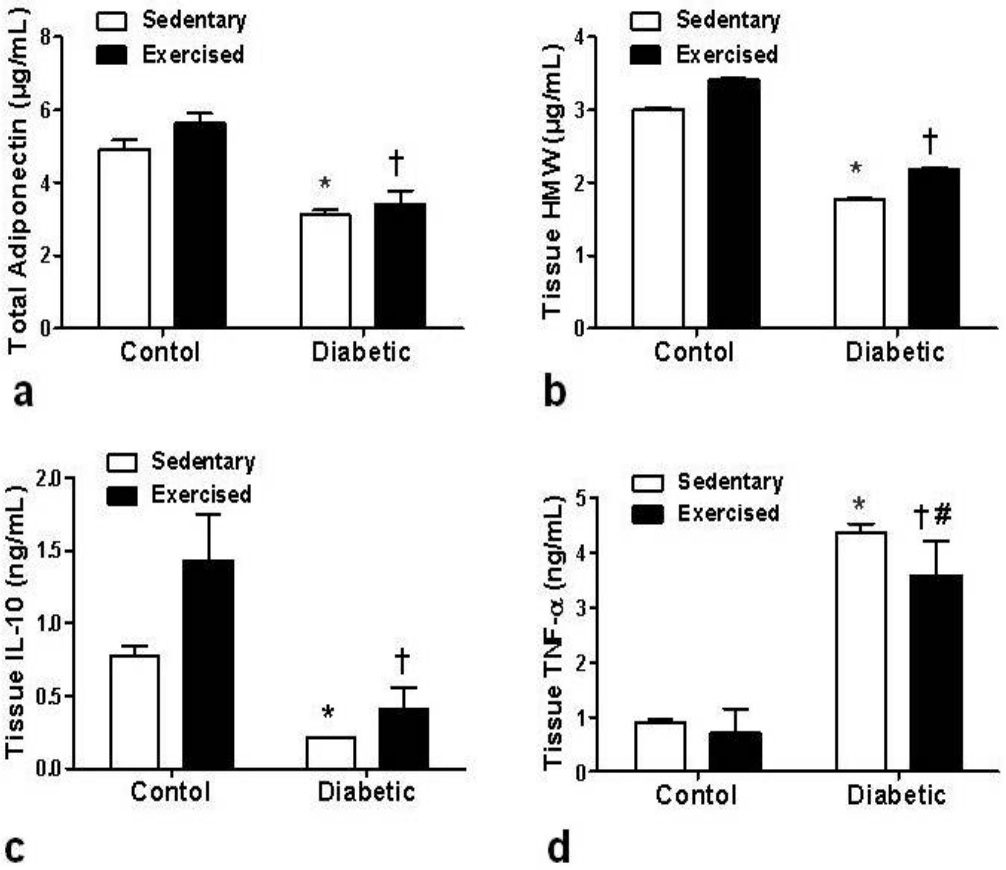

Figure 1. ELISA assay expression of cytokines in the LV of control and diabetic rats. Graphs show the quantitative levels of (a) total adiponectin, (b) HMW adiponectin, (c) IL-10, and (d) TNF-α of the indicated sample groups. Quantitative data are displayed as mean ± SEM (n = 5 animals per group). *Significant difference from sedentary control (SC). †Significant difference from exercised control (EC). #Significant difference from sedentary diabetic (SD) ($p < 0.05$).

Capillary density analysis of the LV showed that diabetic animals presented a lower total capillary density compared SC animals (Fig. 2a-d and 2e, $p < 0.05$). Capillary density of the LV was greater in the ED and EC groups compared to SD and SC, respectively, and ED *vs.* SD (Fig. 2e). Diabetes was associated with reduced levels of myocardial nitric oxide (Fig. 2f, $p < 0.05$) compared with the control groups SC and EC. Interestingly, the exercise training increased NO levels in the LV of EC rats compared with sedentary control (SC *vs.* EC; 210.03 ± 19.87 *vs.* 291.34 ± 13.41, respectively). Swimming training increased NO level by 31% in the diabetic group (SD *vs.* ED), but this did not reach statistical difference ($p = 0.11$). In addition, a

qualitative analysis of the LV revealed greater amounts of collagen fibers around blood vessels characterizing perivascular fibrosis in SD animals, however this was found to a lesser degree in ED animals (Fig. 3).

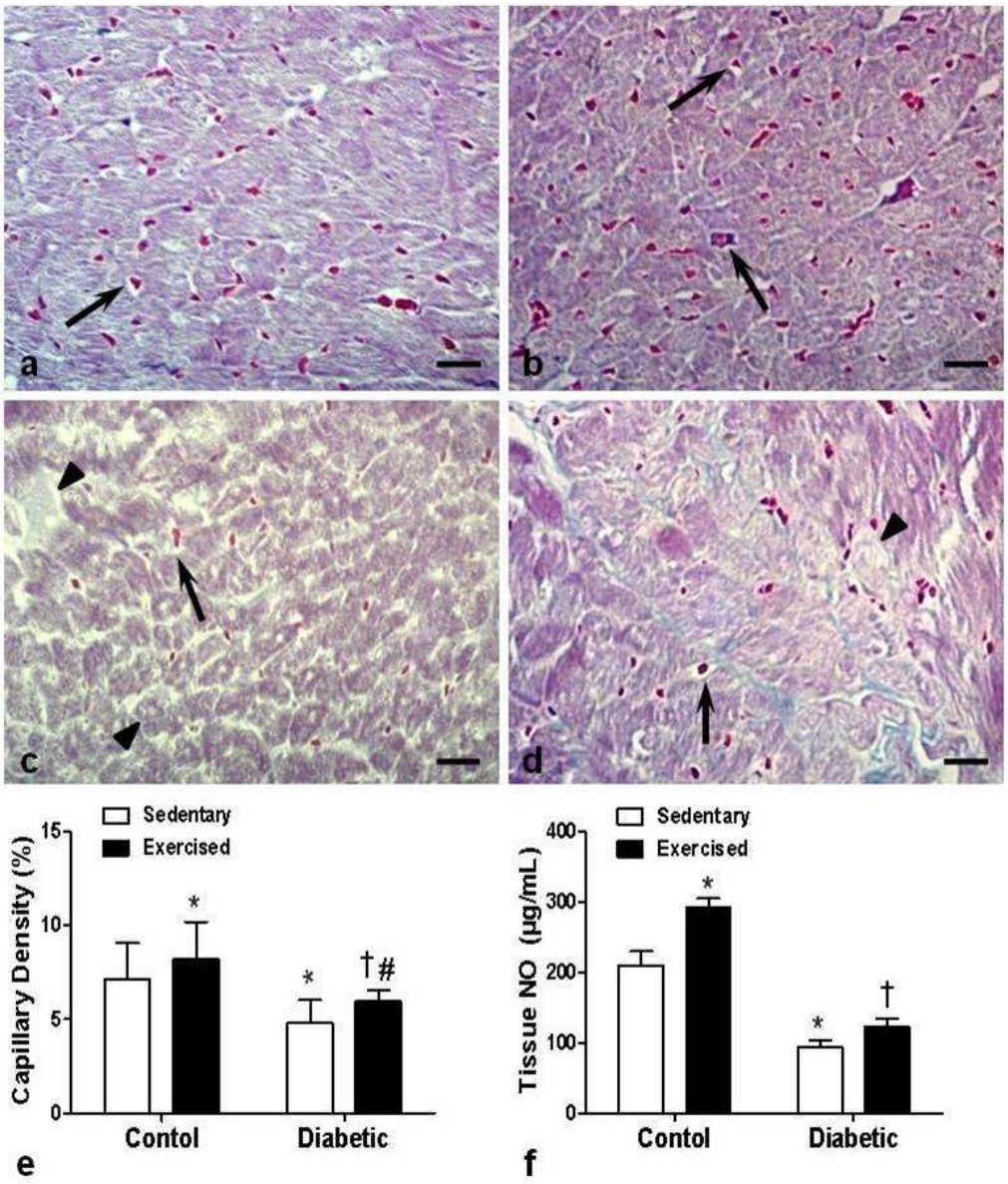

Figure 2. Blood vessels and NO in the LV of control and diabetic rats. Histological sections of myocardium subjected to the Gomori's trichrome technique. (a) Sedentary control, (b) exercised control, (c) sedentary diabetic, and (d) exercised diabetic groups. Arrows = capillaries. Note the increased level of capillary density in panel B and its reduction in panels c and d. The arrowhead in panels c and d indicates cardiomyocyte with vacuolar sarcoplasm (magnification ×400, bar = 30

µm). Graphs show capillary density in panel e and the quantitative levels of LV NO in panel f. Data are displayed as mean ± SEM (n = 5 animals per group). *Significant difference from sedentary control. †Significant difference from exercised control. #Significant difference from sedentary diabetic ($p < 0.05$).

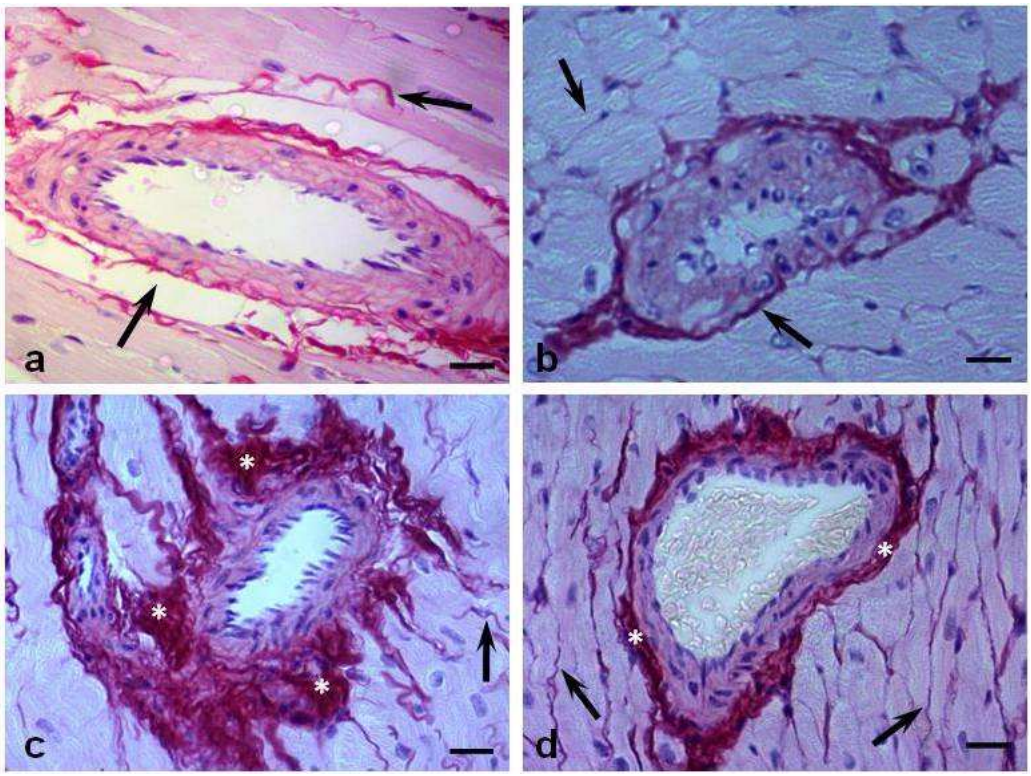

Figure 3. Diabetes-induced cardiac fibrosis. The red color of Sirius red staining under light microscopy indicates total collagen deposition. (a) Sedentary control, (b) exercised control, (c) sedentary diabetic, and (d) exercised diabetic groups. Arrows = collagen fibers. Note the increased amount of collagen fibers in panel c and its reduction in panel d. *Perivascular fibrosis (magnification ×400; bar: 30 µm).

In SC and EC rats, cardiomyocytes were well organized, with myofibrils symmetrically disposed (Figs. 4 a and b). Pathological characteristics from LV of diabetes were observed. Diabetic LV myocardium demonstrated collagen accumulation, perivascular fibrosis and intramyocardial fibrosis (Fig. 3 c), architectural disorganization along with degeneration and atrophy of cardiomyocytes (Fig. 4 c), and evidence of inflammatory-cell infiltrate (not showed in the figure). In

addition, cardiomyocytes from DM rats presented irregular hypertrophy, vacuolar sarcoplasm, reduction of myofibrils, contraction band necrosis, and nuclei with irregular shape (Figures 4 c). Pathological characteristics from LV of diabetes were attenuated by swimming training program (Figs. 3 d and 4 d).

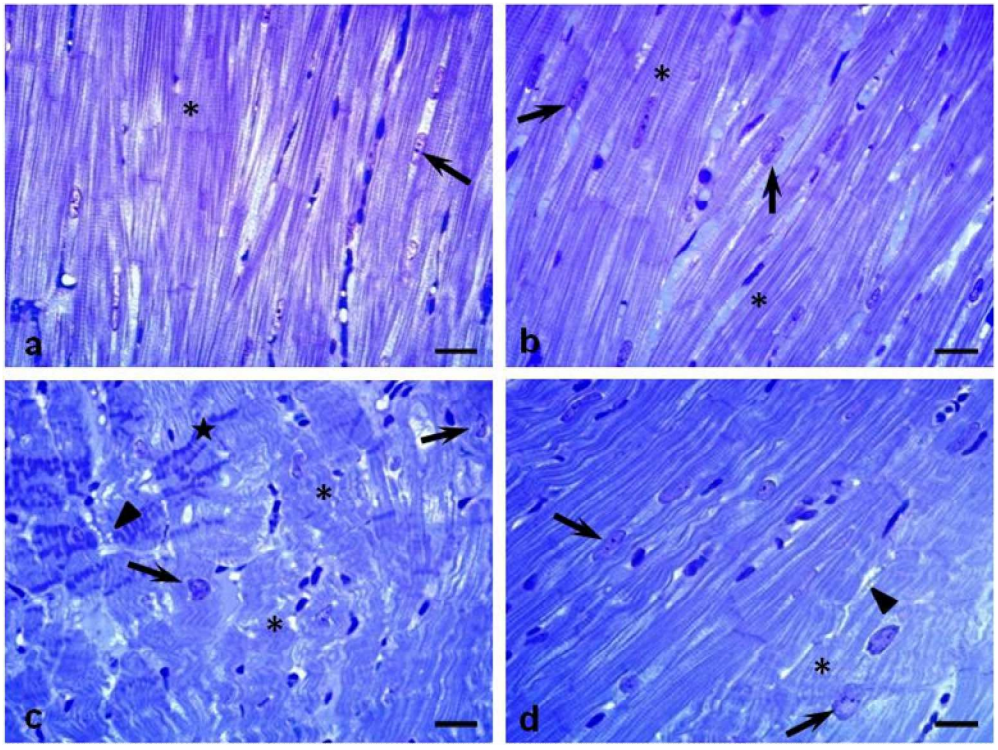

Figure 4. Histological sections stained with toluidine blue/sodium borate 1% showing pathological characteristics from the LV of diabetes. (a) Sedentary control, (b) exercised control, (c) sedentary diabetic, and (d) exercised diabetic groups (magnification ×400, bar = 30 µm). Asterisks = cardiomyocytes, arrows = cardiomyocytes nucleus, arrowhead = vacuoles, and star = contraction band necrosis. Note that pathological characteristics from the LV of diabetes were attenuated by swimming training in panel d (magnification ×400, bar = 30 µm).

Cardiomyocytes contractility

The analysis of cell contractility showed marked changes in the mechanical properties of isolated cardiomyocytes from diabetic animals (Figure 5). In LV cardiomyocytes of animals in the SD group had a significant prolongation of the time to peak of contraction compared to the SC group (300.93 ± 15.26 ms *vs.* 231.25 ±

7.30 ms, respectively, $p<0.05$). The swimming training did not reduce the time to peak contraction in diabetic animals (SD, 300.93 ± 15.26 ms *vs.* ED, 290.65 ± 4.8 ms, $p >0.05$). The effect of swimming program was also not detected regarding the time to peak contraction in LV cardiomyocytes of control animals (EC 227.35 ± 10.93 ms *vs.* SC, 231.25 ± 7.30 ms, $p > 0.05$). In addition, SD *vs.* SC groups (3.05 ± 0.24 and 2.23 ± 0.20, respectively), and ED *vs.* EC groups (2.52 ± 0.22 and 2.60 ± 0.21, respectively) did not exhibit statistically significant differences in cell shortening. Time to half relaxation was greater in the SD compared to SC group (SD, 174.26 ± 12.65 ms *vs.* SC, 146.46±10.06 ms, $p < 0.05$). The swimming training program did not affect the time to half relaxation of cardiomyocytes in diabetic animals (SD, 174.26 ± 12.65 ms *vs.* ED, 209.78 ± 13.37 ms, $p < 0.05$). And the same occurred in the control animals (SC, 146.46±10.06 ms *vs.* EC, 139.12± 12.22 ms, $p < 0.05$).

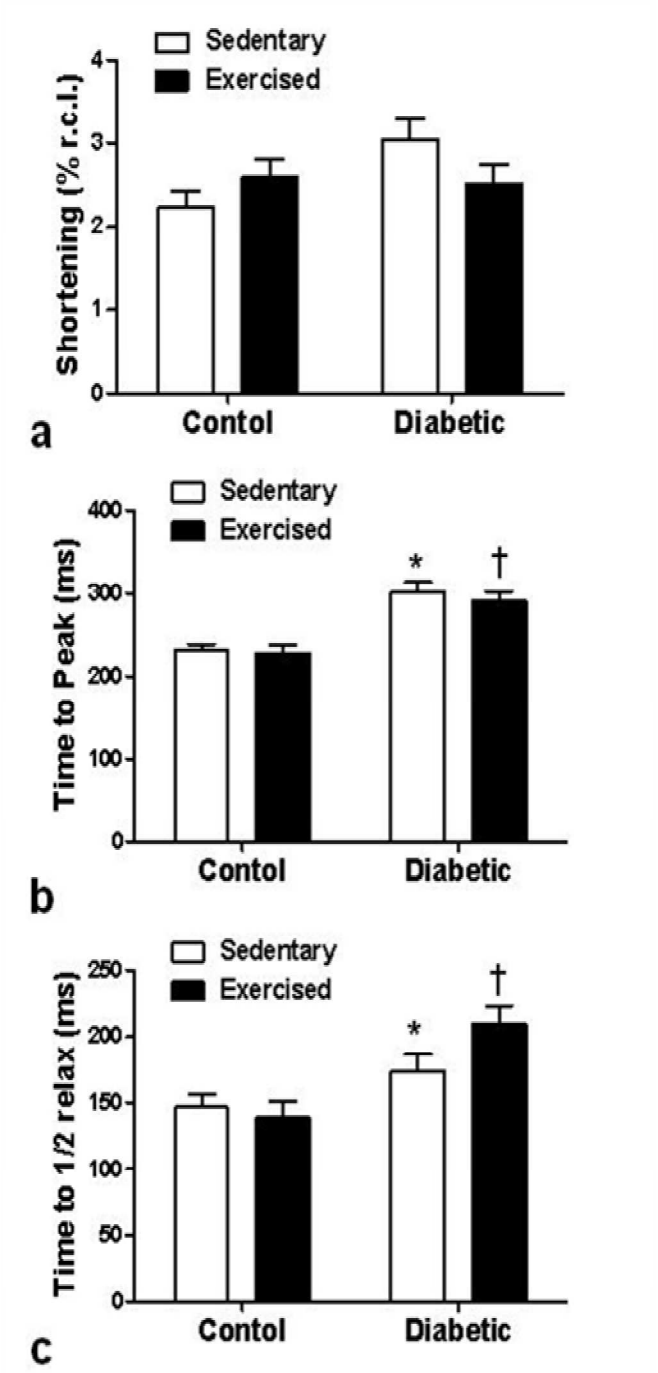

Figure 5. Graphs showing the contractility parameters of the LV isolated cardiomyocytes from control and diabetic rats. (a) Cell shortening expressed as a percentage of resting cell length, (b) time to peak, and (c) time to half relaxation. Data are represented as mean ± SEM of 46–68 cells in each group; % r.c.l., percentage of resting cell length. *Significant difference from sedentary control. †Significant difference from exercised control ($p < 0.05$); Kruskal-Wallis test followed by Dunn test.

Discussion

In the present study we evaluated the effects of low-intensity swimming training on the cardiac structural remodeling, cardiac cytokines and cardiomyocyte contractile function in growing rats with untreated experimental T1DM. The results showed that STZ-induced diabetes promoted marked myocardial morphological reorganization and cardiomyocyte contractile function impairment. The left ventricle of diabetic rats demonstrated pathological characteristics such as histoarchitectural disorganization, collagen accumulation, fibrosis, reduced blood vessels, cardiomyocyte with inflammatory infiltrate, necrosis and irregular hypertrophy. Total adiponectin, HMW adiponectin, IL-10, and NO tissue levels were significantly lower in diabetes, but LV TNF-α levels were increased. However, our exercise training protocol induced a trend towards enhanced IL-10 levels, attenuated the TNF-α level and the pathological characteristics of the LV in diabetes.

Animals with T1DM presented a marked reduction of total adiponectin and HMW adiponectin levels in the LV. As far as we know, this is the first study investigating the levels of cardiac adiponectin in growing rats with diabetes. As of the three major isoforms of adiponectin, low molecular weight (LMW), middle molecular weight (MMW), and high molecular weight (HMW) adiponectin (Hickman and Whitehead, 2012), the HMW is thought to be the major active isoform in peripheral tissues (Maia-Fernandez et al., 2008, Goto et al., 2013). Consistent with previous studies on adult animals, the total and HMW adiponectin levels were inversely associated with T1DM (Pei et al., 2013; Wang et al., 2013). Study by Pei et al., (2013) demonstrated that cardiac adiponectin levels gradually decreased throughout T1DM development in adult mice, which was associated with decreased adenosine monophosphate-activated protein kinase (AMPK) phosphorylation in diabetic hearts. Wang et al. (2011) showed decreased cardiac adiponectin levels and increased inflammatory cytokines TNF-α and IL-6 in diabetic rats. This runs parallel with our results that showed that T1DM rats exhibited reduced cardiac adiponectin levels and local inflammatory response as compared with the control. In contrast with our findings, studies performed by Guo et al. (2007) demonstrated that cardiac adiponectin levels were unchanged in diabetic adult rats, but the adiponectin receptor 1 (AdipoR1) was up-regulated in the heart of STZ-induced diabetes (Guo et al., 2007). Furthermore, increased AdipoR1 expression in cardiomyocyte may be a

compensatory mechanism responsible for the cardioprotective effect of adiponectin in T1DM (Ma et al., 2011; Wang et al., 2013). Despite the increase in AdipoR1 expression, studies showed increased cardiac inflammatory response and decreased GLUT4 protein expression associated with a reduction cardiac adiponectin expression (Sakr et al., 2013). Decreased expression of GLUT4 indicates that glucose metabolism was reduced in diabetic rat hearts (Guo et al., 2007). In our study, the inflammatory status and a possible dysfunction of energy metabolism may be contributed to the deterioration in morphology and cardiac function in STZ-diabetic rats which is in agreement with others (Guo et al., 2007; Wang et al., 2011).

In response to swimming training, total and HMW adiponectin were similarly unchanged in both, diabetic and control groups. These suggest that even if low-intensity aerobic exercise affects the metabolism, probably the production and secretion of total and HMW adiponectin are not locally affected in the heart of growing rats. Thus, the effect of exercise training on adiponectin remains to be elucidated.

In the present study we observed that T1DM caused microvascular damage and reduced NO levels in the left ventricle. Interestingly, swimming training increased capillary density. The attenuation of microcirculation impairment in the ED rats was evidenced by increased capillary density and reduced perivascular fibrosis. These results are exciting as adiponectin is known to exert protective effects through its vasodilator, anti-apoptotic, anti-inflammatory, anti-atherogenic and anti-oxidative activities in both cardiac and vascular cells (Hui et al., 2011; Hickman and Whitehead, 2012). Our findings demonstrate a reduced density of myocardial capillaries in T1DM rats which is in concert with previous reports (Lee et al., 2011; Cosyns et al., 2008). Additionally, a recent study using diabetic rats showed that the microvascular arrangement was very disordered and the surfaces of cardiac microvascular walls were highly irregular, with numerous evaginations and invaginations (Yin et al., 2012). In the current study T1DM affected cardiac levels of NO as shown previously (Wang et al., 2011; Nagareddy et al., 2005). Evidences suggest that in T1DM, the impairment of endothelial function may involve reactive oxygen species such as superoxide that readily react with NO to form peroxynitrate, which results in decreased NO bioavailability (Heidarianpour, 2010). Wang and colleagues (2011) demonstrated that STZ-induced diabetes rats displayed reduced cardiac endothelial nitric oxide synthase (eNOS) activation, which decrease cardiac

NO content (Fuchsjäger-Mayrl et al., 2002). This result was associated with reduced cardiac adiponectin and increased inflammatory cytokines.

We speculated that the reduced levels of both cardiac adiponectin and NO in the current study could be associated with the mechanisms of left ventricular microcirculation impairment in T1DM rats because hyperglycemia and oxidative stress in diabetes can initiate a cardiac structural remodelling that leads to deterioration of arteries, capillaries and venules. On the other hand, adiponectin exerts the protective effects by directly acting on endothelial cells, smooth muscle cells and macrophages (Ohashi et al., 2012) and also stimulates AMPK activation in endothelial cells, leading to activation of eNOS (Maia-Fernandes et al., 2008; Ohashi et al., 2012).

In the present study, the exercise program attenuated significantly reduction in capillary density and perivascular fibrosis in diabetic rats. These are exercise benefits to the myocardium inasmuch as the capillary network participates in maintaining the supply of oxygen and energy substances to the heart. However, in our study, these changes were independent of adiponectin, suggesting that exercise plays an effective role in restoring the capillary net in the myocardium of rats with T1DM via both, the adiponectin-dependent and independent pathways. In addition, exercise training was capable of stimulating angiogenesis in EC animals compared to SC animals. These findings are in agreement with those reported by Ellison et al. (2012) who demonstrated that exercise training induces vascular remodeling of the cardiac muscle by increasing of capillary density. Exercise training can also decrease oxidative stress and improve anti-oxidative capacity of the vascular wall, thus it appears to be beneficial in prevention and improvement of microvascular dysfunction in T1DM (Heidarianpour et al., 2010). Abnormalities in the endothelium/NO pathway have been reported in human and animal models of T1DM in both micro and macro vessels (Khan, 2000; Fuchsjäger-Mayrl et al., 2002). However, the mechanisms underlying the morphological and functional changes of blood vessels are complex and only partially understood.

We observed here increased TNF-α and decreased IL-10 levels in the left ventricle of diabetic rats. In addition, cardiac collagen accumulation (total collagen), local inflammation, degeneration and atrophy of cardiomyocytes, necrosis and myocardial fibrosis were also increased in these animals. Interestingly, our exercise training program attenuated the TNF-α production and left ventricular pathological

remodeling in diabetic animals. Our findings are consistent with previous reports in which intramyocardial inflammation was evidenced by increased levels of cardiac TNF-α (Westermann et al., 2007; Guo et al., 2007; Rajesh et al., 2012; Wen et al., 2013; Huang et al., 2013) and decreased levels of cardiac IL-10 (Huang et al., 2013; Ares-Carrasco et al., 2009). TNF-α is one of the major mediators of inflammation (Ryba et al., 2011) and endogenous TNF-α plays a central role in initiating and sustaining the inflammatory response (Kaur et al., 2006; Hui et al., 2011). Cardiac over-expression of TNF-α has been related to multiple detrimental effects on the heart, including cardiomyocyte hypertrophy, myocardial contractile dysfunction, fibrosis, apoptosis, pathologic heart remodeling and reduction of adiponectin, which leads to heart failure in T1DM (Hui et al., 2011; Rajesh et al., 2012; Nunes et al., 2012; Wen et al., 2013). A recent study revealed intramyocardial inflammation in unmanaged diabetic rats seven weeks after STZ injection, as evidenced by enhanced activity and expression of nuclear factor kappa B (NF-κB), thus leading to increased levels of cardiac pro-inflammatory cytokines (TNF-α, IL-1β), enhanced expressions of cell adhesion molecules (ICAM-1, VCAM-1), and activated invading immunocompetent cells, such as macrophages (CD68+ cells) and T lymphocytes (CD3+ cells), and cardiac collagen accumulation (Wen et al., 2013). The consequence of these abnormal structural alterations in diabetic rats lead to impaired cardiac performance, i.e. increased left ventricular dimensions, reduced ejection fraction and fractional shortening (Wen et al., 2013). The highest VW/BW of hypertrophy of diabetic rats in this study reflects the increased dimensions of the LV.

The increase in pro-inflammatory cytokines in diabetes could also have influenced the levels of cardiac adiponectin and contractile dysfunction in the present study. Inflammatory cytokines can attenuate cardiomyocyte contractility directly through the immediate reduction of the Ca^{2+} transient, via alterations in sarcoplasmic reticulum function and indirectly through attenuation of myofilament calcium sensitivity, through nitric oxide-dependent attenuation (Nian et al., 2004; Duncan et al., 2007; Wen et al., 2013). Sustained expression of TNF-α can also lead to decreased sarcoplasmic reticulum Ca^{2+} ATPases (SERCA2) expression, which is essential for the re-uptake of calcium in an energy-dependent manner after excitation-contraction of the cardiomyocyte (Nian et al., 2004). However, the relationship of these molecular changes with myocardial structural remodeling and

the cellular inflammatory mechanisms in diabetic cardiomyopathy requires further investigation.

We also found that TNF-α production was attenuated in the exercised diabetic animals demonstrating a potential cardioprotective effect provided by exercise training against future cardiovascular injuries. Evidences support the idea that regular exercise may suppress proinflammatory cytokines TNF-α, CRP, and IL-6 levels and also increases anti-inflammatory cytokines such as IL-10, IL-4, TGF-β (Plaisance et al., 2006; Bruunsgaard et al., 2005; Hopps et al., 2011).

In the present study T1DM prolonged the time required for peak cell contraction and the time to half relaxation of left ventricular cardiomyocytes, which is in concert with recent studies conducted in our laboratory (Silva et al., 2011). Cardiomyocytes of diabetic animals may reduce the expression of proteins such as CaMKII, NCX, RyR2, SERCA2 and phospholamban (PLB). These changes lead to a delay the availability of Ca^{2+} for cell contraction and affect the cardiac function of diabetic rats (Bidasee et al. 2003; Bidasee et al. 2004; Bidasee et al. 2008, Stolen et al. 2009, Choi et al., 2002). In addition, cardiomyocytes relaxation depends on the removal of Ca^{2+} from the cytosol: to the sarcoplasmic reticulum (SR) by SERCA2 and PLB; to the extracellular medium by NCX and sarcolemmal Ca^{2+} ATPase; and to the mitochondria by transport of mitochondrial Ca^{2+} (Bers, 2008). In diabetic cardiomyopathy both expression and function of these cellular structures are reduced, and hence the rate of Ca^{2+} removal from the cytosol is diminished (Rajesh et al., 2012; Bidasse et al., 2008; Choi et al., 2002). Taken together, these changes contributes to slow down cell relaxation (Loganathan et al., 2009).

In our study, left ventricular myocyte shortening was not altered by T1DM. Previous studies show inconsistent results since unchanged (Howarth et al., 2002; Howarth 2010) and reduced cardiomyocyte shortening (Silva et al., 2011; Choi et al., 2002) are demonstrated in diabetic rats. Decreased sensitivity of contractile myofilaments to Ca^{2+} and reduced intracellular concentration of Ca^{2+} are probable mechanisms involved in the reduction cell shortening (Bers et al., 2008).

The swimming training employed here did not affect the contractile properties assessed in the present study (cell shortening, time to peak and time to half relaxation) in either control of diabetic rats. However, previous studies have shown that exercise training can restore the contractile function of cardiomyocytes isolated from diabetic animals (Loganathan et al. 2007; Shao et al. 2009; Stolen et al., 2009).

Adaptations to regular exercise can accelerate the availability of cytosolic Ca^{2+} and increase the rate of ATP hydrolysis, increase the expression and/or the activity of RyR2 and sensitivity of contractile myofilaments to Ca^{2+} in diabetic animals (Shao et al., 2009). Moreover, the swimming program applied by Silva et al, (2011) reduced the relaxation time of cardiomyocytes of diabetic animals. This effect has been attributed to the ability of regular exercise to increase the speed of removal of Ca^{2+} from cytosol via increased expression of SERCA2 and PLB (Bers et al., 2008), normalization of NCX expression and function, reduction in the phosphorylation of CaMKII and restoration of the density of transverse tubules (Stolen et al., 2009). Previous studies have demonstrated that cardiac myocyte unloaded shortening may be affected or not by exercise training (Silva et al., 2011; Natali et al., 2002; Howarth et al., 2010). It is worthy to note that, in vivo, cardiomyocytes are connected to each other in an arrangement intimately associated to extracellular matrix and are subject to varying mechanical loads along cardiac tissue. In fact, force development in mechanically loaded cardiomyocytes from trained rats is significantly greater than in those from sedentary rats (Natali et al., 2002). Probably some of the beneficial effects of exercise on myocardial function may be due either to direct effects on cardiac muscle and/or secondary effects including improved glucose metabolism and/or alterations in collagen content of the heart and its associated effects on compliance (Searls et al., 2004; Silva et al., 2011; Silva et al., 2013). Moreover, the absence of effects of exercise training protocols on cardiomyocyte contractile function may be due to insufficient intensity and/or duration of exercise.

In this study, exercise training had no significant effects on body mass in either diabetic or control rats, a result that is consistent with previous studies (Howarth et al., 2010; Silva et al., 2013; Silva et al., 2011). Pathological cardiac hypertrophy induced by experimental diabetes has been related in previous studies. In addition, the apparent absence of an effect of exercise on body mass in either control or diabetic rats probably is due to anabolic and catabolic effects of the exercise regime on muscle protein and fat metabolism, respectively.

Physical exercise is an important factor to improve the balance between glycemic control, inflammation, and cardiovascular risk in T1DM (Rosa et al., 2010). However, we observed that after a 12-hour overnight fast blood glucose was not altered by exercise in either diabetic or control rats. These results are consistent with other reports (Silva et al., 2011; Silva et al., 2013). It is possible that there has

been an increase in glucose uptake through GLUT1 and GLUT4 transporters via activation of AMPK in ED animals and its counterregulatory action of glucagon has helped in the maintenance of hyperglycemia (An and Robrigues, 2006). Studies have demonstrated that exercise was able to improve glucose metabolism in diabetic rats with the reduction of blood glucose levels (Aronson et al., 1997; Gomes et al., 2009). However, it is not clear whether the apparent absence of an effect of exercise on blood glucose level in diabetic rats may be due to the duration and intensity of exercise protocol, the type and severity of diabetes in the animal model or the age of the animals.

In this study T1DM reduced the resting HR in growing rats, but no statistical difference between exercised and sedentary diabetic animals was found. Previous studies reported a reduced HR in adult (De Angelis et al., 2000; Smirnova et al., 2006), adolescent (Lucini et a., 2012) and growing rats with T1DM (Silva et al., 2013), possibly due to autonomic dysfunction. In fact, bradycardia is a very early indication of diabetic cardiomyopathy (Smirnova et al., 2006). Moreover, cardiovascular autonomic neuropathy in diabetes commonly leads to abnormalities in HR control and vascular dynamics (Maser at al., 2003). Additionally, degenerative changes in autonomic neurons have been observed from three days to several weeks after STZ injection in rats (De Angelis et al., 2000). On the other hand, exercise training can prevent cardiac autonomic nervous dysfunction in diabetes (Chimen et al., 2012; Yardley et al., 2012) and reverse bradycardia in these animals (De Angelis et al., 2000).

Conclusion

In summary, our results demonstrate that left ventricle of growing rats with STZ-induced diabetes displays decreased level of total and HMW adiponectin, inflammatory response along with pathological structural remodeling. Low-intensity swimming training promoted a non-significant increasing trend in the levels of IL-10, attenuates TNF-α and pathological remodeling, and increases capillary density in the left ventricle of these animals. These positive changes coexist with cardiomyocyte contractile dysfunction and reduced HMW adiponectin. These results provide insight into the beneficial effects of exercise on the myocardial complications caused by T1DM.

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CONCLUSÕES GERAIS

- Os efeitos crônicos do diabetes induzido por STZ ocorrem no miocárdio do VE de ratos Wistar púberes e promovem cardiomiopatia diabética, comprovada por alterações morfológicas e morfométricas.
- O VE de animais diabéticos apresentou remodelamento estrutural com aumento de colágeno total e de fibras reticulares na matriz extracelular, acúmulo de glicogênio, desorganização da histoarquitetura do miocárdio, infiltrado inflamatório e necrose de cardiomiócitos.
- Os níveis cardíacos de adiponectina total, adiponectina HMW, IL-10 e óxido nítrico foram significativamente menores nos animais diabéticos.
- O exercício físico não alterou de forma significativa os níveis cardíacos de adiponectina total, adiponectina HMW e IL-10 no VE de ratos diabéticos e não diabéticos.
- O diabetes ocasionou resposta inflamatória local com aumento dos níveis de TNF-α no VE.
- O programa de natação de baixa intensidade atenuou a deposição de colágeno total, o acúmulo de glicogênio, os níveis de TNF-α e as alterações histopatológicas avaliadas no miocárdio do VE dos animais diabéticos.
- O percentual de capilares foi significativamente menor no VE dos animais diabéticos.
- O programa de natação induziu aumento no percentual de capilares no miocárdio dos animais diabéticos e não diabéticos e aumento nos níveis de óxido nítrico no VE dos animais não diabéticos.
- Os cardiomiócitos isolados do VE dos animais diabéticos apresentaram prolongamento do tempo para o pico de contração e do tempo para 50% de relaxamento, mas não houve diferença na amplitude de contração.
- O programa de natação aplicado não alterou a amplitude de contração, o tempo para o pico de contração e o tempo para 50% de relaxamento celular em cardiomiócitos isolados do VE de ratos diabéticos e não diabéticos.