UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS - UFSCar-UNESP

Vinicius Guzzoni

Remodelamento da matriz extracelular e respostas cardíacas funcionais em ratos idosos submetidos a treinamento resistido.

SÃO CARLOS - SP

2016

FEDERAL UNIVERSITY OF SÃO CARLOS

BIOLOGICAL SCIENCE AND HEALTH CENTER

INTERINSTITUTIONAL POST-GRADUATION PROGRAM IN PHYSIOLOGICAL SCIENCES - UFSCar-UNESP

Vinicius Guzzoni

Effects of high-intensity resistance training in the extracellular matrix (ECM) and diastolic function in left ventricle of old male rats.

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REMODELAMENTO DA MATRIZ EXTRACELULAR E RESPOSTAS CARDÍACAS FUNCIONAIS EM RATOS IDOSOS SUBMETIDOS A TREINAMENTO RESISTIDO.

Tese apresentado ao Programa de Pós Graduação em Ciências Fisiológicas, para a obtencção do título de Doutor em Ciências Fisiológicas.

Orientadora: Profa. Dra. Heloísa S. Selistre de Araujo
Co-orientadora: Profa. Dra. Ana Paula Davel

SÃO CARLOS - SP
2016

Ficha catalográfica elaborada pelo DePT da Biblioteca Comunitária UFSCar
Processamento Técnico
com os dados fornecidos pelo(a) autor(a)

Guzzoni, Vinicius
G993r Remodelamento da matriz extracelular e respostas cardíacas funcionais em ratos idosos submetidos a treinamento resistido / Vinicius Guzzoni. -- São Carlos : UFSCar, 2016.
67 p.

Tese (Doutorado) -- Universidade Federal de São Carlos, 2016.

1. Envelhecimento. 2. Treinamento resistido. 3. Ventrículo esquerdo. 4. Matriz extracelular. 5. Função diastólica. I. Título.

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas

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TESE DE DOUTORADO

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Universidade Federal de São Carlos

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AGRADECIMENTOS

A DEUS sobre tudo e todos!

À Nossa Senhora e todos os meus Santos Intercessores.

À minha família, meus amados pais, Vitor e Zaira pelo incondicional apoio e amor.

Às minhas irmãs Christina e Viviane Guzzoni pelo respaldo e carinho.

Em especial, agradeço do fundo do meu coração ao meus tios José Pedro Parise e Marcia Gavioli Parise, por me conceder um ambiente muito agradável para residir e extremamente propício para estudar na cidade de São Carlos.

À minha avó Ana Florinda (dona Florinha), mulher indescritível, sábia, que me auxiliou em todos os aspectos. Devo muito a esta mulher a concretização deste trabalho.

Aos meus avós falecidos Leopoldina Motta Guzzoni, Vito Guzzoni e Nelson Parise pela proteção diária.

À todos os membros da minha família em geral, maior graça que Deus me concedeu, o pilar da minha vida. Um eterno obrigado! Tudo foi feito por e para cada um de vocês. Em destaque, ao meu tio Nelson Parise, meu exemplo de vida e profissão e Fran, minha tia, com sua ternura, humildade e inteligência que cativam as pessoas.

À minha afilhada Vitória de França Parise que me trouxe uma alegria indescritível e responsabilidade para as lutas cotidianas.

À UFSCar, na pessoa do Reitor Prof. Dr. Targino de Araújo Filho.

À minha orientadora Profa. Dra. Heloísa Sobreiro Selistre de Araújo pelo total auxílio, supervisão e competência profissional na condução deste estudo. Eternamente grato por seus ensinamentos. Obrigado por me proporcionar um ambiente rico e vasto para execução deste estudo.

À Rita de Cássia Marqueti Durigan e João Luiz Quagliotti Durigan, precursores deste trabalho e pessoas imprescindíveis na minha formação acadêmica e pessoal. Sem a confiança deles, nada do que foi escrito neste trabalho aconteceria.

Aos estudantes de Iniciação Científica Marcelo Mekaro e Tayná Costa pelo total comprometimento em me ajudar em experimentos e atividades relacionadas com este estudo.

À co-orientadora deste estudo, Ana Paula Davel pelo amparo e orientação. Pela dedicação e disponibilidade em vir até São Carlos para conduzir os experimentos.

Ao Prof. Dr. Orlando Moreira Filho pela concessão do microscópio para as análises histológicas no Departamento de Genética e Evolução desta Universidade.

Ao Prof. Dr. José Antunes Rodrigues e aluno André de Souza Mecawi pela colaboração nos ensaios e calorosa recepção em seu Laboratório no Departamento de Fisiologia da Faculdade de Medicina da Universidade de São Paulo (USP), Ribeirão Preto – SP.

Aos Prof. Dr. Hernandes Faustino de Carvalho e Prof. Dr. Carlos Lenz César e ao técnico Vitor pela orientação e solidário oferecimento do microscópio para os experimentos histológicos no Instituto Nacional de Ciência e Tecnologia de Fotônica Aplicada à Biologia Celular (INFABIC) localizado na UNICAMP, Campinas - SP.

Aos amigos de experimento Fabio Bogni, Fernando Fabrizzi e Giselle Lopes. Vocês foram fundamentais na minha adaptação à São Carlos.

À “família LBBM”: Carol, Carmem, Cintia, Kelli, Camila, Uliana, Patty, Araceli, Livia, Patricia Manzini, Sabrina, Monica, Léo, Taís, Milene, Bruna, Wanessa, Grazi, Rafael, Antonio, Tamires e Victoria. Nosso sadio convívio diário foi fundamental para, acima a construção dessa linda amizade. Levo todos, com carinho, para sempre!

Às grandes companheiras, incentivadoras, amigas, confidentes, Cynthia e Karina, mineiras, que sempre estiveram ao meu lado.

Aos meus amigos de Taquaritinga, muitos para nomear, nenhum para esquecer.

À cada professor das disciplinas que frequentei, da banca de qualificação e do estágio PESCD: Sandra Lia do Amaral, Carlos Cressani, Azair de Souza, Carina Andrade, Gerson J. Rodrigues e Gilberto E. Shiguemoto. Docentes que contribuíram fortemente para a concretização deste estudo.

Ao secretário Alexandre pelo pronto auxílio com documentos, dúvidas e burocracia dos trâmites.

À Bety e Márcia, pelo auxílio em diversas atividades.

Em especial e, com eterna gratidão, à John Lawler grande pesquisador e meu supervisor no Texas (EUA) (Redox Biology & Cell Signaling Laboratory) na Texas A&M University (TAMU), onde realizei meu Doutorado Sanduíche. Inesquecível, indescritível! Obrigado por abrir as portas do seu Lab onde pude saborear e vivenciar a ciência em um país-referência no segmento.

À James Matthew Kuczmarski, Jeffrey M. Hord e Curran Reddy, companheiros e “irmãos” de laboratório na TAMU. Vocês foram meus maiores professores. Muito obrigado, sempre!

Aos meus eternos e grandes amigos americanos Andrew Stivers (e família), Enoc Medina, Lyndsay Phillips, Monika Balckwell e brasileiros Paula Cipriano, Vinicius Venancio e

Barbara Castanheira Ferrara Barbosa. Vocês são parte determinante deste trabalho e da minha vida.

À minha professora de inglês nos EUA Geraldine Wick (Geri). Vc foi/é especial. Infelizmente partiu sem que eu pudesse me despedir. Muito disso é pra você, minha querida amiga.

Às minhas escolas de inglês de São Carlos, The FIELDS e The Four.

À minha comunidade católica de São Carlos (São Nicolau de Flue) na pessoa do Pároco Eduardo Malaspina, grande discípulo, e a cada membro da minha Célula Católica Santa Izabel pelas constantes orações, em especial a líder e eterna amiga Maria Clara Santos. Obrigado por sempre estar comigo Clara.

À Paróquia de São Sebastião de São Carlos, lugar maravilhoso para professar a fé. E ao meu querido pai, pároco de minha cidade, padre João.

Aos meus grandes e eternos amigos João e Fabiana Zepon. Sou extremamente grato pelos ensinamentos e carinho para comigo. Conhecer vocês foi uma benção de Deus!

Aos meus amigos de futebol da USP. Obrigado por me proporcionarem um momento de lazer e amizade às terças-feiras. Espero que nossa amizade sempre perdure.

À Igreja A&M Church, em College Station (TX), cidade que residi no período de Doutorado Sanduíche. Pessoas maravilhosas que me acolheram e certamente continuam a orar por mim.

À cada pessoa que trabalhou indiretamente, mas com exímio valor. Ao restaurante universitário, biblioteca, equipe de limpeza, guardas, entre muitos outros que fazem parte desta Instituição.

Agradeço às dificuldades, medos e inseguranças que deparei aos longos desses anos. No enfrentamento destes, guiado pelas bênçãos Divinas, certamente vieram os mais belos aprendizados.

À CAPES, por me conceder as bolsas de estudo (nacional e internacional).

À vida, por me proporcionar muitos desafios e pessoas maravilhosas que me ajudaram e me fizeram um pouquinho mais de mim, de alguma maneira.

“A verdade é um fim inatingível na ciência”
Augusto Cury

**A Tese de Doutorado está redigida na língua inglesa, porém há uma secção em português entre as páginas 12 e 16 contendo: introdução, objetivos e justificativa, como detalhado no Sumário (Table of Contents).*

LISTA DE ILUSTRAÇÕES

LIST OF ABREVIATION

ECM – extracellular matrix;

RT – resistance training;

LV – left ventricle;

BW – body weight;

SBP – systolic blood pressure;

DBP – diastolic blood pressure;

MBP – mean blood pressure;

LVSP – left ventricle systolic pressure;

LVEDP – left ventricle end-diastolic pressure;

ANG-II – angiotensin II-type;

ANP – atrial natriuretic peptide.

Table 1 - List of oligonucleotides primers: **COL1A1**: type-IA1 collagen; **COL3A1**: type-III collagen; **TGF-β1**, transforming growth factor beta-1; **MMP-2**: metalloproteinase 2; **MMP-9**: metalloproteinase 9; **TIMP-1**: tissue inhibitor of metalloproteinases-1; **TIMP-2**: tissue inhibitor metalloproteinases-2; **RPS27a**: ribosomal protein S27a.

Primer name	Forward	Reverse
COL1A1	ATCAGCCCAAACCCCAAGGAGA	CGCAGGAAGGTCAGCTGGATAG
COL-III	TGATGGGATCCAATGAGGGAGA	GAGTCTCATGGCCTTGCGTGTTT
TGF-β1	CCCCTGGAAAGGGCTCAACAC	TCCAACCCAGGTCCTTCCTAAAGTC
MMP-2	CTGGGTTTACCCCCTGATGTCC	AACCGGGGTCCATTTTCTTCTTT
MMP-9	GGATGTTTTTGATGCCATTGCTG	CCACGTGCGGGCAATAAGAAAG
TIMP-1	ATAGTGCTGGCTGTGGGGTGTG	TGATCGCTCTGGTAGCCCTTCTC
TIMP-2	GGACACGCTTAGCATCACCCAGA	GTCCATCCAGAGGCACTCATCC
RPS27a	GACCCTTACGGGGAAAACCAT	AGACAAAGTCCGGCCATCTTC

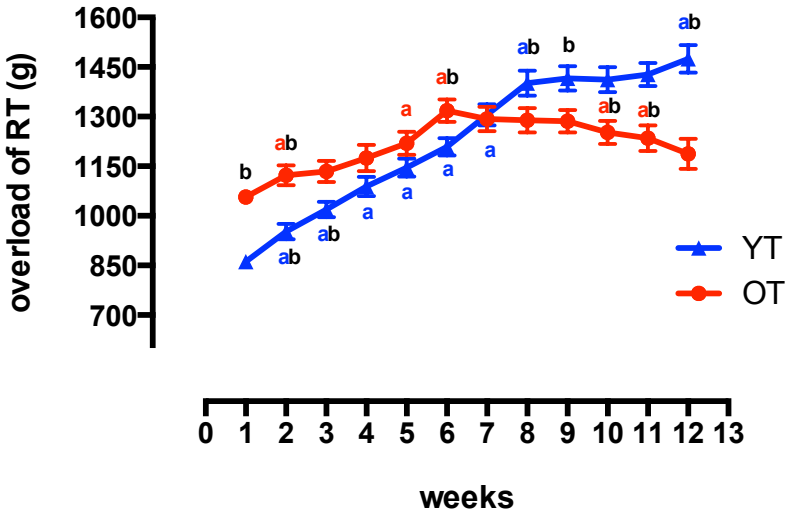
Source: author

Table 2 - Body weight (BW) and cardiac hypertrophy rates of the experimental groups. **BWi**: body weight at the beginning of RT; **BWf**: body weight in the end of RT; **LV**: left ventricle weight; **Whole heart**: whole heart weight; **LV/BW**: LV weight/body weight ratio; **LV/tibia**: LV weight/tibia length; Values are expressed by means ± SEM. Groups: **YS**: young sedentary; **YT**: young trained; **OS**: old sedentary; OT: old trained. Different characters mean statistical difference (ANOVA two-way, Tukey *post-hoc* test $p \leq 0.05$). $^{+}$**YT vs. YS;** ***OS vs. YS;** $^{\#}$**OT vs. YT**. n = 10/group.

	YS	**YT**	**OS**	**OT**
BWi, *g*	313 ± 4	304 ± 6	510 ± 13	519 ± 9
BWf, *g*	486 ^{a} ± 10	426 $^{a\,+}$ ± 9	467 ^{a} ± 15	480 ^{a} ± 9
LV, *mg*	1058 ± 21	902^{+} ± 19	1061 ± 39	1053$^{\#}$ ± 26
Whole heart, *g*	1.34 ± 0.03	1.16^{+} ± 0.03	1.50* ± 0.06	1.47$^{\#}$ ± 0.03
Tibia, *cm*	4.46 ± 0.03	4.43 ± 0.05	4.58 ± 0.04	4.64 ± 0.03
LV/BWf, *mg/g*	2.18 ± 0.02	2.27 ± 0.03	2.20 ± 0.08	2.20 ± 0.04
LV/tibia, *mg/cm*	244 ± 5.7	208^{+} ± 9.7	223 ± 11	236 ± 7.8

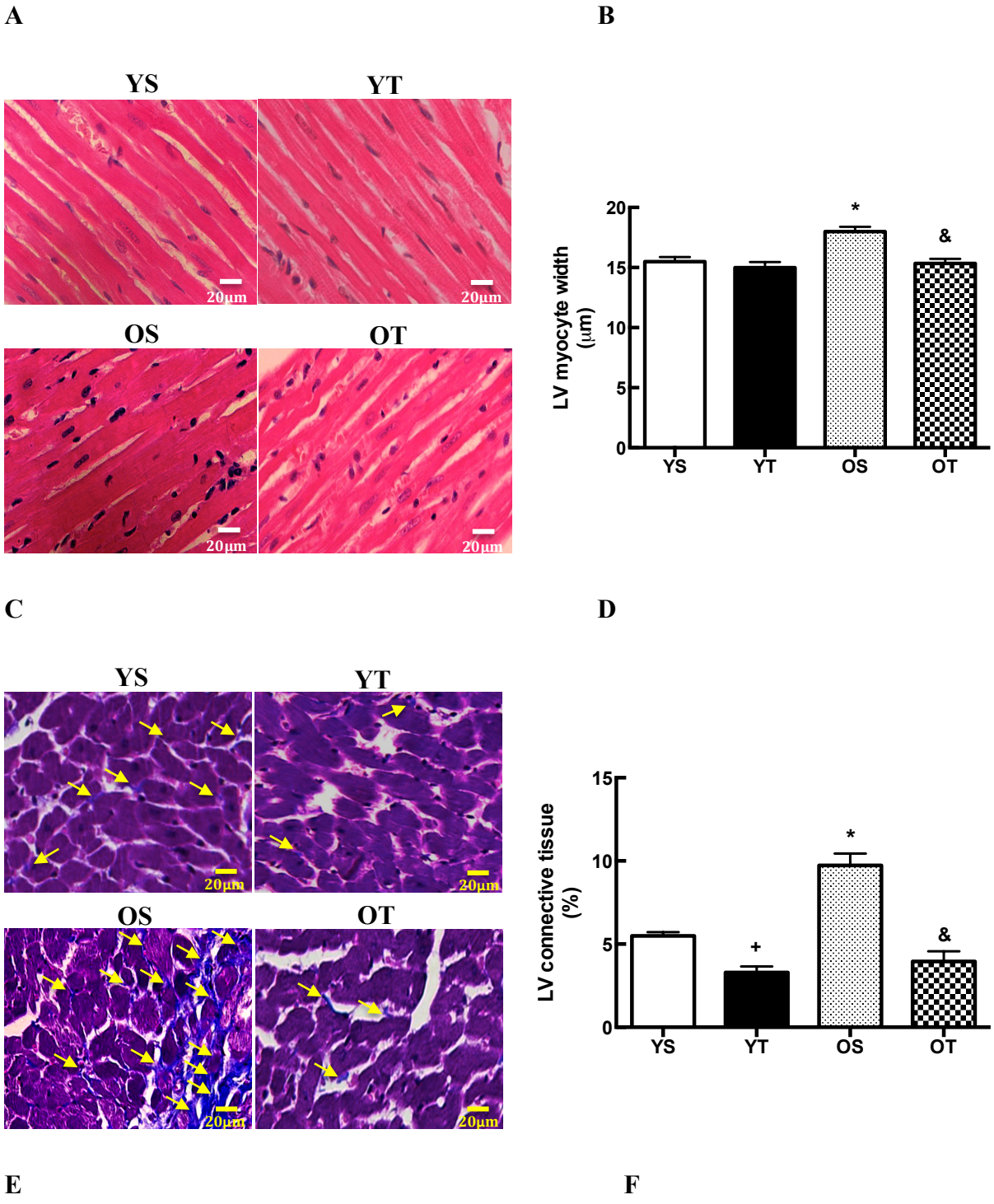
Source: Author

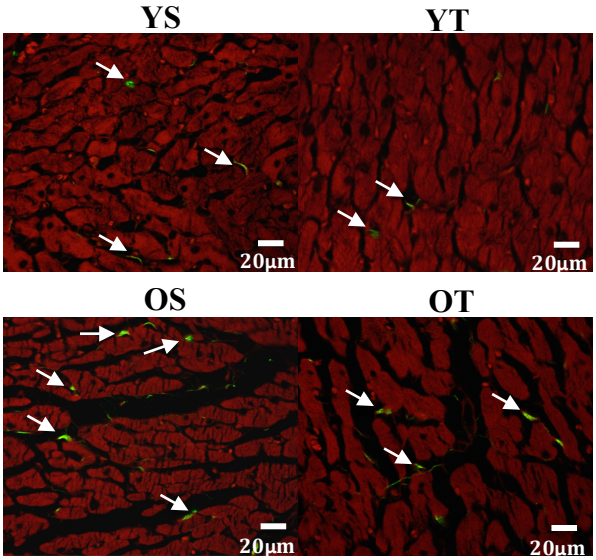
Figure 1 - Overload (g) carried by old and young trained rats along the 12 weeks of RT. Blue and red lines correspond to the young trained rats (YT), and old trained rats (OT) respectively. n=31; [a] significant difference in comparison to the previous week (paired t Student test; $p \leq 0.05$); [b] significant difference between YT x OT (unpaired t Student test; $p \leq 0.05$). Values are expressed as means ± SEM.

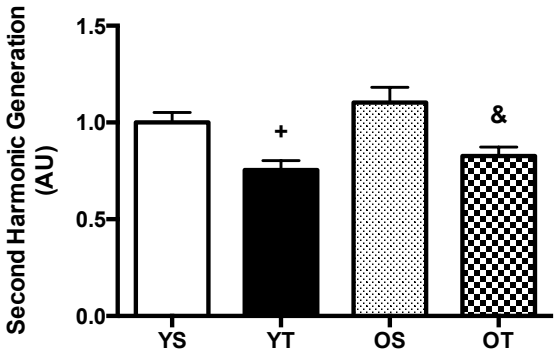


Source: Author

Figure 2 - LV images of hematoxilin-eosin staining (H&E), 20X (**A**), Masson´s trichrome imaging, 20X (**B**) and Second harmonic generation (SHG), 40X (**C**). Connective tissue is staining in blue (yellow arrows, **B**) and in green collagen (white arrows, **C**); Values are expressed as means ± SEM. LV myocyte width (μm) (**A**), % of LV connective tissue (**B**) and %collagen deposition/area (**C**). Groups: **YS**: young sedentary; **YT**: young trained; **OS**: old sedentary; **OT**: old trained. Different characters mean statistical difference (ANOVA two-way, Tukey *post-hoc* test $p \leq 0.05$). $^{+}$**YT vs. YS;** ***OS vs. YS;** $^{\&}$**OS vs. OT**. n = 7/group.

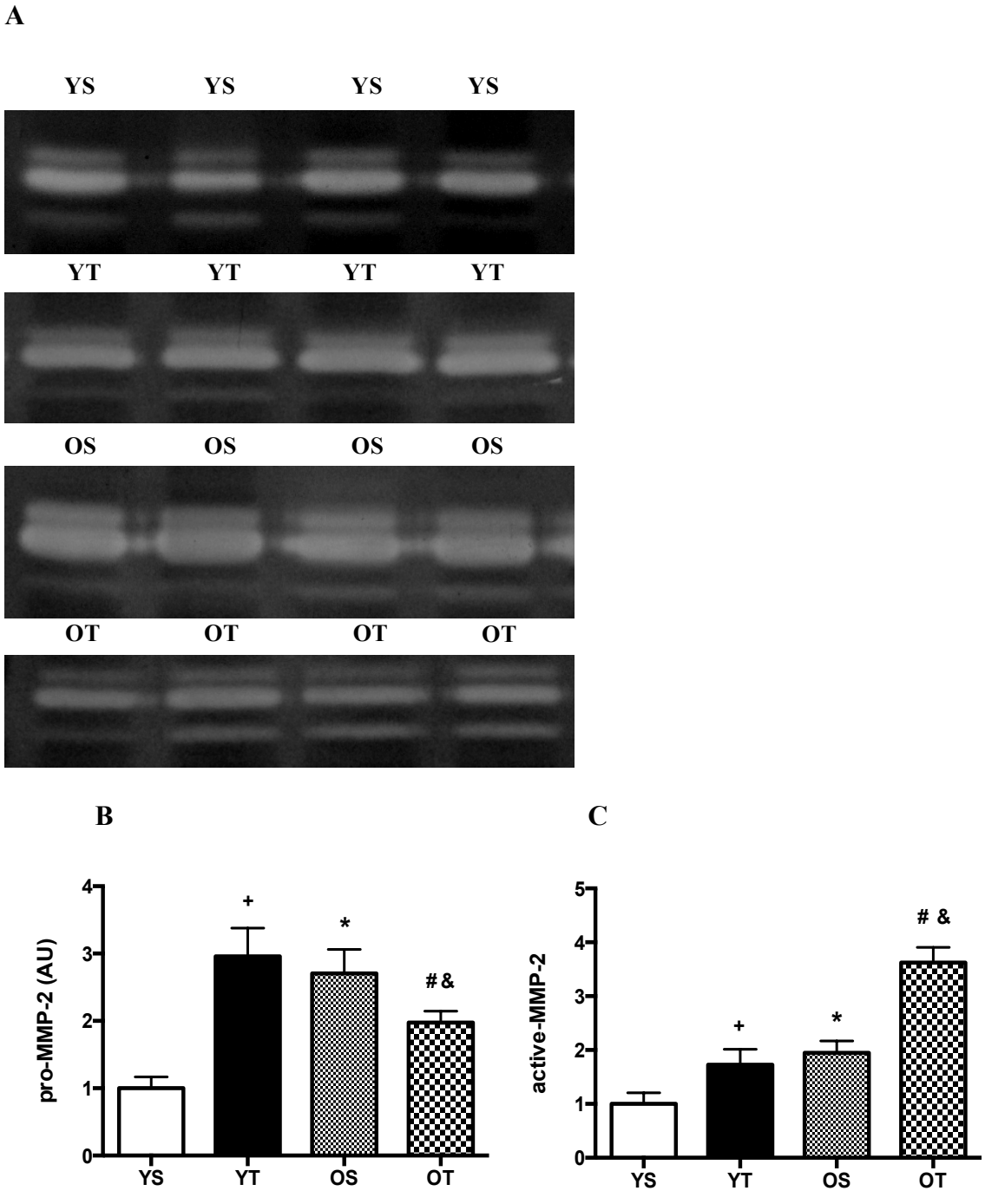






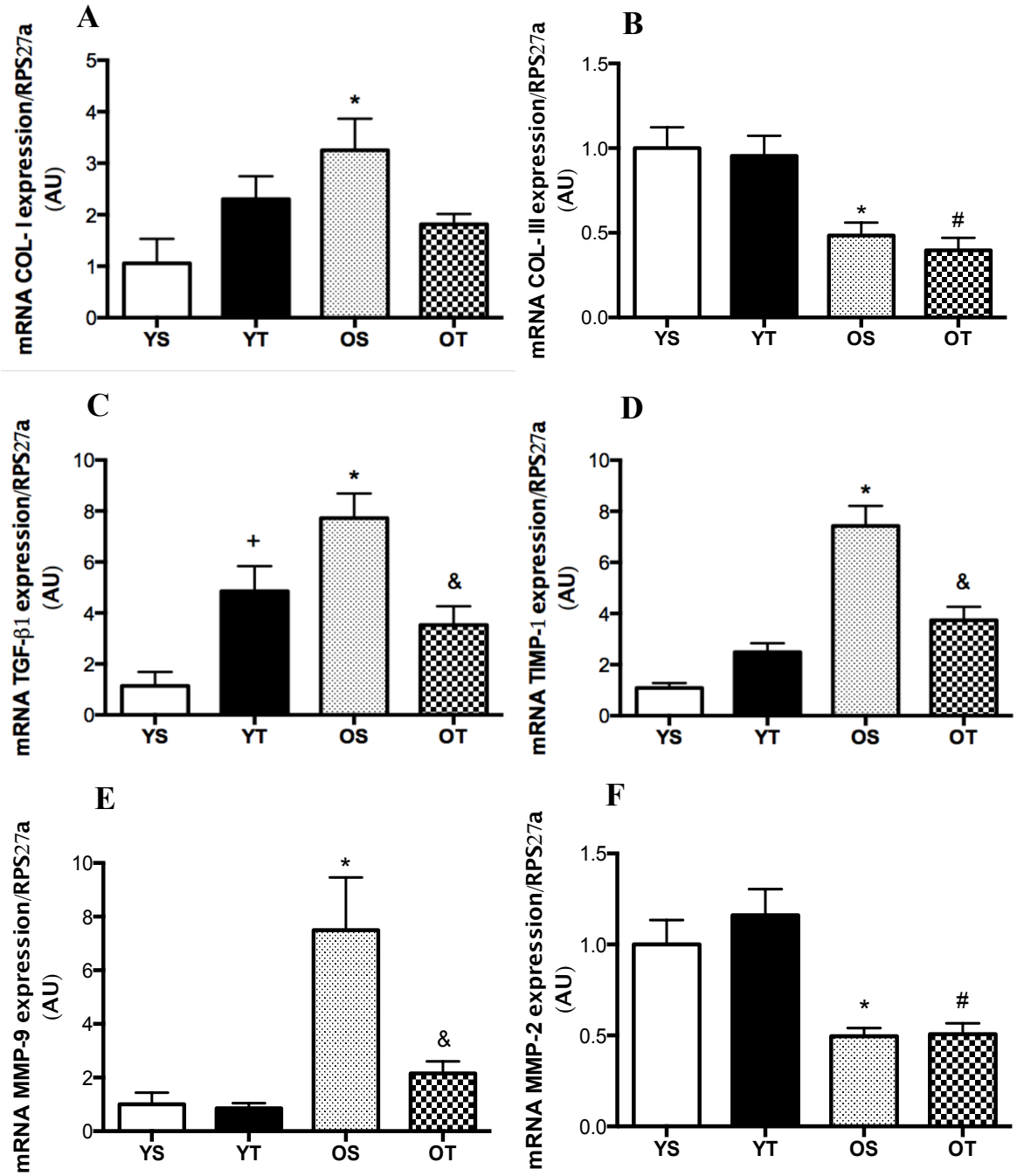
Source: author

Figure 3 - Bands representing the catalytic activity of MMP-2 in the gel (**A**); Densitometry of pro-MMP-2 (**B**) and active-MMP-2 (**C**). Values are expressed as means ± SEM. Groups; **YS**: young sedentary; **YT**: young trained; **OS**: old sedentary; **OT**: old trained. Different characters mean statistical difference (ANOVA two-way, Tukey *post-hoc* test $p \leq 0.05$). **$^{+}$YT vs. YS; *OS vs. YS; $^{\#}$OT vs. YT; $^{\&}$OS vs. OT**. n = 10/group.



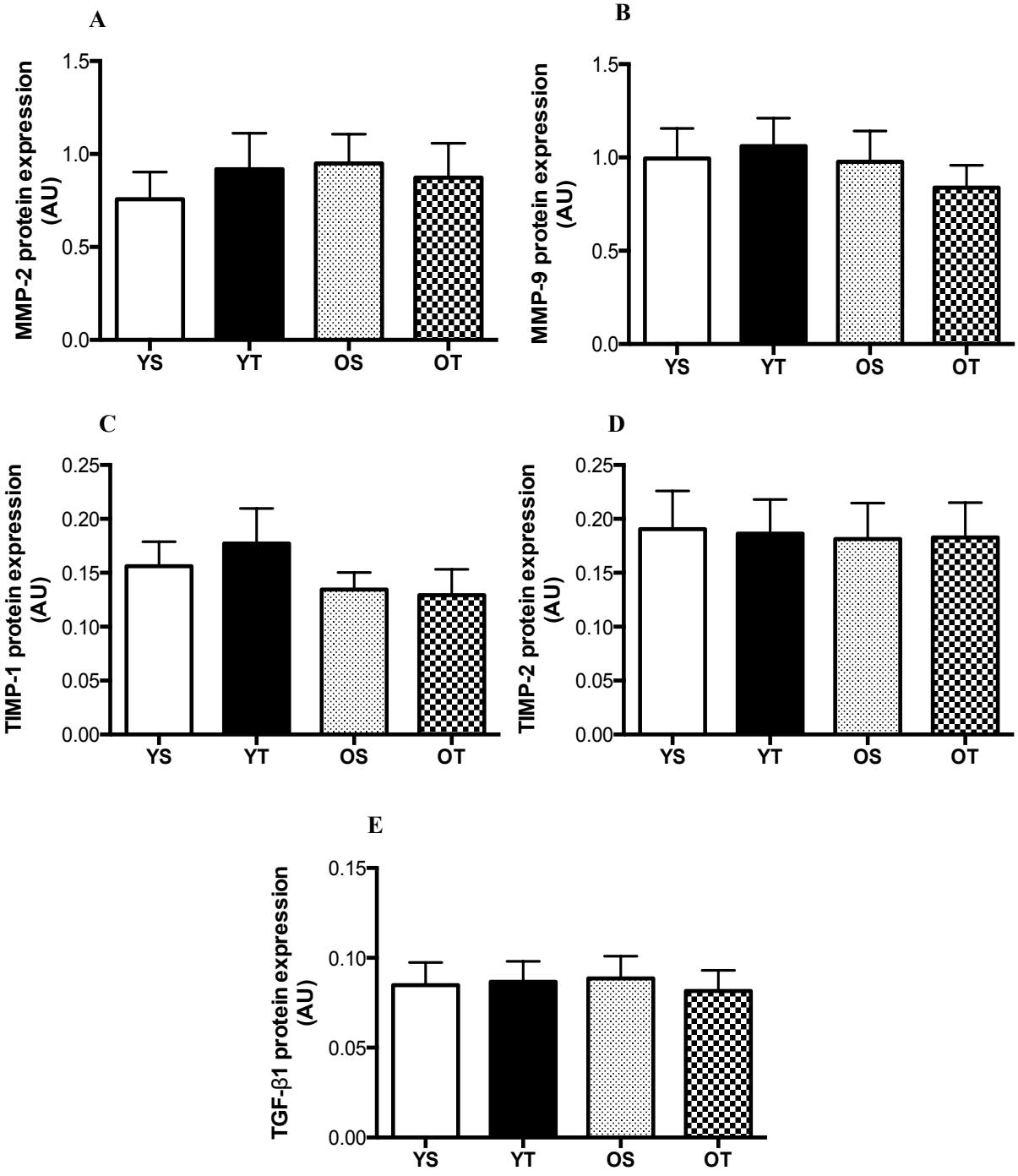
Source: author

Figure 4 - Gene expression of collagen I (COL-I) (**A**), collagen III (COL-III) (**B**), transforming growth factor-β1 (TGF-β1) (**C**), tissue inhibitor of metalloproteinase 1 (TIMP-1) (**D**), metalloproteinase-9 (MMP-9 (**E**) and metalloproteinase-2 (MMP-2) (**F**) in the LV tissues measured by real time PCR. Values are expressed as means ± SEM and normalized by the reference gene (RPS27a). Groups; **YS**: young sedentary; **YT**: young trained; **OS**: old sedentary; **OT**: old trained. Different characters mean statistical difference (ANOVA two-way, Tukey *post-hoc* test $p \leq 0.05$). $^{+}$**YT vs. YS;** ***OS vs. YS;** $^{\#}$**OT vs. YT;** $^{\&}$**OS vs. OT**. n = 8/group.



Source: author

Figure 5 - Protein expression of metalloproteinase-2 (MMP-2) (**A**), metalloproteinase-9 (MMP-9) (**B**); tissue inhibitor of metalloproteinase 1 (TIMP-1) (**C**), tissue inhibitor of metalloproteinase 2 (TIMP-2) (**D**) and transforming growth factor-β1 (TGF-β1) (**E**) in LV tissues, measured by Western immunoblot. Values are expressed as means ± SEM. Groups; **YS**: young sedentary; **YT**: young trained; **OS**: old sedentary; **OT**: old trained. No statistical differences were observed in all proteins. n=9/group.



Source: author

Table 3 - Arterial and intra-ventricular measurements.

	YS	**YT**	**OS**	**OT**
SBP, *mmHg*	122.8 ± 2.7	129.9 ± 4.8	100.5* ± 6.4	109$^{\#}$ ± 4.1
DBP, *mmHg*	87.6 ± 3.1	92.6 ± 2.7	70* ± 4.9	78$^{\#}$ ± 2.8
MBP, *mmHg*	104.3 ± 3.2	108.9 ± 3.2	84.8* ± 5.5	92.8$^{\#}$ ± 3.2
HR *bpm*	204 ± 8	195 ± 6	200 ± 18	189 ± 6
LVSP, *mmHg*	135.8 ± 4	137.7 ± 3	107.6* ± 7.7	99.8$^{\#}$ ± 5.4
+dP/dt, *mmHg/s*	5586.7 ± 347	5613 ± 365	6263.6 ± 590	5298.3 ± 448
LVEDP, *mmHg*	7.3 ± 1	7.3 ± 0.8	10.9* ± 0.4	8.4$^{\&}$ ± 0.9
-dP/dt, *mmHg/s*	-4052.6 ± 308	-4167.5 ± 185	-3232 ± 424	-3335.3$^{\#}$ ± 230
Tau, *s*	0.033 ± 0	0.028 ± 0	0.016* ± 0	0.017$^{\#}$ ± 0

Source: Author

Subtitle: **SBP**: systolic arterial pressure; **DBP**: diastolic arterial pressure; **MBP**: mean arterial pressure; **HR**: heart rate; **LVSP**: left ventricle systolic pressure; **LVEDP**: end-diastolic pressure; +**dP/dt**: positive derivate of LV pressure; **-dP/dt**: negative derivate of LV pressure; **Tau**: time constant of LV relaxation; au: arbitrary unit; s: second. Values are expressed as means ± SEM. Groups: **YS**: young sedentary; **YT**: young trained; **OS**: old sedentary; **OT**: old trained. Different characters mean statistical difference (ANOVA two-way, Tukey *post-hoc* test $p \leq 0.05$). $^{+}$**YT vs. YS;** ***OS vs. YS;** $^{\#}$**OT vs. YT;** $^{\&}$**OS vs. OT**. n = 8/group.

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RESUMO

INTRODUÇÃO: O remodelamento da matriz extracelular (MEC) cardíaca é um evento dentre estas mudanças estruturais no ventrículo esquerdo (VE) que é orquestrado pelos níveis de metaloproteinases (MMP) e seus inibidores endógenos (TIMPs). Na tentativa de prevenir tais efeitos decorrentes da idade avançada, o exercício aeróbico tem sido sugerido por melhorar a fibrose e a função cardíaca. Entretanto, os efeitos do treinamento resistido (TR) nestas variáveis necessitam de melhor compreensão. **OBJETIVO:** investigar os efeitos crônicos do TR de alta intensidade na MEC do VE e a função cardíaca em ratos idosos. **METODOLOGIA:** ratos de 3 e 21 meses de idade foram designados como grupos: jovem sedentário (YS), jovem treinado (YT), idoso sedentário (OS) e idoso treinado (OT). Os grupos treinados foram submetidos à um protocolo de 12 semanas de escalada sob alta intensidade, 3 vezes por semana. Decorridos 48h pós última sessão de treino, medidas hemodinâmicas foram registradas: pressão arterial sistólica (SAP), pressão arterial diastólica (DAP), pressão arterial média (MAP), frequência cardíaca (HR), constante de decaímento da pressão ventricular (Tau), derivada temporal positiva da pressão intraventricular (+dP/dt), razão de decaímento da pressão ventricular (-dP/dt), pressão arterial sistólica máxima do VE (LVSP) e pressão arterial diastólica final do VE (LVEDP). Largura dos cardiomiócitos, % de tecido conectivo e de colágeno intersticial foram analisados no VE. A atividade da MMP-2 foi detectada por zimografia, assim como a expressao gênica e proteica de alguns constituintes da MEC. Moduladores da hipertrofia e fibrose cardíaca, angiotensina II (Ang-II) e peptídeo natriurético atrial (ANP) foram avaliados. **RESULTADOS:** a largura do cardiomiócito e a concentração de colágeno diminuíram em ratos OT comparados ao grupo OS. TR aumentou a atividade da MMP-2 e atenuou os aumentos na LVEDP de ratos idosos. Ratos OT não apresentaram alterações significativas na expressão dos elementos da MEC e nos peptídeos cardíacos Ang-II e ANP. O TR diminuiu significativamente a expressão gênica elevada de TIMP-1, TGF-β e COL-1, observados no grupo OS. **CONCLUSÃO:** o TR foi eficaz em diminuir o colágeno cardíaco o que pode ser associado com a melhora na função diastólica, o que pode estar relacionado com o aumento na atividade da MMP-2 em VE de ratos idosos. O TR atenuou a via de sinalização TGF-β–TIMP-1–COL-1, a nível transcricional. Portanto este estudo revela a importância do treinamento resistido na homesostase da MEC e melhora da função diastólica em modelo experimental de idoso.

Palavras-chave: Envelhecimento. Treinamento Resistido. Ventrículo Esquerdo. Matriz Extracelular. Função Diastólica.

ABSTRACT

INTRODUCTION: It is well documented that aging causes morphological and functional alterations in the heart. Cardiac ECM remodeling is one event of structural changes in left ventricle (LV), which is modulated by MMPs/TIMPs balance and may lead to cardiac fibrosis. To prevent such effects inherent of aging, aerobic exercise training has been suggested to improve the cardiac fibrosis and function. However the effects of resistance training (RT) remains unclear. Whether that RT could alter cardiac function following cardiac ECM remodeling is uncertain. **PURPOSE:** to investigate the chronic effects of high intensity resistance training (RT) in the extracellular matrix (ECM) remodeling of left ventricle (LV) and cardiac function in old rats. **PROCEDURES:** Rats with 3 and 21 months-age were assigned as young sedentary (YS), young trained (YT), old sedentary (OS) and old trained (OT). The trained groups (YT and OT) were submitted to high-intensity RT protocol (3 times a week during 12 weeks). After 48h post-training, hemodynamic and intra-ventricular pressures were recorded. LV myocyte width, LV connective tissue and collagen fibrils were analyzed. MMP-2 activity, gene and protein expression from ECM components as well as angiotensin II (Ang-II) and atrial natriuretic peptide (ANP) were evaluated **FINDINGS:** LV myocyte width and connective tissue were reduced in OT rats. RT increased the MMP-2 activity in OT rats and improved the age-related increase in the left ventricle end diastolic pressure (LVEDP). The RT unchanged Ang-II and ANP in LV of old rats**. CONCLUSION:** RT was effective to decrease LV connective tissue, which was associated with increased ECM remodeling by MMP-2 activity in LV tissue and improvement of LVEDP in aging rats. Our results point out the importance of RT in ECM homeostasis and diastolic function in experimental aging model.

Key-words: Aging. Resistance Training, Left Ventricle, Extracellular Matrix, Diastolic Function.

INTRODUÇÃO

O envelhecimento é um processo inerente a todos os seres vivos que se estabelece como um fator de risco independente para a manifestação de doenças cardiovasculares (LAKATTA; LEVY, 2003; NICCOLI; PARTRIDGE, 2012; STERN; BEHAR; GOTTLIEB, 2003). Entretanto, é difícil determinar precisamente as adaptações cardíacas frente ao processo natural do envelhecimento e àquelas ocasionadas por doenças cardiovasculares decorrentes.

A fibrose cardíaca é uma característica do processo natural de envelhecimento que vem sendo documentado ao longo dos anos (BOLUYT et al., 1994; FEINBERG; VOGELSTEIN, 1984; GOLDSMITH; BRADSHAW; SPINALE, 2013; HORN, 2015; (OLIVETTI et al., 1991ab; QUEREJETA et al., 2004) por favorecer uma maior rigidez da câmara cardíaca asssociado com piora do relaxamento ventricular, o que contribui para o desenvolvimento da disfunção diastólica (HORN, 2015; LAKATTA et al., 1987; REED et al., 2011). A fibrose intersticial constitui um dos eventos do remodelamento cardíaco (HORN, 2015) cuja definição é a resposta compensatória decorrente do aumento da sobrecarga de pressão imposta ao ventrículo esquerdo (VE) - devido ao enrijecimento arterial decorrente da idade - o que leva à alguns eventos celulares, tais como: apoptose e necrose, diminuição do número de cardiomiócitos com consequente hipertrofia das células remanescentes, deposição de gordura visceral epicárdica e acúmulo de proteínas da MEC – a fibrose intersticial (ANVERSA et al., 1990; HORN, 2015; OLIVETTI et al., 1991ab). O acúmulo de tecido fibroso estabelece-se por 2 processos: fibrose reativa ou reparativa. A fibrose reativa ocorre em resposta à um aumento do estresse da parede miocárdica com consequente espessamento das fibras de colágeno já existentes dentro do espaço intramuscular. Por outro lado, fibrose reparativa é a síntese de novas redes de colágeno nos espaços criados por células necróticas e apoptóticas (BURSTEIN; NATTEL, 2008; TARONE et al., 2014; WEBER, 1989). Entretanto, é difícil estabelecer se estes processos contribuem de forma conjunta ou isolada na manifestação da fibrose cardíaca em decorrência do envelhecimento.

De uma forma geral, o acúmulo de colágeno configura a fibrose cardíaca (HORN et al., 2012; JUGDUTT, 2003; LAKATTA; LEVY, 2003). Entretanto o colágeno está inserido dentro de uma uma vasta rede de macromoléculas extracelulares, constituída de moléculas fibrilares e não fibrilares, a matriz extracelular (MEC) que compoe o tecido conjuntivo. A

principal função da MEC é fornecer suporte para as células contráteis – os cardiomiócitos (JOURDAN-LESAUX et al., 2010). Juntamente com a proteína contrátil titina, a MEC também possui papel fundamental na elasticidade e complacência do músculo cardiaco e transmissão do estresse mecânico durante o ciclo cardíaco (GIELEN et al., 2005; NAGUEH et al., 2004; PUGH; WEI, 2001; STEWART et al., 2003). Além do mais, a MEC é um ambiente rico de moléculas sinalizadoras, tais como citocinas, fatores de crescimento e um reservatório de proteases (GOLDSMITH; BORG, 2002; KASSIRI; KHOKHA, 2005). Dessa forma, ela está em constante processo de síntese, degradação e resíntese de proteínas (*turnover* protéico). Por sua vez, o termo remodelamento da MEC é estabelecido como uma sequência de processos de degradação, remoção dos subprodutos, reposição celular e consequente mudança estrutural do tecido (APTE; PARKS, 2015). Frente às mudanças estruturais que ocorrem no coração envelhecido, principalmente hipertrofia e fibrose cardíacas, utilizaremos aqui o termo remodelamento da MEC.

O balanço entre a síntese e degradação das proteínas da MEC é orquestrado por proteases dependentes de zinco, denominadas metaloproteases de matriz (MMPs) cuja atividade é modulada principalmente por seus inibidores endógenos, os chamados inibidores teciduais de metaloproteases, os TIMPs (CREEMERS et al., 2003; JAYASANKAR et al., 2004; MOTT; WERB, 2004; SPINALE et al., 1998). As gelatinases (MMP-2 e MMP-9) são enzimas que degradam gelatinas, os subprodutos da degradação de colágeno devido à ação da colagenase MMP-1 (KASSIRI; KHOKHA, 2005). Entretanto, Foi demonstrada diminuição e aumento na expressão protéica das MMPs e TIMPs respectivamente, em coração de ratos idosos (KWAK et al., 2011). Além do mais, o TGF-β1 é um importante regulador do TIMP-1 e está relacionado com a fibrose no coração envelhecido (BONNEMA et al., 2007; CHEN et al., 2000; TSUTSUI et al., 2007). Angiotensina II (Ang-II) e peptídeo natriurético atrial (ANP) são importantes sinalizadores da fibrose cardíaca e hipertrofia cardíaca, respectivamente (ASAKURA et al., 2002; CHEN et al., 2000; GONZÁLEZ; LÓPEZ; DíEZ, 2004; KNOWLES et al., 2001; LIJNEN; PETROV; FAGARD, 2001; OLIVER et al., 1997; TAKAHASHI et al., 1994; TSUTSUI et al., 2007). A Ang-II atua como uma peptídeo de sinalização *upstream* ao TGF-β1, mediando as respostas relacionadas à fibrose cardíaca (BAKER; ACETO, 1990; SADOSHIMA; IZUMO, 1993; SCHUNKERT et al., 1990; THANNICKAL et al., 2003) assim como da produção de MMPs (KYRIAKIS; AVRUCH, 2001). A ação direta da Ang II no miocárdio também modula a

função cardíaca (KJAER; HESSE, 2001). O ANP é um peptídeo cardíaco associado com as adaptações à sobrecarga cardíaca de pressão (JIN et al., 2000) sendo, portanto, considerado um marcador de hipertrofia patológica (DIETZ, 2005). Embora sua função esteja relacionada com limitação da fibrose cardíaca (LEVIN; GARDNER; SAMSON, 1998; VARDENY; TACHENY; SOLOMON, 2013), concentrações de ANP aumentam no VE de ratos envelhecidos (WU; KWAN; TANG, 1997).

Na tentativa de amenizar a excessiva fibrose cardíaca relacionada à idade avançada, o treinamento aeróbico tem sido amplamente estudado (KWAK et al., 2011; THOMAS et al., 2001; WRIGHT et al., 2014). Os trabalhos, em geral, demonstraram seus efeitos benéficos por promover redução da fibrose cardíaca relacionada com a idade avançada (KWAK et al., 2011; THOMAS et al., 2000), associado com o aumento na expressão MMP-2 e redução na expressão de TGF-β1 e TIMP-1 em VE de ratos idosos (KWAK et al., 2011). Outros estudos sugerem que este efeito possa promover melhora nos parâmetros funcionais cardíacos (CHOI et al., 2009; KWAK; SONG; LAWLER, 2006; LAKATTA; LEVY, 2003; RAYA et al., 1997; TSURUDA et al., 2004). Entretanto, os efeitos do treinamento resistido nesse parâmetros ainda necessitam ser melhor compreendidos. Ainda, se essas alterações estruturais da MEC cardíaca em idoso poderiam ser moduladas pelo treinamento físico resistido e as consequentes respostas cardíacas funcionais são ainda pouco conhecidas. Embora haja poucos estudos sobre os efeitos do treinamento resistido nos parâmetros de remodelamento cardíaco em modelo de idoso, este tipo de treinamento físico tem sido amplamente recomendado para este segmento da população por sua eficácia em mimizar as perdas de força, massa muscular e óssea inerentes ao avanço da idade (EVANS, 1999; HÄKKINEN et al., 1998). Considerando que o treinamento resistido impõe uma sobrecarga de pressão – e de volume – sobre o VE (BERNARDO et al., 2010; GROSSMAN; JONES; MCLAURIN, 1975), nós hipotetizamos que o treinamento resistido aumenta o remodelamento da MEC do VE e melhora a função cardíaca em ratos idosos.

JUSTIFICATIVA

Diante das previsões de um ascendente aumento da população idosa, devido à melhora da expectativa de vida por influência de diversos fatores, estudos contemplando temas sobre o envelhecimento mostram-se relevantes, principalmente no que tange à envelhecer com qualidade de vida.

Clinicamente, a prática de exercício físico com características aeróbicas tem se mostrado eficaz nos aspectos morfológicos e funcionais cardíacos na população idosa. Embora o treinamento físico resistido seja extremamente recomendado para este tipo de população, visto seus benefícios músculo-esqueléticos, ainda se faz necessário uma melhor compreensão dos efeitos deste nos parâmetros cardíacos tanto no aspecto morfológico quanto no funcionamento da bomba cardíaca. Portanto este estudo possui relevância experimental com a intenção de elucidar alguns pontos importantes dentro do tema *remodelamento cardíaco na idade avançada*, especificamente da MEC, de modo que possa contribuir com novas pesquisas na área. Visto a dificuldade em se adequar protolocos de treinamento resistido para modelos experimentais e a escassez de trabalhos científicos até o momento, acreditamos que esta pesquisa venha acrescentar à literatura científica na tentativa de avançar as fronteiras do conhecimento na área em questão.

OBJETIVO GERAL

Testar os efeitos crônicos do treinamento físico resistido de alta intensidade sobre as adaptações morfológicas cardíacas e o remodelamento da MEC em VE de ratos idosos, bem como a função cardíaca.

OBJETIVOS ESPECÍFICOS

Avaliar os aspectos morfológicos do VE de ratos idosos; avaliar a atividade enzimática da MMP-2 do VE em ratos idosos; investigar a expressão genética e protéica dos elementos envolvidos no remodelamento da MEC de VE de ratos idosos e identificar as vias de sinalização *upstream* do remodelamento da MEC em VE de ratos idosos.

INTRODUCTION

Progressive fibrosis is a hallmark of the aging heart, as confirmed in animal and human studies (NEILAN et al., 2013). Left ventricular (LV) hypertrophy and myocardial fibrosis are compensatory responses to changing load conditions in the aged heart which are caused by diminished vascular compliance and elevated cardiac pressure overload (GRAHAM et al., 2011; O'ROURKE; HASHIMOTO, 2007). These changes elevate the ventricular work required to eject blood during systole, resulting in cardiac remodeling (CHEN et al., 1998). In general, cardiac remodeling stands for morphological, cellular, molecular and functional changes in the myocardium caused by alterations in overload placed into the heart (COHN et al., 2000; HORN, 2015; QUARLES et al., 2015). Pathological cardiac remodeling is associated with myocardial infarction, inflammatory myocardial disease, hypertension (pressure overload), aortic regurgitation (pressure overload) and cardiomyopathies. The increased pressure overload elicits compensatory responses to the myocardium, including reduced cardiomyocyte numbers, lengthening of the remaining cardiomyocytes and proliferation of cardiac fibroblasts followed by collagen accumulation in the ventricular chamber (COHN et al., 2000; JANSSENS; LIJNEN, 2006; LAKATTA; LEVY, 2003; TORELLA et al., 2004). Myocardial collagen accumulation, or fibrosis, is managed by balanced equilibrium between extracellular matrix (ECM) synthesis, maturation, processing and degradation – the ECM turnover (ANVERSA et al., 1990; CAPASSO; FITZPATRICK; ANVERSA, 1992; CAPASSO et al., 1990; CENTURIONE et al., 2003; EGHBALI et al., 1989; FRATICELLI et al., 1989; HORN, 2015; SUSSMAN; ANVERSA, 2004; KWAK et al., 2011).

Matrix metalloproteinases (MMPs) are endopeptidases involved in ECM turnover and cardiac remodeling for degrading ECM proteins (AHMED et al., 2006; CHAKRABORTI et al., 2003; TSURUDA et al., 2004). Paradoxically, increased MMP levels mediate LV hypertrophy by enhancing ECM constituents, including collagen, fibronectin, elastin and laminin, leading to fibrosis with subsequent LV chamber dilatation and impaired LV function (JANSSENS; LIJNEN, 2006; OPIE et al., 2006). MMPs are synthesized by myofibroblasts, inflammatory cells, and myocytes (CLEUTJENS et al., 1995; COKER et al., 2001; HORN, 2015; MA et al., 2013) which are expressed constitutively at low levels in an inactivate form (zymogen

or pro-MMP) (TURNER; PORTER, 2012). MMP-2 and MMP-9 (gelatinases) are the main enzymes of cardiac remodeling because they break denatured collagen (gelatin) (COKER et al., 1998; HADLER-OLSEN et al., 2011; SPINALE et al., 1998), fibronectin, elastin and laminin in the rat myocardium (CHEUNG et al., 2000; TYAGI; RATAJSKA; WEBER, 1993; SPINALE, 2007). MMP activity is regulated by endogenous inhibitors, the tissue inhibitor metalloproteinases (TIMPs), which interact with MMPs at a 1-to-1 stoichiometric ratio (BONNEMA et al., 2007). Whereas MMPs have been associated with collagen degradation, there is recent evidence showing fibrosis following MMP activity (APTE; PARKS, 2015; GOLDSMITH; BRADSHAW; SPINALE, 2013). Expression of MMPs is regulated transcriptionally by growth factors, hormones and inflammatory cytokines (DESCHAMPS; SPINALE, 2006; FANJUL-FERNÁNDEZ et al., 2010). Angiotensin (Ang-II), atrial natriuretic peptide (ANP) and transforming growth factor beta (TGF-β) have been reported as one of the key upstream signaling pathways of cardiac hypertrophy and fibrosis (ASAKURA et al., 2002; CHEN et al., 2000; GONZÁLEZ; LÓPEZ; DÍEZ, 2004; LIJNEN; PETROV; FAGARD, 2001; KWAK, et al., 2011; ROSENKRANZ, 2004; TSUTSUI et al., 2007).

The ECM provides scaffolding for myocyte alignment, crucial for systolic function and diastolic function (due to increasing stiffness and decreasing compliance) (KHAN; SHEPPARD, 2006; KWAK et al., 2013). While LV systolic function is maintained (LAKATTA; LEVY, 2003), diastolic function is compromised with aging. One likely reason for impaired diastolic function is collagen accumulation in LV, which results in myocardial passive stiffness, decreased ventricular filling and impaired contractile function (BOLUYT et al., 1994; CIESLIK et al., 2011; LAKATTA; LEVY, 2003; MATSUBARA et al., 2000; YAMAMOTO et al., 2002; ZILE; BRUTSAERT, 2002). Decline in age-related cardiac function, associated or not associated with cardiac fibrosis, were demonstrated in humans (SCHULMAN et al., 1992; STEWART et al., 2003; SUSIC; FROHLICH, 2008) and animals (CHOI et al., 2009; DE SOUZA, 2002; LIU et al., 2000). Therefore, age-related LV structural alterations may induce functional cardiac changes, which evoke a significant impact on the health of the elderly (CIESLIK et al., 2011; LAKATTA; LEVY, 2003; ZILE; BRUTSAERT, 2002). However, there is controversy about to what extent these adaptations are part of natural aging process or a response to pathological stimulus since aging is a risk factor for cardiovascular disease (ARBAB-ZADEH et al., 2004;

LAKATTA; LEVY, 2003; NICCOLI; PARTRIDGE, 2012; STERN; BEHAR; GOTTLIEB, 2003).

Exercise training has been postulated for its beneficial cardiovascular effects (ELLISON et al., 2012) and the reduced risk of cardiac events (NACI; IOANNIDIS, 2013) especially in the elderly (CONONIE et al., 1991). As a whole, exercise training provides enhancements in maximal cardiac output, increase in stroke volume and decrease in resting heart rate (MIHL; DASSEN; KUIPERS, 2008). For that reasons, unlike pathological remodeling, exercise training is a potent stimuli for physiological cardiac remodeling (MIHL; DASSEN; KUIPERS, 2008). Endurance training or aerobic exercises (e.g., walking, running and swimming) involves isotonic contractions of large skeletal muscle mass and are performed for extended periods (e.g., 30-60min) using oxygen as the main energy supply for sustaining repetitive high-intensity, low-resistance exercise (MORGANROTH et al., 1975; NADER, 2006). It triggers substantial skeletal muscle vasodilatation and cardiac volume overload (pre-load) caused by increasing venous return (PLUIM et al., 2000). The volume overload lead to increased stretching force on the myocardium (ANVERSA; OLIVETTI, CAPASSO, 1991), which stimulates the ventricular dilatation (VINEREANU et al., 2000), characterized by increasing left ventricular internal width and left ventricular wall thickness (PLUIM et al., 2000). The endurance exercise results in eccentric cardiac hypertrophy with normal or improve of ventricular function (HOSSACK, 1987; MELO et al., 2009). Such adaptations have been reported both in aging humans (ARBAB-ZADEH et al., 2004; TAKEMOTO et al., 1992) and experimental animal models (BRENNER; APSTEIN; SAUPE, 2001; JIN et al., 2000).

However, studies addressing the effects of the resistance training on cardiac remodeling, ECM turnover and cardiac functional are scarce. Although endurance exercise training has been shown to improve the aged-associated LV remodeling, cardiac fibrosis and cardiac function, the effects of high-intensity resistance training in cardiac ECM remodeling and its implications in the cardiac function are not well understood.

Resistance training (RT), also known as strength-or-weight lifting (HASS; FEIGENBAUM; FRANKLIN, 2001) has been suggested to the elderly populations for preventing disabilities, maintenance of independence (OKAMOTO; MASUHARA; IKUTA,

2006; WHARBURTON; GLEDHILL; QUINNEY, 2001), reduction of muscle mass (sarcopenia) and loss of strength inherent of aging (KRAEMER, 2002; HURLEY; ROTH, 2000; ROTH et al., 2002; WINETT; CARPINELLI, 2001). It involves smaller muscle mass with few repetitions of muscle contractions (usually <20) until exhaustion at high or maximal exercise intensities during short-duration periods (NADER, 2006). The American Heart Association (AHA) recommends strength-training exercises at least 60% of 1 RM (one Repetition Maximum) of intensity (FIATARONE et al., 1990; HAGERMAN et al., 2000). Recommendations by the majority of studies and health organizations state that the RT must be progressive, executed at low repetitions and moderate volume, with overload against the concentric phase of movement (KRAEMER, 2002). Pollock et al (1994) recommends resistance training for elderly persons performing 1-8 sets, 12 repetitions and 8-10 exercises twice a week. Unlike the endurance exercise training, RT induces pathological cardiac remodeling, which could potentiate, to some extent, the compensatory responses of natural aging process. Resistance-type exercise imposes high pressure overload to the heart due to increased myocardial wall stress (FAGARD, 1996; MELO et al., 2015) inducing cardiac concentric hypertrophy (MACDOUGALL et al., 1985; MIHL; DASSEN; KUIPERS, 2008). However, Pluim et al (2000) concluded in a meta-analysis that ventricular hypertrophy, eccentric or concentric, is not dependent upon type-specific exercise training (endurance or resistance). Elevations in blood pressures (systolic and diastolic), cardiac output and heart rate have been documented during RT execution (FISMAN et al., 1997; HOOGSTEEN et al., 2004), although Fleck and Kraemer (2004) reported reduces in rate-pressure product (product of heart rate and blood arterial pressure) following RT programmes, which may reduce cardiac demands during daily activities (MCCARTNEY et al., 1993). Furthermore, heavy RT may lead dramatic acute increases in both systolic and diastolic blood pressure when Valsava manoeuvre is evoked (MACDOUGALL et al., 1985 MCCARTNEY, 1999) as well as increases in systemic vascular resistance due to isometric contraction imposed by heavier loads (FERNANDES et al., 2015).

The study of Kwak et al (2011) revealed that aerobic exercise training attenuates cardiac fibrosis in the aging rat's heart, linked to increased active MMPs expression but with no cardiac function evaluation. Therefore, the major aim of this study was to investigate the chronic effects of high-intensity resistance training in the ECM remodeling of LV from older rats. We

hypothesized that high-intensity resistance training enhances the ECM remodeling in the LV of aging rats with improvement of diastolic function. Such assumption is based on pressure overload placed in LV due RT.

MATERIAL AND METHODS

Experimental Group

Twenty male Wistar rats Rattus norvegicus albins with 3 (young) and 21 (old) months' age were used at the beginning of resistance training (RT) protocol. The animals were housed at a constant room temperature (22±2°C) and light cycle (12:12-h light-dark cycle) with free access to standard rat chow and tap water. All procedures were performed in accordance with the USA Guide for care and use of laboratory animals. The study received approval from the Ethics Committee on Animal Experimentation of Federal University of Sao Carlos, SP, Brazil (protocol 015/2012) according to the Ethics Committee on Animal Use (Federal Law 11.794) and Protection Code of Animals (State Law 11.977). Rats were randomly divided into four groups: YS (young-sedentary), YT (young trained), OS (old sedentary) and OT (old trained). Animal studies may provide similar benefits to cardiovascular health of the growing aged population (HACKER et al., 2006). Animals of the 6-month-olds represent a group of mature rats, as indicated by the fact that their rapid growth phase, and rats with 24 months of age show low mortality rate. Thus, the effects of aging can be investigated independently of confounding factors, such as growth or overt pathology (TURTURRO et al., 1999). Basically, one day of human's life corresponds to thirty days of a rat, which imply rat with 24 months of age (720 days of life) is similar to a elderly from 58-60 years-old (BENEDICT; SHERMAN, 1937).

Resistance Training

The RT was adapted to Hornberger and Farrar (2004) study. Rats were adapted to climbing a ladder during 3 days with a load apparatus attached to their tails. They were initially placed at the bottom of the ladder and familiarized with climbing. We eventually stimulate the animals to initiate climbing in order to learn the upward movement. On the last familiarization day, the maximal load carried through entire length of the ladder was considered the start overload to start the resistance training protocol. This RT was performed in alternative days, three times per week during 12 weeks. The length of the ladder allowed the animals to make 8–12 dynamic movements per climb, with 4-9 climbs/session. The climbs consisted of carrying a

progressive load of 65, 85, 95 and 100% of the maximal carrying capacity of each animal. At the end of these 4 climbs, an additional 30-g weight was added to the load apparatus until animal were not able to climb the entire ladder successfully. At the top of the ladder the rats arrived in a housing chamber where they rest for 120 seconds. RT session consisted of 5-8 climbs per climb over 6-8 seconds. At the end of the ET, the young and old animals achieved 6-mo and 24 mo-age respectively.

Measurements of hemodynamic parameters

Forty-eighty hours after the last exercise training session, rats were anesthetized with a ketamine-xylazine mixture (95 and 12mg/kg respectively, ip) as described for mice and adapted here for rats (KOCH et al., 1995; ROCKMAN et al., 1996) and a polyethylene catheter (PE-50, 8cm, filled with heparinized saline) was introduced through the right carotid artery into the left ventricle (LV). Arterial blood pressure – systolic, diastolic and mean arterial (SBP, DBP and MBP respectively) – heart rate (HR), maximal left ventricular systolic pressure (LVSP), LV end-diastolic pressure (LVEDP) and time constant of isovolumic relaxation (Tau) were obtained by a pressure transducer (Transpac IV-Abbbot, Critical Care Systems, Nashua, NH) and recorded continuously in unconscious rats. The positive and negative first derivatives of LV pressure vs. time (+dP/dt and -dP/dt, respectively) were also recorded (Mc Lab 8E, AD Instruments). In general, LVSP is related to the pressure load (or afterload) (DOUGHTY et al., 1997) and +dP/dt (a cardiac contractile index) with systolic performance (MASON, 1969). LVEDP, -dP/dt and Tau are used to measure the diastolic function (BOUDEWIJN et al., 2002). The LV catheter was then pulled out, and arterial blood pressure was measured – systolic and diastolic arterial pressure (SBP and DBP respectively). The maintenance of diastolic blood pressure at proper values was the guarantee that the aortic valve was not damaged during the procedure (DAVEL et al., 2008). After arterial and LV pressure recordings, animals were euthanized and the heart was isolated and weighed. The LV weight was normalized to body weight. This ratio was used as an index of LV hypertrophy. Of note, we were not able to measure the arterial pressure in conscious animals because they were rapidly driven to another experiment where it was necessary the euthanasia.

Histological analysis and Second Harmonic Generation (SHG)

The LVs were fixed with 4% paraformaldehyde in phosphate-buffered saline for 24h. After dehydration in ethanol, the material was embedded in paraffin for histological determinations. Serial 6μm sections were cut, stained with hematoxylin-eosin. Different cuts were stain with Masson's trichrome to visualize collagen and myocytes. Colorimetric staining for connective tissue was an adaptation of the Masson's trichrome technique (KIM et al., 2008). Afterwards, the cuts were analyzed under a light microscope. Histological sections were visualized with an Olympus Upright light Microscope (DP2-BSW 2.2 software). ImageJ 1.45S software (Media Cybernetics, Silver Spring, MD) was used to evaluate LV myocyte width. Five images of the myocardium were randomly acquired. Seven cells of each image, for a total of 45 LV myocytes per animal, were acquired and measured at 40x magnification. To quantify the width of myocytes, longitudinal myocytes with discernable lateral borders were visualized from microscopic images of H&E-stained histological sections. The distance spanning the lateral borders and perpendicular to the longitudinal axis of each identified myocyte was measured recorded as the LV myocyte width. Although measurements of myocyte width have methodological limitations we used such parameter to determine myocyte hypertrophy (KELLY et al., 2009).

SHG was identified using an Olympus confocal system (IX-81 inverted microscope, the FV300 scan head, the FV-5 COMB2 laser combiner, and two Hamamatsu model 3896 PMTs). SHG was examined by excitation with a Titanium sapphire laser (Tsunami, Spectra Physics) at 800 nm and 80 MHz repetition rate, coupled to the scan head by an external port, and collected by the condenser in the forward direction with a bandpass filter at 400 nm (Oriel Corporation, Stratford, CT) and a blue shortpass filter to reject any fluorescence signal. The Fluoview software was used to reconstruct the images (BRUNI-CARDOSO et al., 2010).

Gelatin zymography

Gelatin zymography of MMP-2 was performed as previously described for cardiac muscle extracts (SNOEK-VAN BEURDEN et al., 2005). Frozen LVs (25mg/1ml buffer) were

incubated in extraction buffer (10 mM cacodylic acid, pH 5.0; 150mM NaCl; 1 µM ZnCl2; 20 mM CaCl2; 1.5 mM NaN3 and 0.01% Triton X-100 [v/v]) overnight (18h) at 4°C with continuous mixing. The solution was centrifuged for 10 min (13000 g at 4°C) and total protein was determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) in a spectrophotometer at 562 nm, according to manufacture's instruction. Twenty micrograms (20µg/mL) of the solution was loaded into a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel prepared with 1 mg/mL gelatin. After electrophoresis, the gels were washed twice for 20 min in 2.5% Triton X-100 to remove the SDS. Gels were rinsed and incubated in buffer substrate [50 mM Tris-HCl (pH 8.0); 5 mM CaCl2; 0.02% NaN3] at 37°C for 20 h. Afterward, gels were stained with Coomassie brilliant blue for 1.5 h and destained with acetic acid: methanol: water (1:4:5) for visualization of the activity bands. Gelatin-degrading enzymes were visualized as clear white bands against a blue background, indicating proteolysis of the substrate protein. The samples were also performed in presence of 15 mM EDTA that inhibited the MMPs activity confirming their existence. The molecular mass of gelatinolytic activities was determined by comparison to reference protein molecular mass marker PageRulerTM Prestained Protein Ladder (Fermentas Life Sciences, Burlington, ON). Activity bands were identified according their molecular weights (72 kDa: pro-MMP-2 and 57 kDa: cleaved or active-MMP-2). The gels were then photographed with a Canon G6 Power Shot 7.1 mega pixels camera. Densitometric quantitative analysis of the MMP-2 protein bands seen in the zymography gels was performed using the Gene Tools version 3.06 software (Syngene, Cambridge, UK).

RNA isolation and Analysis

One frozen fragment of each muscle was homogenized and the total RNA isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The extracted RNA was dissolved in tris-HCl and ethylene-diaminetetracetic acid (TE) pH 7.6 and quantified spectrophotometrically. The purity was assessed by determining the ratio of the absorbance at 260 nm to that at 280 nm. All samples had ratios above 2.2. The integrity of the RNA was then confirmed by inspection of the ethidium bromide (Invitrogen, Carlsbad, CA) stained 18S and 28S ribosomal RNA under violet ultra light.

Reverse Transcription

We reverse transcribed 1 mg of RNA to synthesize cDNA. A reverse transcription (RT) reaction mixture containing 1 mg of cellular RNA, 5x reverse transcription buffer, a dNTP (Promega, Madison, WI) mixture containing 0.2 mmol·L-1 each of dATP, dCTP, dGTP and 0.1 mol·L-1 of dTTP, 1 ml of oligo (dT) primer (Invitrogen, Carlsbad, CA) and 200U of M-MLV RT enzyme (Promega, Madison, WI) was incubated at 70ºC for 10 min, 42ºC for 60 min and finally heated at 95ºC for 10 min before quick chilling on ice.

Oligonucleotide primers

Oligonucleotide primers were designed using the Primer Express Software 2.0 (Applied Biosystems, Foster City, CA) (MENON; SINGH; SINGH, 2005). All of sequences were synthesized using Imprint. The sequences primes used are depicted in the Table 1. RPS27a works as the best reference gene due to constant amplification among the groups. The list of oligonucleotide Primers is described in Table 1.

Real-time polymerase chain reactions

The RNA transcript levels for the different experimental and control muscles were analysed simultaneously and the reactions carried out in duplicate in the C1000 Thermal Cycler (CFX 96TM Real Time System, Bio-Rad) using fluorescent dye SYBR green detection (Applied Biosystems, Foster City, CA) and 180 nM of each primer in a final volume of 50 µl. Thermal cycling conditions to MMP-2 and RPS27a included 10 min at 95°C, and then 40 cycles each of 15 s at 94°C, 30 s at 48°C to MMP-2 and at 56°C to RPS27a, respectively, and 1 min at 72°C, then 10 min at 72°C. For each gene, all samples were amplified simultaneously in duplicate in one assay run. Data were analysed using the comparative cycle threshold (Ct) method. The target gene expression was normalized to RPS27A (ribosomal protein S27a). Different normalization tests were applied in order to choose RPS27A as a reference gene. This gene encodes a fusion protein consisting of ubiquitin and ribosomal protein S27a. Ubiquitin is a highly conserved

protein found in every eukaryotic cell, which plays an important role in degradation of proteins. It is induced by cell stress such as hypoxia to help in protecting cells from damage (EL-KASHEF et al., 2015).

Western immunoblot

Protein abundance in the whole extract of ventricular muscle was determined with western immunoblotting. Samples were homogenized using tissue homogenizer for small tissues in ice-cold RIPA buffer (10 µl/mL of proteinase inhibitor). Samples were then centrifuged at 1000g for 10 minutes. Protein concentration was determined using a BCA assay (Thermo-Fisher, Waltham, MA) and tissue lysates were stored at -80°C.

For gel electrophoresis, all homogenates were suspended in sample buffer (Tris HCl, pH 6.8 with 2% SDS, 60 mM DTT, and 25% glycerol) and heated for 5 minutes at 95°C. Protein (30 µg/ml) was loaded onto 10% polyacrylamide gels and separated in a Bio-Rad Protein III gel-box (Hercules, CA; 60V for 1.3-1.5 hours). Gels were transferred (100V for 1.5 hours) to nitrocellulose membrane and stained with a 0.1% Ponceau red solution in 5% acetic acid to confirm equal loading. Ponceau red stained membranes were digitized and then washed in Tris buffer saline + 0.1% Tween-20 (TTBS) for further antibody detection. Membranes were blocked in 5% non-fat dry milk in TTBS and then probed for various proteins using primary antibody solutions in blocking buffers (KWAK et al., 2011). Primary antibodies used included: mouse monoclonal anti- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:30.000; ABCAM); MMP-2 (1:2500; ABCAM); MMP-9 (1:3000; ABCAM); TIMP-1 (1:2500; ABCAM), TIMP-2 (1:2500; ABCAM) and TGF-β (1:3000; ABCAM). Anti-Mouse specie-specific secondary antibody (1:15000; ABCAM) was incubated with membranes following primary antibodies. Membranes were then stained with chemilluminenscent peroxidase substrate (#CPS160-Kit Sigma-Aldrich) and imaged using a Molecular Imager ChemiDocTM XRS+ Imaging System (Bio-Rad).

ImageJ (1.48 version, National Institutes of Health, USA) and Image Lab (Version 5.2.1 Bio-Rad Laboratories, 2014) softwares were used for densitometry analysis. Protein abundance was expressed as relative units normalized to GAPDH. Ponceau red was a staining used to determine transfer efficiency and equal loading as a loading control.

Determination of cardiac peptides concentration

For measurements of cardiac angiotensin II-type (Ang-II) and atrial natriuretic peptide (ANP) concentrations LVs were manually dissected and homogenized with 0.045N HCl in ethanol (10ml/g of tissue) containing peptidase inhibitors (1mm p hydroxymercury benzoate, 30 mm 10-phenanthroline, 1mm phenylmethanesulphonylfluoride, 1mm pepstatin A and 7.5% ethylenediaminetetraacetic acid 50µl/ml of blood) and 0.0043% protease-free bovine serum albumin. Protein concentrations in the plasma and in crude homogenates were determined by the Bradford method (Bio-Rad). These homogenates were centrifuged (1600g for 40min at 4°C), and the supernatants were evaporated, followed by dilution of the pellet in 2ml of 0.1% trifluoroacetic acid (TFA). Next, tissue peptides were extracted onto a Bond Elut SPE-Column (Peninsula Laboratories Inc., Belmont, CA, USA). The columns were pre-activated by sequential washes with 4ml of 60% acetonitrile/0.1% TFA and 20ml of 0.1% TFA. After sample application, the columns were washed with 20 ml of 0.1% TFA. The adsorbed peptides were eluted with 3ml of 60% acetonitrile/0.1% TFA into polypropylene tubes. After evaporation, angiotensin peptide levels were measured by radioimmunoassay (MECAWI et al., 2013). The antibodies for Ang-I and Ang-II were obtained from Peninsula Laboratories (Ang I: T4166 and Ang II: T4007) and the ANP antibody was kindly donated by Jolanta Gutkowska (Hotel Dieu, University of Montreal, Canada). The sensitivity of the radioimmunoassay and the coefficients of intra-and inter-assay variability were 1.2pg/mL, 9.85 and 7.4% for Ang-I, 0.39 pg/mL, 7.9% and 5.4% for Ang-II and 0.7 pg/mL, 5.5% and 8.1% for ANP.

STATISTICS

Results were expressed as means ± SEM. The RT overload and rat's body weight were analyzed by nonparametric-paired t Student test in order to compare the differences among the weeks. To verify the differences in the RT overload between the groups (YT x OT), we used one-way ANOVA. Shapiro-Wilk's W and Levene's tests were applied to evaluate the normality of means and homogeneity of the variances, respectively. All the other results were analyzed by two-way ANOVA followed the Tukey's *post hoc* test. Significance level was set at 0.05 ($p \leq 0.05$).

RESULTS AND DISCUSSION

RT performance and body weight

In order to investigate the chronic effects of RT, rats were euthanized 48h-post exercise training period. The heart was excised and dissected into LV for cardiac hypertrophy analysis.

The RT was described as a progressive resistance training (Figure 1) whose intensity was controlled by increasing overloads. The American College of Sports Medicine (ACSM) states that resistance training should target major muscle groups for 2 and/or 3 days a week at intensity upper to 65% of subject's one-repetition maximum (DONNELLY et al., 2009). Thus, we used the Hornenberger and Farrar (2004) exercise training protocol in an attempt to mimic such progressive resistance training with some modifications (intensity and frequency of exercise training) for healthy elderly). The overload of RT corresponds the sum of rat's weight and the absolute load of carrying. For that reason, old rats initiate the RT with higher overload than that of young ones in the first week of training (b, $p \leq 0.05$). In the second week, OT rats maintained the higher overload of training compared to young-matched group (b, $p \leq 0.05$) and this difference was observed at week 6 again. Moreover, in the second week, the overload significantly increased for both groups when compared to the respective week 1 (a, $p \leq 0.05$). The overload continued increasing over the weeks, which means the RT was performed at maximal intensity. Also, this RT might be a strength exercise training protocol with muscular hypertrophic adaptations, since we found hindlimb hypertrophy of both trained animals groups (data not shown). In the eighth week, the overload was higher in young rats than that for old animals meaning that old rats' performance remained constant in the following weeks, supposing lower tolerance to a high intensity exercise training of old rats compared to young rats at a specific time course.

The body weight of young animals (YS and YT) increased after 12 weeks. However, old rats (OS and OT) showed lower body weight in relation to their initial body weight ($p<0.05$) (Table 2). The RT did not affect the BWf of old rats whereas young trained rats showed lower BWf than sedentary young animals. Exercise training reduces the body mass index by

decreasing adipocyte mass and total triglycerides (SWIFT et al., 2014). However, RT appears to contribute minimally for reduction of body fat (DONNELLY et al., 2009). The weight loss observed in YT rats could be associated with increased energy expenditure once intramuscular triglycerides utilization and fat oxidation are increased due to exercise energetic demand at the rest periods after maximal-intensity exercise training regimens (KANG et al., 2009; KIENS; RICHTER, 1998). Goto et al (2007) shown the resistance exercise training prior to an aerobic workout has increased lipolysis during subsequent aerobic exercise, suggesting its pivotal role in energetic metabolism. Here, the maintenance of body weight following 12 weeks in old rats could be related to the lower tolerance showed in OT rats (Figure 1) or due to decreased food ingestion of old rats, even though we did not control such variable. It has been reported that energy expenditure declines with aging (WEINSIER et al., 2000; WESTERTERP, 2000) which not explain our findings in these aging rats.

Aging and RT effects on cardiac weight rates

Given that the cardiac hypertrophy is a compensatory response to the overload placed on aged LV, we sought to investigate the cardiomyocyte width, heart weight and the left ventricle-to-body weight ratio, as an index of LV hypertrophy (CHOI et al., 2009). In OS rats, whole heart weight was greater than that for YS rats, suggesting aged-related cardiac hypertrophy ($p \leq 0.05$, Table 2). However, LV/BWf ratio revealed no changes among all experimental groups, which is in accordance with Hacker et al (2006). We would expect to find increased LV mass in old groups such as in other studies using rats in sedentary (CHOI et al., 2009; RINALDI et al., 2006; THOMAS et al., 2001) or training conditions (KWAK; SONG; LAWLER, 2006). However, Raya et al (1997) detected decreases in LV-to-body weight ratio with aging. These divergences could be related to the differences in the age and strain of the rats. The RT reduced significantly the absolute LV weight in YT rats when compared with YS group (Table 2), whereas in other studies there was no significant differences on absolute LV mass between young and old rats (KWAK; SONG; LAWLER, 2006) and after exercise training (CHOI et al., 2009).

We also analyzed the left ventricle-to-length ratio (LV/tibia) to investigate the cardiac hypertrophy since such normalization avoids external factors and minimally displays

changes in weight (ROSSONI et al., 2011). Nevertheless we did not find differences among the experimental groups (Table 2). Early studies had not find increases in heart weight/tibia length ratio, even after exercise training (BRENNER; APSTEIN; SAUPE, 2001; ROSSONI et al., 2011).

Many investigators have assumed that an increase in the heart weight-to-body weight ratio is the main evidence of hypertrophy. Since the exercise training programs cause a decrease in the body weight in male rats, this change in body weight is frequently responsible for increasing the heart weight-to-body weight ratio. In attempt to clear the divergences here and among the other studies, it makes necessary histological studies that define the normal myocardial cell size for a given age of the animal.

Effects of RT and aging on LV structure

A relevant consequence of age-induced remodeling of the heart is the accumulation of connective tissue (CENTURIONE et al., 2003; GAZOTI et al., 2001), with increases in myocardial collagen concentration in the tissue (NGUYEN et al., 2001). Some microscopy experiments have proposed in order to examine tissues morphology. We sought to detect insoluble proteins including collagen by SHG microscopy which identifies the morphology and arrangement of collagen fibers (TSAI et al., 2014) as well as LV myocyte width and connective tissue were quantified.

LV myocyte width was larger in the OS than that in YS rats. Furthermore, we observed that RT significantly reduced the age-related LV myocyte width (Figure 2A and 2B). Contrary to LV/BWf and LV/tibia ratios, we were able to observe evidences of cardiac hypertrophy by microscopic analysis. Studies of Kwak (2006, 2011) also have demonstrated larger cardiac myocyte as a function of aging and exercise training.

Moreover, we noted marked increase of LV collagen concentration in OS *vs.* YS group (Figure 2B and 2E), which is in accordance with others (CHOI et al., 2009; KWAK et al., 2011; THOMAS et al., 2001; WRIGHT et al., 2014). Such findings suggest remarkable LV remodeling in OS animals.

Resistance training was able to attenuate the age-associated collagen accumulation in LV tissues (Figure 2C and 2D). Similarly, Kwak et al (2011) have documented greater connective tissue in LV of old rats (vs. young group) and that aerobic exercise training prevented such age-induced connective tissue accumulation in LV tissue. We presume that exercise training, independent of its characteristics (volume, overload, number and intervals between of sets) can be a relevant physiological agent for old populations against cardiac fibrosis. We must emphasize the crucial role of RT in cardiac adaptations since LV collagen tissue decreased in young rats as well.

To scrutinize the collagen content and its conformation in the LV of old groups, we used second-order harmonic generation (SHG) microscopy. Although we did not find high levels of collagen in OS rats through SHG microscopy, the RT was able to attenuate the interstitial collagen of LV from both young and old rats (Figure 2E and 2F). Taken together, the RT showed to be an effective intervention to lower the LV fibrosis as we observed by two reliable microscopy techniques.

MMP-2 activity in LV

As MMP-2 has been shown for exerting an important role in aging cardiac tissue (HORN et al., 2012; MA et al., 2013;), we aim to evaluate whether the increases of age-related LV collagen deposition could be related with diminished MMP-2 activity in old groups. High-intensity resistance training was able to increase the MMP-2 activity (Figure 3C), which was associated with reduced collagen levels in LV of trained rats (young and old).

Consistent with Horn et al (2012), we observed increased zymographic activity of MMP-2 in LV tissues (Figure 3C). MMP-2 activity increased in OS (1.9-fold) vs. YS rats assuming, in turn, augmented ECM remodeling in the LV. Other aging experimental model has also shown increased MMP-2 activity in LV tissues (HORN et al., 2012). Likewise, pro-MMP-2 was greater in OS (2.7-fold) *vs.* YS group (Figure 3B). Once we observed increased collagen deposition in OS groups, we would expect to find reduced MMP-2 activity in LV, which was not observed. To address this question, we choose "*ECM remodeling*" terminology because we are considering a replacement - with subsequent accumulation of collagen and ECM proteins -

followed by tissue connective breakdown in LV from OS rats. *ECM turnover* could be applied to ECM catabolism (breakdown and clearance of ECM) with physiologic breakdown and replacement. Finally, *ECM Degradation* also manages ECM proteolysis, but it is described for excessive matrix destruction, as exhibit in diseases. Of note, remodeling may not include systematic replacement of the ECM that was cleared (APTE; PARKS, 2015). Therefore, considering marked increases of LV collagen in OS rats, remodeling might be the suitable terminology in this study.

MMP-2 activity increased in old sedentary group (Figure 3C), hypothesizing increased proteolysis in EMC from LV. MMP activity is required to maintain increased LV size as well as increased MMPs levels correlates with decreased insoluble collagen levels in LV from aging mice (LINDSEY et al., 2005). Other studies, in turn, did not show increases on MMP-2 activity with aging. In 24 month-old rats the MMP-2 activity decreased in 40% suggesting age-associated fibrosis following reduced MMP-2 activity (ROBERT et al., 1997). However we would expect observe decreased MMP-2 activity in OS rats once we documented elevated collagen deposition in this group (Figure 2E). One potential explanation for that could be associated with connective tissue synthesis by some bioactive molecules produced by MMP-1 (matrikines) (KAKKAR; LEE, 2010). Furthermore, in post-infarcted rats, activity of MMPs induced the formation of new collagen with altered crosslinkings (WOODIWISS et al., 2001). In accordance with our results, we might suggest accumulation of interstitial collagen (and connective tissue as well) following increased MMP-2 in old sedentary rats (PAGE-MCCAW; EWALD; WERB, 2007; PARKS, 1999). There is evidence that matrix degradation is not the predominant function of these enzymes, given that several reports demonstrated that some MMPs act as substrates for cytokines, chemokines, receptors, antimicrobial peptides (KHOKHA; MURTHY; WEISS, 2013). We were not able to analyze the collagenase activity (MMP-1) to yield a better understanding of this issue. Our findings are consistent with a recent study of Wright et al (2014) demonstrating increased MMPs activity as an effect of aging. Whereas it seems divergent, other possible explanation is that the own ECM components can promote pro-MMP activation by binding of insoluble elastin to the proMMP-2 leading to its auto-activation (EMONARD; HORNEBECK, 1997). Also, TIMP-2 plays a role in the conversion of proMMP-2 to its active form by creating MT1-MMP (also called MMP-14) complex with (JEZIERSKA; MOTYL, 2009; NAGASE; VISSE; MURPHY, 2006; SUTTON; SHARPE, 2000).

Although the pro-MMP-2 was reduced in OT *vs.* OS group, the MMP-2 activity elevated in OT rats. We could assume that activation of MMP-2 was followed by decreases on pro-MMP-2 from OT group. On the other hand, the increased MMP-2 activity could be associated with decreased collagen levels in old trained rats. Thus, we may assume that RT in old animals might be modulating some proteins and signaling pathways that active MMP-2. Decreased TIMPs activity, elevated inflammatory cytokines and enhanced pro-MMP-2 proteolytic cleavage by MT1-MMP are possible candidates triggered by RT in the MEC (BERGMAN et al., 2003; JEZIERSKA; MOTYL, 2009; JACOB-FERREIRA et al., 2013; KWAK; SONG; LAWLER, 2006; KWAK et al., 2011; NAGASE; VISSE; MURPHY, 2006; SIWIK; PAGANO; COLUCCI, 2001; SUTTON; SHARPE, 2000). Other factors, such as oxidative stress (peroxynitrite), endothelin-1, Ang-II, IL-1B, hypoxia, phosphorylation status are also potential contributors of aging and exercise training effects (BENIGNI et al., 2009; LAWLER et al., 2009; SARIAHMETOGLU et al., 2007; TAKENAKA et al., 2006).

In young groups, the RT increased significantly both pro-MMP-2 and MMP-2 activity. For our understanding, it is the first time that high intensity RT promoted increases in MMP-2 activity both in young and in older rats.

Bellafiore et al (2013) reported reduced pro-MMP-2 activity after aerobic exercise training at low-moderate intensity hearts but with no detection of active MMP-2 in hearts from mouse. In agreement with our study, MMP-2 was detected in rat cardiac muscle after 72, 96 and 120 hours of acute swimming training with an anaerobic component (VERZOLA et al., 2006). Therefore, there are still divergences of the effects of exercise training on MMP activity. It could be hypothesized some activators of MMP-2 modulated by RT as aforementioned, even in young trained group.

Gene expression of ECM elements

We observed that COL-I mRNA significantly elevated in OS *vs.* YS rats (Figure 4A), which is consistent with Horn et al (2012). The increased COL-1 synthesis could explain our observations of collagen deposition in microscopic analyses (Figure 1C and 1E). However, Masson et al (2000) reported no changes in COL-I mRNA in rats with 18-mo of age while Thomas et al (2000) observed declines in COL-I and III mRNA with aging. Increases in COL-I

mRNA could be associated with upregulated TGF-β1 and TIMP-1 mRNAs observed in OS rats here (Figure 4C and 4D). Recently, it was shown increased TIMP-1 gene expression with aging (WRIGHT et al., 2014). At a transcription level, our findings are consistent with the signaling pathway assembled by Kwak et al (2011) since TGF-β1 is a potent stimulator of TIMP-1 and a contributor to fibrosis in the aging heart (BONNEMA et al., 2007; TSUTSUI et al., 2007). Furthermore, over-expression of TGF-β in the heart increases matrix fibrosis and provoke systolic and diastolic dysfunction (KHAN; SHEPPARD, 2006).

Gelatinases (MMP-2 and MMP-9) gene expression exhibited opposite behaviors with aging. While MMP-9 elevated with aging, MMP-2 mRNA decreased when compared to YS rats (Figure 4E and 4F). Although we found increased MMP-9 synthesis in OS rats, fibroblasts produce less MMP-9 with aging (LINDSEY et al., 2005). By contrary, MMP-2 and COL-III mRNA significantly decreased in OS group, either in sedentary or trained groups when compared to young-matched groups (Figure 4B and 4F). Down-regulation of COL-III and MMP-2 mRNAs here are in accordance with others (MASSON et al., 2000; ROBERT et al., 1997). There are controversies between mRNA expressions of gelatinases with aging. It has been shown either downregulation (ROBERT et al., 1997; THOMAS, et al., 2000) or gene upregulation with age (BÁTKAI et al., 2007) as well as no alterations of MMP-2, TGF-β1 and TIMP-2 mRNAs expression in LV from rats (ROSSONI et al., 2011). The decreased myocardial collagen synthesis suggests that age-related collagen accumulation occurs predominantly via posttranslational modifications and/or reduced collagen degradation (HORN 2015; THOMAS, et al., 2000). Mechanisms for regulation of MMP gene activation include the transcription factor nuclear factor-κB (NF-κB) and/or activated protein-1 (AP-1) (TSURUDA et al., 2004). Indeed, RT was able to reduce only MMP-9 mRNA expression in old rats while MMP-2 mRNA reduction was an age-effect, even after RT (Figure 4E and 4F). Contrasting low levels of MMP-2 synthesis along with augmented MMP-2 activity, we hypothesize that the ECM remodeling by MMP-2 is modulated at posttranscriptional level in LV tissues of aging rats.

The RT was able to reduce the TGF-β1, TIMP-1 and MMP-9 mRNA expression in OT *vs.* OS rats (Figure 4C, 4D and 4E). The RT effect in decreases of COL-I and COL-III did not reach statistical significance. On the other hand, Thomas et al (2000) found that 10 weeks of exercise training reversed the age-associated declines in myocardial type-I and III procollagen (mRNA levels) of septum and free wall of LV. Our exercise training protocol was different from

that used by Thomas' research group, leading us to suppose distinct effects between the protocols (endurance x resistance training) on the transcriptional factors that modulate the gene upregulation. The microRNAs has emerged as a potential modulator of gene transcription and pretranslational synthesis to the cardiac remodeling (FERNANDES et al., 2015; MELO et al., 2015) which could affect results in this study.

Increased collagen synthesis may be matched with a greater increase in collagen degradation by metalloproteinases (LINDSEY et al., 2005) in aging rat model, which is in accordance with our results of increased COL-I RNA expression (Figure 4A), LV collagen accumulation (Figure 2) and MMP-2 activity in OS group (Figure 3 C).

In young groups, the RT increased significantly TGF-β1 mRNA levels compared to YS rats (Figure 4C). Calderone et al (2001) observed increased TGF-β1 mRNA levels in the LV of trained female rats for 3 or 6-wks. The author suggests that the increases in TGF-β1 mRNA may contribute to myocardial remodeling. If considering the augmented MMP-2 activity after RT, our observations in upregulated TGF-β1 mRNA might support the LV remodeling observed here in young trained rats (Figure 3C). Differently, TGF-β1, did not alter in LV from trained rats after 16-weeks at high-intensity endurance exercise. Pro-collagens (I and III), MMP-2 and TIMP-1 mRNA expressions showed unchanged after endurance training (BENITO et al., 2011), which is in accordance with our findings even considering the differences of exercise training features.

Mechanical stimuli gives rise to bioactive molecules and cause oxidative stress, which in turn stimulate MMP induction. Mechanical stretch increased MMP-2 mRNA levels, which was dependent upon ROS signaling cascade (GROTE et al., 2003). Once aging has been associated with increased oxidative stress and significant MMP-2 activity in heart, the increased MMP-2 activity observed here could be modulated by oxidative stress even with reduced MMP-2 mRNA level in old groups, which it corroborates with Wang et al (2010) report.

Protein expression of ECM elements

We were not able to find any changes in protein expression of gelatinases, TIMP-1, TIMP-2 and TGF-β1 among the experimental groups (Figure 5). We assume two possible reasons for explain such findings. First, it could be related with MMPs localization among the

cellular fractions (ECM x membrane x cytoplasmic). As we analyzed the whole LV fraction, elegant studies had shown a shift localization of MMPs and TIMPs due to aging (LINDSEY et al., 2005). However, our results of MMP-2 protein expression are consistent with Lindsey et al (2005), whereas these authors used mice as experimental groups, and Kwak et al (2011) who did not observe alterations in TIMP-2 levels with aging. Also Horn et al (2012) found differences in TIMP-1 and TIMP-2 proteins levels in LV of old female sheep compared to young group. However, MMP-9 and TIMP-1 were decreased in middle-adult mice (15 months old) compared to young mice (LINDSEY et al., 2005). On the other hand, TIMP-1 increased substantially in old sedentary rats (KWAK et al., 2011).

Second, there might be a delayed time course between the proteins translation and the time-point detection when using Western Blotting methodology. Ultimately, controversial results around the protein expression of ECM in aging models can be observed among the studies.

Diminished ECM degradation has been correlated with lower MMPs protein levels followed by ECM accumulation, mostly insoluble collagens deposition (LINDSEY et al., 2005). In this regard, Kwak et al (2011) observed decreases in pro and active MMP-2 protein expression in LV from old sedentary rats compared with young-matched rats (KWAK et al., 2011). Moreover, the aerobic exercise training resulted in elevated pro and active MMP-2 protein expression in old trained rats, suggesting the protective role of aerobic exercise training in age-related MMPs dysfunction. The increased MMPs proteins levels were related to the reduced TIMP-1 after aerobic exercise training in old rats even though no changes were observed in TIMP-2 (KWAK et al., 2011).

Considering the recent studies, the current literature is unclear about MMPs or TIMP expression with aging in the heart (KWAK, 2013). Although our results did not show differences in MMPs, TIMPs and TGF-β1 levels with aging and resistance training, we may emphasize the augmented ECM remodeling in LV by increased MMP-2 activity observed in this study, corroborated by other studies. To our understanding, there are no studies about the chronic effects of high intensity resistance training in MMP proteins levels and gene expression of cardiac ECM in aging rat models.

It was recently discovered two different intracellular MMP-2 isoforms: MMP-2_{NTT-50} and MMP-2_{NTT-76} (ALI et al., 2012), which could further explain the divergences in and among the studies.

Effect of RT on aging cardiac function

The cardiac function declines with aging mainly due to prolongation of contractile duration and time to peak tension with diminished ability to generate force (ASSAYAG et al., 1997). Blood pressure, in turn, rises with age (FRANKLIN et al., 1997) determined by peripheral vascular resistance (i.e., vascular stiffening) and stroke volume (DART; KINGWELL, 2001). However, some rodent models of aging did not present change in blood pressure (CAPASSO et al., 1990; LIN et al., 2008).

Also, aging confounds the effects of anesthetic agents, which may be difficult to investigate solely the aging effects. As ketamine/xylazine anesthesia depresses cardiac function in young mice (ROTH et al., 2002) and increases heart rate in older mice at low doses of the anesthesia (CHAVES et al., 2003), we recognize that anesthesia conditions could be a limitation of this study, despite of others have used such anesthesia to assess *in vivo* cardiac function using cardiac catheterization in intact anesthetized old rats. (KOCH et al., 1995; RINALDI et al., 2006; ROCKMAN et al., 1996).

Despite of anesthetic conditions to measure the cardiac function, we observed that systolic (SBP), diastolic (DBP) and mean (MBP) blood pressures were attenuated with aging in relation to matched young groups (OS *vs.* YS; OT *vs.* YT) (Table 3). HR unchanged among the groups, which it is consistent with Rinaldi et al (2006) study even though our HR values are lower than that found by these authors. Later, it was demonstrated that HR and MBP were lower in old rats than those for young rats (CHOI et al., 2009), which is in accordance with our results. Such findings may represent cardiac output impairment with aging as aftermentioned.

Although in humans (CARTER et al., 2003; KELLEY; KELLEY, 2000; RAY; CARRASCO, 2000; WILEY et al., 1992) and rats (BARAUNA et al., 2005) there have been studies suggesting that RT may reduce the arterial blood pressure, others indicate that does not affect the resting blood pressure (FLECK, 1988) or still may cause hypertension - increase in

SBP and DBP blood pressures (FLECK; DEAN, 1987; SALE et al., 1994), higher pulse pressure and arterial stiffness (BERTOVIC et al., 1999). Due to predominantly static exercise component within, RT acutely increases the blood pressure followed by increasing cardiac output and heart rate which in turn favors an overload (pressure overload) upon the heart (MACDOUGALL et al., 1985). Despite of controversial results, this study showed that RT did not affect SBP, DBP and MBP in both young as old rats when compared to their match sedentary groups.

The LVSP fell in the old groups compared with their matched young groups (Table 3), suggesting a decreased systolic performance with aging, which is in accordance with Choi et al (2009). However, other study reported decreases in LVSP from old rats in relation to young ones (RINALDI et al., 2006). The RT did not affect LVSP whereas Rinaldi et al (2006) reported that exercise training on treadmill was effective to decrease the LVSP in old rats. No significance difference on +dP/dt measures was observed among the experimental groups while Choi et al (2009) reported that +dP/dt was attenuated with aging. The +dP/dt is a classic sensitive parameter to changes in contractility but depend upon changes in volume overload (preload) (KASS et al., 1987; LITTLE, 1985). Moreover, +dP/dt is an important index of myocardial contractility because it is directly influenced by cardiac inotropism (FRANK; LEVINSON, 1968). Alterations on LVSP are directly relationship to the ventricular dimensions (or volume) alterations (KASS; MAUGHAN, 1988; SAGAWA, 1981) and pressure load (or afterload) (DOUGHTY et al., 1997). Our results suggest impaired contractility with aging but with no RT effect. Paradoxically, most recent evidences have shown that MMPs degrade microfilaments (SAWICKI et al., 2005) and sarcomeric proteins (ALI et al., 2010), which could affect the contractile function (SAWICKI et al., 2005; WANG et al., 2002) and myocardial compliance (CHUNG et al., 2013; HAMDANI et al., 2013). In this context, we might suppose the decreased LVSP with aging observed here also could be related with increased MMP-2 activity in LV form OS rats.

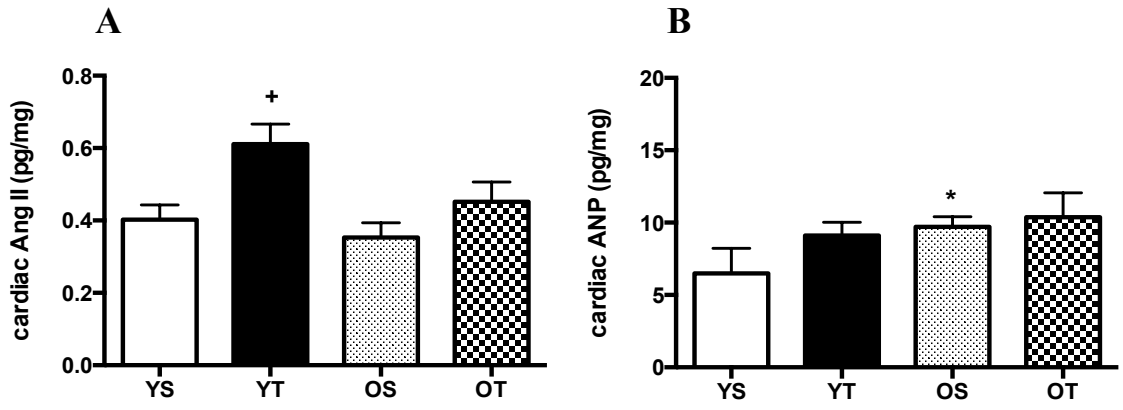
We demonstrated that LVEDP increased in OS rats compared with YS rats (Table 3). Such observation is in accordance with Choi et al (2009) whereas Raya et al (1997) did not find significant differences in aging rats. Diastolic dysfunction is composed by an active and a passive component (CHOI et al., 2009). Isovolumic relaxation, as a part of active component, can be quantified by calculating -dP/dt and Tau (MAURER et al., 2004). We only observed decrease in -dP/dt in OT *vs.* YT group, which was consistent with Choi et al (2009) study. Moreover, Tau,

a classic parameter of active relaxation, was reduced with aging whereas it was increased in old rats in others studies (RAYA et al., 1997; CHOI et al., 2009). However, Tau and -dP/dt were not different between OS *vs.* OT rats (Table 3). We presume the decreased Tau and increased LVEDP could result in impairment LV relaxation and thus contribute with diastolic dysfunction with aging. Moreover, impaired LVSP and LVEDP in OS rats could be closely associated with collagen accumulation observed in LV of this group (Figure 2C and 2D).

Consistently, the RT was able to attenuate the increased age-associated LVEDP (Table 3) suggesting that RT could act at the passive component of relaxation, but not at active relaxation (measured by Tau), in old rats. While the abnormality of active relaxation is an early sign of diastolic dysfunction in cardiovascular diseases, it is further common in the elderly (YAMAMOTO et al., 2002). However, passive stiffness is not precisely separated from active relaxation and these changes may reflect LV stiffness (CHOI et al., 2009). Exercise training either in older people or in animals reverses the aging-associated reductions in cardiac filling phases (LEVY et al., 1993). Here, we emphasize the effect of RT in diastolic function of old rats. Such improvement has been related to exercise training-induced decreases in LV collagen content (KWAK et al., 2011; THOMAS et al., 2001) and cross-linkings (CHOI et al., 2009; THOMAS et al., 2000). In fact, we also demonstrated reduced interstitial collagen in old trained rats, which is in accordance with them.

Cardiac peptides

Figure 6 - Angiotensin-II (Ang-II) (**A**) and atrial natriuretic peptide (ANP) (**B**) concentrations in the LV tissues detected by radioimmunoassay. Values are expressed as means (pg/mg tissue) ± SEM. Groups: **YS**: young sedentary; **YT**: young trained; **OS**: old sedentary; **OT**: old trained. Different characters mean statistical difference (ANOVA two-way, Tukey *post-hoc* test $p \leq 0.05$). $^{+}$**YT vs. YS;** ***OS vs. YS;** n = 9/group.



In attempt to investigate whether some of upstream pro-fibrotic factors could be mediate the age-related responses, we sought to quantify angiotensin-II (Ang-II) and atrial natriuretic peptide (ANP) of LV tissues (Figure 6A and 6B). We did not observe statistical differences in Ang-I among experimental groups (data not shown).

Ang-II stimulates synthesis of collagen (MANN, 2003), laminin, fibronectin (KAWANO et al., 2000; LIJNEN; PETROV; FAGARD, 2001; OLSON et al., 2005), and induces TGF-β1 upregulation in myofibroblasts and cardiac fibroblasts (CAMPBELL; KATWA, 1997; LEE et al., 1995). Unexpectedly, Ang-II did not alter with aging and RT (Figure 6A).

In young groups, RT was able to increase Ang-II in LV tissue compared to matched-sedentary group (Figure 6A), while other study did find any effect of RT (BARAUNA et al., 2008). Differences on RT protocols and methodologies of Ang-II measurements (ELISA x radioimmunoassay) could explain the differences between. These authors suggest that Ang-II does not exert a role in the cardiac hypertrophy after RT. Ang-II has been associated with cardiomyocyte hypertrophy acting at AT1-receptors *in vitro* (LU; YANG, 2009) and in senescent rats (JONES; BLACK; WIDDOP, 2004). We would expect to find increases of Ang-II concentration in OS rats since studies have reported that Ang-II facilitates the collagen synthesis and myocardial fibrosis by TGF-β1 (BRIEST et al., 2001; DOSTAL, 2001; KUPFAHL et al., 2000; SCHULTZ et al., 2002; TSURUDA, et al., 2004) as well as MMP activity and TIMP production (CHUA; HAMDY; CHUA, 1996; SADOSHIMA; IZUMO, 1993).

On the other hand, natriuretic peptides are active peptides that induce degradation of collagen (GONZÁLEZ; LÓPEZ; DÍEZ, 2004). ANP is associated with pathological cardiac hypertrophy to pressure overload (BARAUNA et al., 2008; JIN et al., 2000) and is released by volume overload (MORI et al., 2004). ANP has paracrine effects in the heart (DIETZ, 2005) and limits cardiac hypertrophy and fibrosis (LEVIN; GARDNER; SAMSON, 1998; VARDENY; TACHENY; SOLOMON, 2013) for inhibiting secretion and proliferation of collagen (CAO; GARDNER, 1995; MAKI et al., 2000). It increases in LV with aging (WU; KWAN; TANG, 1997). Even though we would expect to find reduced ANP concentration, even considering cardiac hypertrophy observed in OS group, ANP was elevated in OS *vs.* YS rats (Figure 6B), which it is consistent with other studies (WU; KWAN; TANG, 1997; YOUNES et al., 1995). Since we found increased LV collagen in OS rats *vs.* YS (Figure 2D), this augmented ANP concentration in LV could be associated with reduced collagen synthesis - and not associated

with increased degradation capacity - and supported by its capacity to inhibit proliferation of, and collagen secretion from, cardiac fibroblasts (CAO; GARDNER, 1995; MAKI et al., 2000). In fact, we did observe reduced COL-III synthesis, but not COL-I, which could be associated with ANP concentrations in OS group (Figure 4A and 4B).

CONCLUSION

Although there are some controversies about “intrinsic” aging-induced remodeling and overlap cardiac adaptations between aging and cardiovascular disease (HORN, 2015) our results point out the relevance of chronic effects of resistance training in aging experimental model. The RT triggers potential effects in ECM homeostasis of LV in aged rats with substantial changes in passive component of diastolic relaxation. Of note, clinical trials and others studies are required to address this approach and further understanding of this issue.

STUDY LIMITATIONS

We know the collagen corresponds the most abundant fibrous protein of ECM and should be investigated. However, our first goal was to investigate the overall response in the LV. Moreover, picrosirius red staining could be used as suitable histological technique to observe the differences of interstitial collagen-types among the fibers.

Moreover, this study did not evaluate the LV collagen cross-linking, which have been attributed to myocardial stiffness in aging animals models (NORTON et al., 1997) These cross-linkings can be modulated by aerobic exercise training (THOMAS et al., 2000) and would be interesting to investigate the effects of RT in order to contribute with the findings of this study.

ACKNOWLEDGEMENTS

We would like to kindly thank Dr. James M Kuczmarski for providing invaluable advice and feedback in the writing of this study.

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