PROGRAMA INTERINSTITUCIONAL DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS
UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
DEPARTAMENTO DE CIÊNCIAS FISIOLÓGICAS
LABORATÓRIO DE FISIOLOGIA DO EXERCÍCIO

EFEITO DO TREINAMENTO RESISTIDO E REPOSIÇÃO DE HORMÔNIO SOBRE O TRANSPORTADOR DE GLICOSE HEPÁTICO (GLUT2) DE RATAS OVARIECTOMIZADAS.

Tese de doutorado de Luciane Magri Tomaz

Orientador: Prof. Dr. Sérgio E. A. Perez

Co-Orientador: Prof. Dr. Jean Marc Lavoie

São Carlos - SP
Outubro de 2016

UNIVERSDIDADE FEDERAL DE SÃO CARLOS

PROGRAMA INTERINSTITUCIONAL DE
PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

EFEITO DO TREINAMENTO RESISTIDO E REPOSIÇÃO DE HORMÔNIO SOBRE O TRANSPORTADOR DE GLICOSE HEPÁTICO (GLUT2) DE RATAS OVARIECTOMIZADAS.

Tese apresentada ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas, do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Doutor em Ciências Fisiológicas. Área de Concentração: Fisiologia.

14 de Outubro de 2016.

São Carlos – SP

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

Tomaz, Luciane Magri
T655e Efeito do treinamento resistido e reposição de hormônio sobre o transportador de glicose hepático (GLUT2) de ratas ovariectomizadas / Luciane Magri Tomaz. -- São Carlos : UFSCar, 2016.
65 p.

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

1. Acúmulo de gordura. 2. PEPCK. 3. Glicogênio hepático. 4. Exercícios. 5. Glicose. I. Título.

UNIVERSIDADE FEDERAL DE SÃO CARLOS

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

Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado da candidata Luciane Magri Tomaz, realizada em 14/10/2016:

Prof. Dr. Sergio Eduardo de Andrade Perez
UFSCar

Prof. Dr. Fabio Santos de Lira
UNESP

Profa. Dra. Fulvia de Barros Manchado Gobatto
UNICAMP

Profa. Dra. Márcia Regina Cominetti
UFSCar

Prof. Dr. Cleiton Augusto Libardi
UFSCar

Apresentação

História acadêmica

Iniciei a prática de esportes aos 10 anos, me encontrei na natação. Esporte este que tomei como paixão e profissão por 12 anos. Desde que me encontrei na natação, descobri uma graduação, motivada pelo meu primeiro treinador Fabiano Carvalho. Acho que naquela época eu nem sabia o que era uma graduação, mas a admiração pelo trabalho e o quanto ele me transformou era tão grande que quando me perguntavam o que eu gostaria de ser quando crescesse, logo respondia; - "Profissional de Educação Física!" Ao longo destes 12 anos nadando e estudando, terminei a graduação em Educação Física. – "E agora? Qual o caminho?" Tantas possibilidades. De novo, depois de tanto tempo, meu grande primeiro treinador me ajudou a encontrar o caminho, mais que isso, me mostrou que o caminho eu já sabia, mas era preciso acreditar que eu era capaz de fazer o que quer que eu me propusesse a fazer, se fosse com a mesma vontade e disciplina que eu tive por 12 anos de natação!

Tomei coragem e mergulhei nos mares da fisiologia do exercício, não foi nada fácil, tive de dar alguns "Sprints de 100 m nos estudos" para conseguir acompanhar a 9ª turma do curso de fisiologia do exercício da UFSCar. Foi em 2009 que iniciei a especialização em fisiologia do exercício e me deparei com um oceano de informações e possibilidades. Mais que isso, em pouco tempo descobri que apesar das dificuldades de viajar final de semana, assistir aula do professor Vilmar Baldissera sobre fisiologia cardiorrespiratória, fazer o teste de lactato mínimo, sair da piscina praticamente carregada, de novo descobri o que eu queria! Estava apaixonada pela fisiologia do exercício. Estava pronta para novas braçadas. Mestrado será? Eu gostei muito de fisiologia, mas fazer um mestrado era um empenho muito grande! Deixar tudo e mudar para São Carlos? Será que dou conta? Sabia que dava, só não sabia que tão rápido e tão intenso.

Terminei a especialização em Julho de 2010 e em Agosto deste mesmo ano me mudei para a cidade de São Carlos. Cinco meses de muito estudo, com muita disciplina e foco, passei em primeiro lugar com o professor Sérgio Perez e em 6^0 na classificação geral de bolsas do programa de pós-graduação em ciências fisiológicas. Eu não acreditava, só conseguia explicar o que eu sentia para os meus amigos da natação, foi como abaixar uns 2 segundos na prova de 100m e pegar primeiro lugar! Muito merecido, eu sabia o quanto tinha me esforçado. Em março de 2011 começou a rotina de pós-graduação. Projetos, aulas, estudo, artigo em inglês para estudar e que inglês? Difícil, como se não bastasse me propus a ir a um congresso internacional apresentar, em Inglês, um trabalho em que eu era coautora. Fizemos um tour pela Europa com um orientador turístico sensacional! A viagem foi inesquecível e

por fim, a apresentação do trabalho também foi! Apesar do empenho do Sérgio em me ajudar a apresentar o trabalho, foi ruim, muito ruim e voltando desta viagem incrível, eu fiz uma promessa pessoal e verbalizei a ele; - "Próximo congresso internacional que eu for, meu inglês será bom, pelo menos!" Em pouco tempo terminei o mestrado e já estava com o projeto do doutorado em andamento. Uma etapa tão importante concluída, mas que passou tão rápido quanto as provas de 50 m.

Tive tempo de analisar meus erros, acertos e em Outubro de 2013 me matriculei no doutorado. Com uma visão mais ampla sobre a importância da pós-graduação para a pesquisa científica, procurei me aprofundar na linha de pesquisa que estava trabalhando durante o mestrado. Neste período, estavam abertos editais do governo federal brasileiro para o desenvolvimento de parte do projeto de doutorado no exterior. Por que não? Tentar é o mínimo. Arrumei tudo o que era preciso e 6 meses depois eu estava embarcando para o Canadá! Um pouco de tudo eu levava na minha mala, menos o inglês, em contrapartida, tinha muita força de vontade e disciplina, mas muita, muita ansiedade também! Primeiro dia na Universidade de Montreal, ao ir até a sala do professor Jean-Marc Lavoie, sétimo andar, um cheiro de cloro que eu não conseguia me conter, fui em direção ao cheiro e lá estava a porta da arquibancada daquela piscina gigantesca. Respirei fundo muitas vezes antes de ir falar com o professor. Acho que se eu entendi ⅓ do que ele me disse foi muito. O tempo passou, muitas braçadas eu dei naquela piscina e em pouco tempo eu estava falando inglês, aprendendo e pesquisando muito! Encerrei meu período no Canadá apresentando meus dados em um congresso Canadense e uma apresentação do meu projeto para graduandos, pós-graduandos e professores do departamento, em inglês! Foi uma experiência incrível.

De volta ao Brasil, ao laboratório, muita vontade de aplicar tudo que vi e experimentei. Cursei as disciplinas oferecidas no programa com um olhar muito mais profundo e crítico. Senti o quão enriquecedor foi ter esta experiência no Canadá. Foi sensacional entender como as coisas funcionam no exterior e assim, conseguir valorizar o que o Brasil e o Canadá tem de melhor.

Após exatamente 1 ano voltei à Montreal, desta vez para finalizar a escrita do artigo para publicarmos, 1 mês apenas e não consigo nem expressar o quão intenso foram aqueles dias. Muito frio e muita coisa para acontecer; artigo para escrever, trabalho para apresentar em congresso, rever alguns amigos e um computador quebrado. Desta vez não consegui nem mesmo sentir o cheiro da piscina. Foco, artigo, muito foco! Valeu a pena, voltei do Canadá com um artigo na mala. Entre algumas submissões e rejeições, parte importantantíssima do processo de aprendizagem, o nosso artigo foi aceito na revista científica "Journal of Exercise

Nutrition and Biochemistry". Felicidade indescritível, acho que naquela linguagem da natação, seria algo como aquele momento em que você fica em 2^0 lugar por alguns décimos, mas abaixou alguns segundos do seu melhor tempo. Só quem acompanhou do início ao fim para entender a felicidade que é isso! Em fim alguns dos projetos em que participei no decorrer desta minha formação de professora e pesquisadora em ciências fisiológicas, estão nos itens anexos da tese, assim como o Artigo!

Agora estou aqui, em mais uma etapa desafiante. Apresentando a tese em inglês, no meu país, e o que vai ser daqui pra frente não sei. Quanto mais me aprofundo, percebo que cada braçada é como uma gota no oceano, insignificante talvez, mas essencial. Nestes anos todos, me alegra lembrar que depois que aprendi a nadar, seja em um rio de águas calmas ou em mar aberto, a direção a ser tomada está em minhas mãos. Hoje posso dizer que não me preocupo em saber até onde vou chegar, preciso apenas de oportunidades!

AGRADECIMENTOS

Tão desafiante quanto a produção desta tese, foi utilizar duas página para agradecer as pessoas que fizeram parte desta minha trajetória. Sempre achei muito difícil esta parte dos agradecimentos, talvez porque a vida não se coloca em análise de regressão e não é pelo valor de *P* que descobrimos a significância das pessoas na nossa trajetória. Mas vamos lá, este é o momento.

Primeiramente agradeço a Deus por me guiar, iluminar e me dar serenidade para seguir em frente e não desanimar, digo, persistir apesar das dificuldades. Agradeço aos meus pais Aparecido Tomaz de Brito e Tereza Martins Magri Tomaz, e familiares pela paciência, carinho, atenção e estímulos por que não é fácil. Obrigada Natalia, Carolina e Rafaela, minhas irmãs!

Agradeço imensamente meu marido, assim que tudo der certo por aqui, oficializaremos o que já é muito oficial pra nós. João Paulo você é a melhor companhia que pude imaginar em ter. Esta tese com certeza não é só minha, se tem alguém que faz parte disso, com certeza é você. É até engraçado quando as pessoas falam que eu tenho facilidade com tecnologia ou que sou bem dinâmica com algumas coisas, mal sabem não é mesmo? Não só pelo direcionamento "tecnológico" que você me proporciona, mas principalmente pelas discussões acerca do metabolismo hepático e treinamento que você se esforça tanto pra entender e me ajudar. Obrigada pela tão gigantesca paciência e carinho que você tem por mim!

Dentro da pós-graduação fiz sinceras amizades, Marina Barbosa minha mentora desde o mestrado, obrigada pelas discussões, ensinamentos e companhia. Luísa Cedin que chegou no laboratório como IC foi ficando e cativando com seu bom humor e sinceridade! Gustavo Canevazzi, nossa loucura de tentar mudar o mundo, independente do caminho que trilharmos, sabemos qual será nossa estratégia pra isso! Vocês são muito importantes, muito obrigada pela amizade.

Aos meus orientadores; Sérgio Perez, agradeço por me dar carta branca e suporte em tudo que eu inventava de fazer, ainda bem que tudo deu certo não é mesmo?! Agradeço imensamente ao professor Jean Marc que também abriu as portas do seu laboratório, pelo acolhimento, amizade e todo o suporte que me proporcionou enquanto eu estava em Montreal e até por aqui no Brasil. Muito Obrigada mesmo!

Agradeço a todo o pessoal do laboratório, pelo convívio em todos esses anos, alguns chegando, outros que já se foram mas de cada um de vocês guardo um sentimento especial e

aprendizado, obrigada Natalia Santanielo, Maria Fernanda Rodrigues, Danilo Bertucci, Cristiani Lagoeiro, Júlio Cesar, Giovanna Togashi, Jeferson Teixeira, Guilherme Borges, José Neto, Leandro Ruffoni, Daiana Viana, Anderson Lino, Fabiano Ferreira, Mateus Domingos, Rodrigo Magosso, Thiago, João, Markus Campos e Fernanda Daniele.

A Zahra Farahnak que me acolheu no laboratório em Montreal, me ensinou de tudo, desde técnicas de laboratório, pesquisas e até que o "outro lado do sol" era a sombra! Ótimos momento, ótimos aprendizados e amizade, meu muitíssimo obrigada por tudo que fez por mim.

Agradeço aos técnicos de laboratório, Pierre Corriveau, da UdeM que só de olhar minhas amostras sabia o que eu tinha feito certo ou errado, e a Tatiana Passos pela ajuda no andamento das análises por aqui.

Ao Programa Interinstitucional de Pós-Graduação em Ciências Fisiológicas UFSCar/UNESP. A todos os professores do departamento de Ciências Fisiológicas que sempre nos auxiliaram tanto no processo de aprendizado e formação quanto no andamento e viabilidade das nossas pesquisas. À Secretaria de Pós-Graduação do Departamento de Ciências Fisiológicas da UFSCar, Alexandre e a Carmen. Ao departamento de Cinesiologia da Universidade de Montreal, por facilitar a parceria e disponibilizar infraestrutura e equipamentos para o desenvolvimento dessa tese de doutorado. A todas estas instituições a baixo por, de alguma forma, viabilizar a conclusão desta tese. Aos membros da banca examinadora, por aceitarem o convite em participar da defesa e promover importantes contribuições acadêmica e científica.

De tudo ficaram três coisas
A certeza de que estamos começando
A certeza de que é preciso continuar
A certeza de que podemos ser interrompidos...
antes de terminar.

Façamos da interrupção um caminho novo
Da queda, um passo de dança
Do medo, uma escada
Do sonho, uma ponte
Da procura, um encontro!

Fernando Sabino

SÍNTESE DA TESE EM LÍNGUA PORTUGUESA

Tanto a menopausa quanto a retirada do hormônio estradiol de ratas, pela ovariectomia (Ovx), são associadas com várias alterações metabólicas, tais como a perda de densidade mineral óssea, diabetes, perturbações da função muscular, doença cardíaca coronária, (LEITE et al., 2009; MALTAIS, 2009; JELENIK, 2013), incluindo o aumento da deposição de gordura no fígado (NEMOTO et al, 2000; PAQUETTE et al, 2007; VÖLZKE et al, 2007). O acúmulo de gordura no fígado é conhecida como um componente de síndrome metabólica hepática (MARCHESINI et al., 2005). A esteatose hepática é duas vezes mais comum no período pós-menopausa do que no período pré-menopausa (BARSALANI et al, 2008; CLARK, 2002). O mecanismo geral do acumulo de gordura no fígado envolve um desequilíbrio entre a disponibilidade e eliminação de lipídios. O acúmulo excessivo de gordura no fígado pode ocorrer como um resultado do aumento da entrega de gordura para o fígado e o aumento da síntese de gordura no fígado, reduzido a oxidação das gorduras e reduziu a exportação de gordura sob a forma de triglicerídeos de lipoproteínas de baixa densidade. Neste contexto, é importante considerar estratégias para prevenir ou reverter essas mudanças metabólicas no período pós-menopausa. Existem estratégias consolidadas para tentar solucionar estes problemas; a terapia de reposição hormonal e exercícios são ótimas estratégias (SAENGSIRISUWAN et al., 2009). A reposição hormonal (E2) é uma das terapias mais eficazes utilizados para reduzir essas complicações pós-menopáusicas desfavoráveis a saúde da mulher. Mas apesar desta eficácia na prevenção de muitas doenças metabólicas, há um maior risco de seus efeitos adversos, tais como o aumento da incidência de câncer de mama e um aumento transitório de tromboembolismo venoso (SKOUBY et al., 2005; SCARABIN; OGER; PLUMESA, 2003). A prática de exercício de *endurance* também tem sido importante estratégia para evitar a acúmulo de gordura e a regulação positiva de diversos genes envolvidos na lipogênese *de novo* (PIGHON; GUTKOWSKA; JANKOWSKI,

2010). Há também evidências de que o treinamento resistido (RT) em ratas poça ser uma alternativa para o treinamento de *endurance* por impedir ou reduzir o conteúdo de gordura hepático (CORRIVEAU et al, 2008; LEITE et al, 2009) sem aparente evidências de efeitos adversos. Se, de facto, a lipogênese de *novo* é aumentada no fígado das ratas Ovx, as moléculas de glicose adicionais devem estar disponíveis como substratos para a síntese de lipídios. Mas, ainda há pouca informação sobre como o fígado gorduroso pode afetar o metabolismo de glicose hepático.

Assim, o objetivo deste estudo foi avaliar o efeito da Ovx e 12 semanas de treinamento resistido (RT) sobre a expressão gênica e proteica do transportador de glucose 2 (GLUT2), o principal transportador de glicose hepático, e sobre o fator de transcrição PPARγ, conhecido por modular a expressão de GLUT2. Para esta proposta, foram utilizadas quarenta ratas da espécie Holstman, separadas em 5 grupos: Sham-Sedentário (Sed), Sham treinamento resistido (RT), Ovx-Sed, Ovx-RT, e Ovx-reposição hormonal (E2). O protocolo de RT foi constituído por sessões a cada 72 h, durante 12 semanas, o qual os animais realizavam de 4 a 9 escaladas verticais (1,1 m) com pesos progressivos (até 30 g), atados à cauda e com intervalos de 2 min. A reposição do estradiol foi realizado por meio do implante de uma cápsula Silastic (5% de 17β-estradiol em 10µl de solução) no dorso das ratas.

Além da gordura hepática, a concentração proteica de GLUT2 e a transcrição de PPARγ foram aumentados ($P < 0,05$) em comparação entre animais Ovx e Sham-SED, sugerindo o aumento da absorção de glucose hepática decorrente a retirada do estrogênio. O RT, e a E2 em ratas Ovx reduziu o acúmulo de gordura no fígado, bem como a expressão de GLUT2 e PPARγ para valores próximos ao do grupo controle Sham-Sed. Os resultados deste estudo sugerem que o acúmulo de gordura no fígado de ratas Ovx é acompanhada por um aumento da captação de glicose proporcionando assim, um aumento da lipogênese *de novo*. O RT parece ser um modelo de exercício apropriado para contornar estes efeitos.

EFEITO DO TREINAMENTO RESISTIDO E REPOSIÇÃO DE HORMÔNIO SOBRE O TRANSPORTADOR DE GLICOSE HEPÁTICO (GLUT2) DE RATAS OVARIECTOMIZADAS.

RESUMO

A retirada do estrogênio através da ovariectomia (Ovx) em ratas, tem sido constantemente associado a distúrbios do metabolismo lipídico, dentre eles, o acúmulo de gordura hepático que já é bem documentado. Por outro lado, ainda há pouca informação sobre como o fígado gorduroso pode afetar o metabolismo de glicose hepático. Assim, o objetivo deste estudo foi investigar o efeito da Ovx e 12 semanas de treinamento resistido sobre a expressão gênica e proteica do transportador de glucose 2 (GLUT2), o principal transportador de glicose hepático, e sobre o fator de transcrição PPARγ, conhecido por modular a expressão de GLUT2. O treinamento resistido (RT) em ratas é uma categoria de exercícios que recebia pouca atenção, mas tem mostrado resultados positivos sobre as alterações metabólicas de ratas Ovx, bem como em mulheres pós-menopausadas. Quarenta ratas da espécie Holstman foram separadas em 5 grupos: Sham-Sedentário (Sed), Sham treinamento resistido (RT), Ovx-Sed, Ovx-RT, e OVX- reposição hormonal (E2). O protocolo de RT foi constituído por sessões a cada 72 h, durante 12 semanas, o qual os animais realizavam de 4 a 9 escaladas verticais (1,1 m) com pesos progressivos (até 30 g), atados à cauda e com intervalos de 2 min. Além da gordura hepática, os níveis proteicos de GLUT2 e a transcrição de PPARγ foram aumentados ($P < 0,05$) em comparação entre animais Ovx e Sham-SED, sugerindo o aumento da absorção de glucose hepática decorrente a retirada do estrogênio. O treinamento resistido, bem como a reposição do hormônio em ratas Ovx reduziu o acúmulo de gordura no fígado, bem como a expressão de GLUT2 e PPARγ para níveis próximos ao do grupo controle Sham-Sed. Os resultados deste estudo sugerem que o acúmulo de gordura no fígado de ratas Ovx é acompanhada por um aumento da captação de glicose proporcionando assim, um aumento da lipogênese *de novo*. RT parece ser um modelo de exercício apropriado para contornar estes efeitos.

Palavras chaves: Acúmulo de gordura, PEPCK, Glicogênio Hepático, Exercício e Glicose.

INTERINSTITUTIONAL PROGRAM OF POST GRADUATION IN PHYSIOLOGICAL SCIENCES
FEDERAL UNIVERSITY OF SÃO CARLOS
BIOLOGICAL SCIENCES AND HEALTH CENTER
DEPARTMENT OF PHYSIOLOGICAL SCIENCES
EXERCISE PHYSIOLOGY LABORATORY

Doctoral Thesis of Luciane Magri Tomaz

Supervisor: Professor. Ph.D. Sérgio E. A. Perez

Co-Supervisor: Professor. Ph.D. Jean Marc Lavoie

EFFECTS OF RESISTANCE TRAINING AND HORMONE REPLACEMENT ON HEPATIC GLUCOSE TRANSPORTER 2 (GLUT2) IN OVARIECTOMIZED RATS.

ABSTRACT: Oestrogens withdrawal in rats, through ovariectomy (Ovx), has been repeatedly associated with disturbances in lipid metabolism. Among them, liver fat accumulation is well substantiated. On the other hand, there is few information on how liver fat in Ovx rats may affect glucose metabolism in liver. The purpose of the study was to investigate the effects of Ovx and 12 weeks resistance training (RT) program on gene expression of GLUT2 the main glucose transporter in liver and on PPARγ a transcription factor known to target GLUT2 gene expression. RT in rat is an exercise training model that has received little attention but that has been shown to result in metabolic adaptations in Ovx rats as well as in post-menopausal women. Holstman rats were divided into 5 groups: Sham-sedentary (Sed), Sham-resistance trained (RT), Ovx-Sed, Ovx-RT, and Ovx-Sed with hormone replacement (E2). The RT protocol consisted of resistance training sessions held every 72 h for 12 weeks, during which session the animals performed 4 to 9 vertical climbs (1.1 m) with progressive weights (up to 30 g) tied to the tail at 2 min intervals. In addition to liver fat, GLUT2 protein levels and PPARγ transcripts were all increased ($P < 0.05$) in Ovx compared to Sham-Sed animals, suggesting an increase hepatic glucose uptake under oestrogens withdrawal. Resistance training as well as oestrogens replacement in Ovx rats decreased liver fat accumulation as well as GLUT2 and PPARγ gene expression to the level of Sham-Sed group. The present results suggest that liver fat accumulation in Ovx rats is accompanied by an increased glucose uptake thus providing substrate for an increased *de novo* lipogenesis. RT appears to be an appropriate exercise model to circumvent these effects.

Key Words: Liver fat, PEPCK, Hepatic glycogen, Exercise and Glucose.

FIGURE LIST

Figure 1: Effects of estradiol in many tissue about glucose and lipid metabolism. 18

Figure 2: Metabolic effects of E2 in hepatocyte, adipocyte and myocyte. 20

Figure 3: Schematic representation of E2 signaling in liver metabolism. 22

Figure 4: Resistance training effects in different tissues. 23

Figure 5. Organogram of the experimental groups 27

Figure 6. Materials used to construct the silastic capsule. 28

Figure 7. Rat climbing the ladder during the training session. 29

Figure 8. Maximum load determination from 75% of rat's body mass. 31

Figure 9. Carried load per training session. 31

Figure 10. Graphical simulation of agarose gel and integrity number 34

Figure 11. Color and size of liver. 38

Figure 12. Carried Load on trained groups throughout training. 39

Figure 13. Hepatic mRNA and protein levels of the GLUT2. 40

Figure 14. Liver TAG and hepatic mRNA expression of PPARγ. 41

Figure 15 Glycogen levels and mRNA expression of PEPCK. 42

Figure 16. Coefficient correlation and linear regression of GLUT2 and PPARγ gene expression. 43

TABLE LIST

Table 1. Training schedule. 29

Table 2. Oligonucleotides primers used to RT-PCR..... 34

Table 3. Anthropometric parameters and food intake..... 39

ABBREVIATION LIST

GLUT2: Glucose transporter 2

PPARγ: Peroxisome proliferator-activated receptor gamma

Ovx: Ovariectomy

RT: Resistance training

Sed: Sedentary

E2: Estrogen Hormone replacement

PEPCK: Phosphoenolpyruvate carboxykinase

SREBP-1c: sterol regulatory element-binding protein 1c

SCD-1: stearoyl-CoA desaturase-1

VLDL-TG: very low density lipoprotein triglycerides

cDNA: complementary DNA

RIN: RNA integrity number

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

ACTB: Beta-actin

Liver TAG: Hepatic Triacylglycerol

NAFLD: Non-alcoholic fatty liver disease

SUMMARY

1. INTRODUCTION 18
2. OBJECTIVE 24
2.1 Especific Objective 24
3. HYPOTHESIS 25
4. METHODS 26
4.1 Ethics Committe Approve 26
4.2 Animal Care 26
4.3. Experimental Groups 26
4.4 Surgical procedures of Ovariectomy and Sham 27
4.5 Hormone Replacement (E2) 28
4.6 Resistance training 28
4.6.1 Apparatus to training sessions 28
4.6.2 Familiarization Step. 30
4.6.3 Maximal load determination step. 30
4.6.4 Training Session. 30
4.7 Food intake and body weight control 32
4.8 Animals euthanasia and tissue sampling 32
4.9 17β-estradiol plasma level 32
4.10 Quantitative Real Time Polymerase Chain Reaction (RT-PCR) 33
4.11 Protein Assay 35
4.12 Liver Triglycerides Assay 36
4.13 Hepatic Glycogen Assay 36
4.14 Statistical Analysis 36
5. RESULTS 38
6. DISCUSSION 44
6.1 Ovariectomy 44
6.2 Estrogen Replacement 46
6.3 Resistance training 47
7. CONCLUSION 48
8. STUDY LIMITATIONS 49
9. FUTURE PERSPECTIVES 50
10. ATTACHMENTS 51
10.1 Published Article 51
10.2 Published Articles and in submission process related to this Project 58
10.3 Abstracts Published in International Conferences 59
10.4 Ethics Committee's opinion on the Animal's Use 60
11. REFERENCE 61

1. INTRODUCTION

Estrogens are steroid hormones primarily known for their role in promotion of female sex characteristics and reproductive capability. Estrogens are produced by the ovaries and adrenal glands and circulate throughout the body, where they have effects on most organ systems, including brain, breast, cardiovascular (heart and vasculature) immune, reproductive (ovaries and uterus), bladder, skin, and bone (JELENIK; RODEN, 2013; MESSIER et al., 2011). There are three forms of estrogens in the female body: estrone (E1), estradiol (E2), and estriol (E3). During a woman's reproductive years, the principal circulating estrogen is 17β-estradiol (E2). 17β-estradiol is involved in gene regulation and has an important role in several physiological and pathological states in both men and women (BARROS; MACHADO; GUSTAFSSON, 2006)

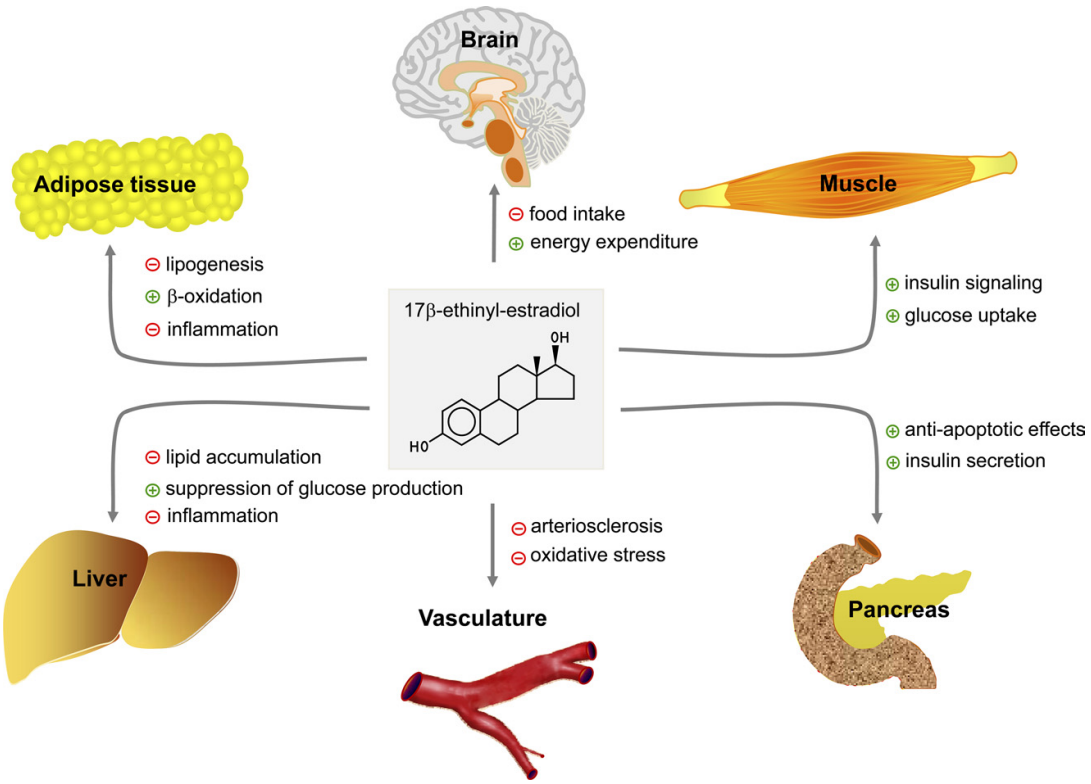


Figure 1: Effects of estradiol in many tissues about glucose and lipid metabolism.
From JELENIK; RODEN, (2013) Tissue-specific stimulatory (+) and inhibitory (-)

Around 40s age the estrogens level starts to decrease and menstrual cycles start to be irregular, this period is called climacteric. In this period many hormonal changes happen, predominantly caused by a marked decline in the ovarian follicle number. When there are the cessation of follicular activity associated to the cessation menstruation it marks the end of natural female reproductive life and the postmenopausal period (MESSIER et al., 2011).

Both menopause and estrogen withdrawal in rats, through ovariectomy or other animal models to simulate menopause, are associated with several metabolic changes, such as loss of bone mineral density, diabetes, impairment of muscle function, coronary heart disease, (LEITE et al., 2009; MALTAIS; DESROCHES; DIONNE, 2009; JELENIK; RODEN, 2013) including increase of liver fat deposition (NEMOTO et al., 2000; PAQUETTE et al., 2007; VÖLZKE et al., 2007). Liver fat accumulation is recognized as the hepatic component of metabolic syndrome (MARCHESINI et al., 2005).

Hepatic steatosis is twice as common in post-menopausal period as in pre-menopausal time (BARSALANI et al., 2008; CLARK; BRANCATI; DIEHL, 2002). The general mechanism of liver fat accumulation involves an imbalance between lipid availability and lipid disposal. Excessive fat accumulation in liver can occur as a result of increased fat delivery into liver and increased fat synthesis in liver, reduced fat oxidation, and reduced fat exportation in the form of very low density lipoprotein triglycerides (VLDL-TG) (LAVOIE; PIGHON, 2012; MUSSO; GAMBINO; CASSADER, 2009). Measurements of molecular markers in different studies suggest a reduction in lipid oxidation and in the production of VLDL and an increase in *de novo* lipogenesis (BARSALANI et al., 2008; PAQUETTE et al., 2009).

The specific origin of the lipids that accumulate in rat liver under estrogen withdrawal remains unclear. However, several molecular markers of *de novo* lipogenesis in the liver, including the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) and its downstream enzyme stearoyl-CoA desaturase-1 (SCD-1), have been reported to be greatly increased in ovariectomized rats (DOMINGOS; RODRIGUES; STOTZER,

2011; PAQUETTE et al., 2008). If, indeed, *de novo* lipogenesis is increased in the livers of Ovx rats, then extra glucose molecules must be available as substrates for lipid synthesis.

When glucose concentration outside the liver rises, glucose is rapidly taken up into hepatocytes via GLUT2. GLUT2 is known to transport glucose across the hepatic plasma membrane in a bidirectional manner (WOOD; TRAYHURN, 2003) and are not sensitive to insulin. Thus, the process of glucose uptake and phosphorylation by the hepatocyte depends on the glucose concentration outside of the cell. Glucose is temporarily 'trapped' by phosphorylation to glucose-6-phosphate and is unaffected by insulin, at least in the short term (CHOUKEM; GAUTIER, 2008). However, insulin is crucial in the subsequent steps of the storage process, like glycogen synthase and inhibition of glycogen phosphorylate (PETERSEN et al., 1998).

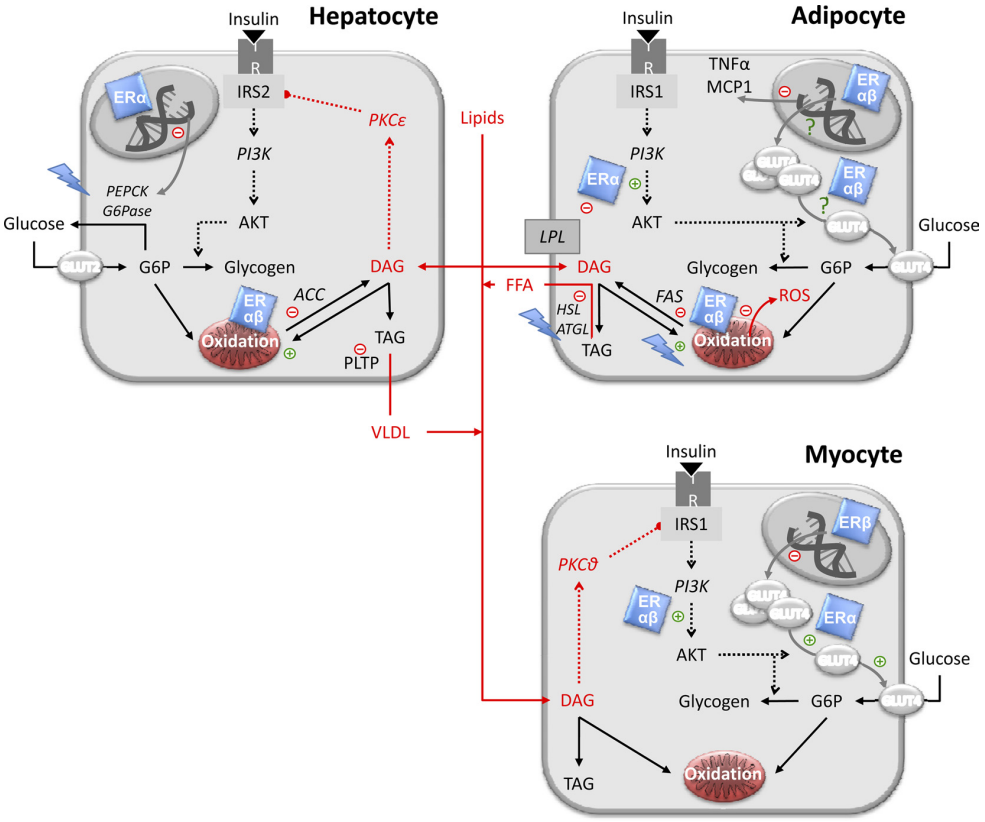


Figure 2: Metabolic effects of E2 in hepatocyte, adipocyte and myocyte.
From (JELENIK; RODEN, 2013). Ovariectomized female mice with direct (peripheral) effects are indicated by blue diamonds showing the responsible ER whereas indirect (central nervous) effects are indicated by blue flash symbols.

Several studies have shown that liver GLUT2 expression is regulated under different metabolic states. Liver GLUT2 expression is decreased during starvation and is restored to normal levels by a high-carbohydrate diet (BURCELIN et al., 1992; THORENS; CHARRON; LODISH, 1990) and GLUT2 are increased in streptozotocin-diabetic rats associated with SREBP-1c expression increased in liver (IM et al., 2005b). In addition, we also targeted the transcription factor PPARγ.

PPARγ acts as a critical transcription factor in the regulation of adipose differentiation, lipid storage, and of genes involved in energy storage and utilization. It can also affect insulin sensitivity by regulating hormones, cytokines, and proteins that are involved in insulin resistance (STAELS; FRUCHART, 2005). Gene expression of PPARγ is low in liver tissue, it represent only 10% - 30% of the level in adipose tissue (MATSUSUE et al., 2003; ROGUE et al., 2010). Nevertheless, there is some evidence that liver GLUT2 is a direct target of PPARγ, thus contributing to glucose transport into the liver (IM et al., 2005a). PPARγ also targets genes involved in glucose metabolism as the phosphoenolpyruvate carboxykinase (PEPCK) (NAKAMURA; YUDELL; LOOR, 2014). PEPCK is an enzyme in the lyse family used in the metabolic pathway of gluconeogenesis. It converts oxaloacetate into phosphoenolpyruvate and carbon dioxide. Pyruvate dehydrogenase catalyzes an irreversible degradation step of pyruvate to acetyl CoA, however PEPCK induction switches the metabolic fate of pyruvate from oxidation to G3P synthesis and has been almost exclusively linked to gluconeogenesis to the point that changes in the levels of PEPCK mRNA or its activity are associated with the control of hepatic glucose output with alterations in life span (YANG; KALHAN; HANSON, 2009).

Thus, it is important to consider strategies to prevent or revert these metabolic changes in postmenopausal period. Nowadays there are consolidated strategies to try to solve this problems; Hormone replacement therapy and exercises (SAENGSIRISUWAN et al.,

2009). Hormone replacement therapy is one of the most effective therapies used to reduce unfavorable postmenopausal complications such as osteoporosis (STEVENSON, 2005), hypercholesterolemia (ŽEGURA et al., 2006), coronary heart disease (COLLINS et al., 2006), and risk of developing type 2 diabetes mellitus (SKOUBY et al., 2005), despite its adverse effects, such as an increased incidence of breast cancer and a transient increase in venous thromboembolism (SCARABIN; OGER; PLU-BUREAU, 2003). In Ovx rodents, estrogen replacement decreases fat accumulation, improves serum lipid profiles (SHINODA; LATOUR; LAVOIE, 2002), and restores insulin action on muscle glucose transport.

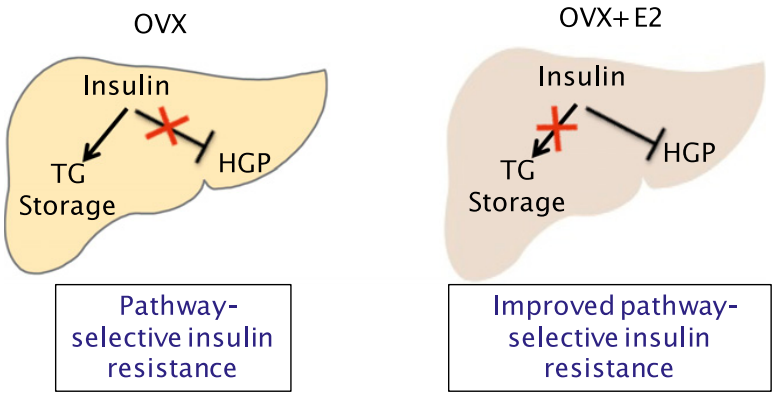


Figure 3: Schematic representation of E2 signaling in liver regulation of glucose and lipid metabolism. Figure from (ZHU et al., 2013).

Furthermore, there is accumulating evidence that endurance exercise training overcomes several of the metabolic effects of ovariectomy in rats. For instance, Ovx animals can benefit from exercise training by a reduction in fat gain (RICHARD; ROCHON; DESHAIES, 1987; SHINODA; LATOUR; LAVOIE, 2002) and insulin resistance in skeletal muscle (SAENGSIRISUWAN et al., 2009).

Endurance exercise training has also been reported to prevent liver fat accumulation in rats (PIGHON et al., 2010) as well as the up regulation of several genes involved in *de novo* lipogenesis (PIGHON; GUTKOWSKA; JANKOWSKI, 2010). There is also evidence that resistance or strength training in rats, as an alternative to endurance training, prevents or

decreases liver fat content (CORRIVEAU et al., 2008; LEITE et al., 2009). Thus, RT has been reported to promote muscle strength and hypertrophy in Ovx animals providing protection against menopause-associated sarcopenia and osteopenia (CORRIVEAU et al., 2008; LEITE et al., 2009).

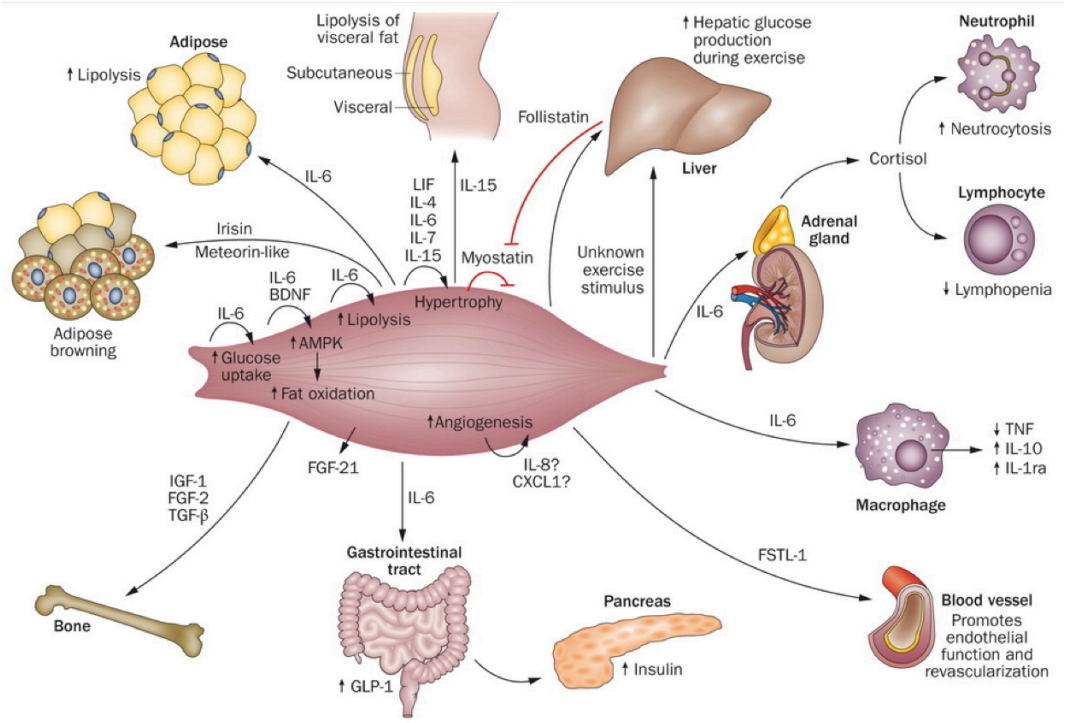


Figure 4: Resistance training effects in different tissues.
Figure from (BENATTI; PEDERSEN, 2015).

More recently, it was reported that resistance training restored the gene expression of key molecules involved in *de novo* lipogenesis in livers of ovariectomized rats (DOMINGOS; RODRIGUES; STOTZER, 2011). We, therefore, used a resistance training program, which can be considered a model of strength training, to test the hypothesis that resistance training ameliorates the increase in GLUT2 expression in livers of Ovx rats.

2. OBJECTIVE

The aim of this study was to use the resistance training program and estrogen replacement to promote alterations on hepatic GLUT2 and liver TAG of ovariectomized rats.

2.1 Especific Objective

Analyze the gene and protein expression of GLUT2 in trained ovariectomized rats or with hormone replacement.

Analyze the lipid content and PPARγ gene expression in trained ovariectomized rats or with hormone replacement.

Quantify the liver glycogen and PEPCK gene expression in trained ovariectomized rats or with hormone replacement.

Present a correlation of GLUT2 and PPARγ gene expression results in ovariectomized rats trained, sedentary or with hormone replacement.

3. HYPOTHESIS

We postulated that Ovx-induced liver fat accumulation would also result in increased hepatic glucose uptake through increased expression of GLUT2. The first purpose of the present study was to test the hypothesis that hepatic expression of GLUT2 is increased in Ovx rats. Thus, higher liver TAG as well PPARγ will be presented by Ovx rats.

To test the treatment of Ovx condition, we used the RT and E2 in Ovx rats to test the hypothesis that both will revert the Ovx effects in liver.

The RT as well E2 will act antagonistically to the effects of ovariectomy. Both interventions decrease GLUT2 and liver TAG and PPARγ expression and increase gene expression of PEPCK and glycogen amount in ovariectomy rats.

4. METHODS

4.1 Ethics Committe Approve

All experiments described in the present report were conducted according to the (Guide for the Care and Use of Laboratory Animals, 1996) and approved by the Ethics Committee on Animal Use from the Federal University of São Carlos (CEUA-UFSCar) Protocol n° 005/2013.

4.2 Animal Care

Female Holtzman rats *(n* = 40) from the animal facility of the University of São Paulo State (UNESP, Araraquara, Brazil) weighting ~ 220 g upon arrival were housed in collective cages. The animals had *ad libitum* access to food and tap water. All animals were fed with commercial rodent chow. Their environment was controlled in a reverse light cycle (12 h dark starting at 08:00 AM). Food intake was monitored daily over the entire experimental period. Body mass was measured 3 times/week at the same time of day.

Upon arriving at Exercise Physiology Laboratory from Federal University of São Carlos, (UFSCar, São Carlos, Brazil) the animals have remained for two weeks in acclimatization condition and inverted cycle. After this period, the animals were divided into five groups according to Figure 5.

4.3. Experimental Groups

Rats were randomly distributed into five experimental groups (*n* = 8/group). Rats first underwent a bilateral ovariectomy *(n* = 24) or a bilateral sham operation (Sham, *n* = 16). Thereafter, one group of Ovx and one group of Sham rats remained sedentary while one

group of Ovx and one group of Sham rats were submitted to a 12-week resistance training program. The third group of Ovx rats remained sedentary and was given 17β-estradiol replacement. Altogether, five groups were compared: Sham-sedentary (Sed), Sham-resistance trained (RT), Ovx-Sed, Ovx-RT, and Ovx-Sed with hormone replacement (Ovx-E2).

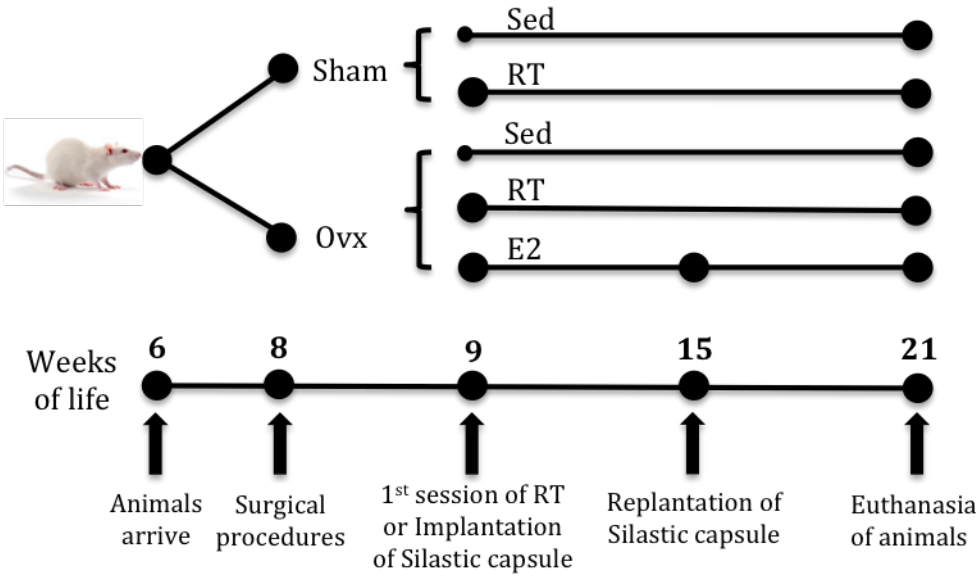


Figure 5. Organogram of the experimental groups
Adapted from BARBOSA et al., 2016

4.4 Surgical procedures of Ovariectomy and Sham

Ovariectomy and sham surgeries were performed when the rats reached 250 g of body mass, according to the technique described by KALU (1991). To perform this procedure, rats were first anesthetized with a mixture of ketamine-zylazine (61.5 - 7.6 mg/kg, ip). A bilateral incision (1.0 - 1.5 cm) was made through the skin and muscle layers. The ovaries were removed and the skin and muscles were sutured. The sham surgery was performed using the same procedure but the ovaries were not removed. The analgesic tramadol hydrochloride (20 mg/kg) was injected every 24 h for five days after surgery. The rats were allowed to recover from surgery for seven days, which also permitted us to ensure the systemic effects of Ovx. At the end of this period, rats in the Ovx-E2 group underwent a

second surgical procedure to insert Silastic capsules under the skin of the neck to provide hormonal replacement.

4.5 Hormone Replacement (E2)

Silastic capsules (15 mm) with internal and external diameter of 1.02 mm and 2.16 mm, respectively (Dow Corning VWR International, Buffalo Grove, IL, USA) were used for E2 replacement. Sunflower oil was used as the vehicle, with a 5% concentration of 17β-estradiol (50 mg/ml oil). Ten µL of this solution was pipetted into each capsule and then both sides were sealed with Silastic glue. The capsules were stored for 24 h to allow the glue to dry. After drying, the capsules were kept in saline (0.9%) solution (CAMPOS et al., 2014).

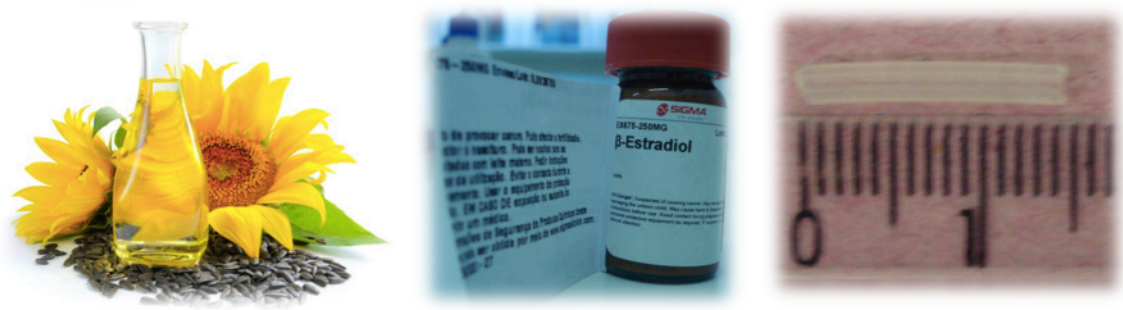


Figure 6. Materials used to construct the Silastic capsule.

4.6 Resistance training

4.6.1 Apparatus to training sessions

The training sessions consisted of climbing a vertical wooden and metal ladder with the following dimensions: 80° of inclination, 110 cm height x 18 cm width, and 2 cm spacing between the ladder rungs. At the top of the ladder, the animals reached a 20 x 20 x 20 cm cage, which allowed them to rest between climbing sessions. The first three training sessions were used to allow the animal to adapt to the process of climbing and to determine the

maximum possible weight load. The load consisted of fishing sinkers that were previously weighed, labeled, and stored in falcon tubes. The falcon tubes were tied to proximal portion of the rats' tails. The present RT protocol is an adaptation of the program described by Hornberg & Farrar (2004).

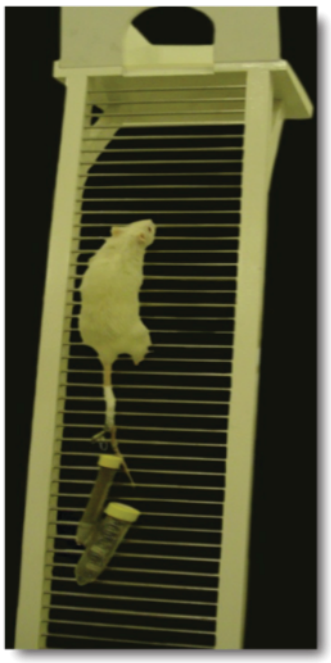


Figure 7. Rat climbing the ladder during the training session.
From Leite, R.D. Thesis, 2009.

The training sessions were conducted every 72 hours (Table 1) with no fixed weekly days for training. The training schedule was 12 weeks, totaling 27 strength training sessions.

Table 1. Training schedule.

Training Schedule						
Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
X	Familiarization	Familiarization	Determination of maximum load	X	X	1º Training Session
X	X	2º Training Session	X	X	3º Training Session	X
X	4º Training Session	X	X	5º Training Session...	X	X

Familiarization, maximum load determination and training sessions every 72 hours.
(X) Days recovery.

4.6.2 Familiarization Step.

The animals were first familiarized with the training procedures over two consecutive days. The overload apparatus was attached to the rat's tail without any weight. The rats were placed at the bottom of the ladder and familiarized with the climb. Once they reached the cage at the top of the ladder, the rats were allowed a 2 min rest period. If necessary, a physical stimulus with fingers or tweezers was applied to the animal's tail to initiate climbing movements. The rats were considered to have adapted to the ladder protocol when voluntary climbing was performed three consecutive times without any stimulus.

4.6.3 Maximal load determination step.

After 2 days of familiarization to training session, the third day was the maximal load determination. It was initiated with a load equivalent to 75% of the rat’s body mass. After each successful climb, the rats recovery for 2 minutes and 30 g were added to the apparatus until the rat was unable to climb the entire length of the ladder even after three successive stimulus at the tail. The highest carried load was considered the maximum carrying capacity of each rat as shown figure 8.

4.6.4 Training Session.

The training sessions consisted of 4 to 9 climbs with progressively increasing weights. The size of the ladder required the animals to perform 8 - 12 movements per climb. The first four climbs were carried out with loads corresponding to 50, 75, 90, and 100% of the rat’s maximal carrying capacity as determined in the last training session. For the 5 subsequent climbs, additional 30 g loads were progressively added until the rat was unable to climb the

entire ladder as shown figure 9. The new maximal load determined during the training program was used for the next session.

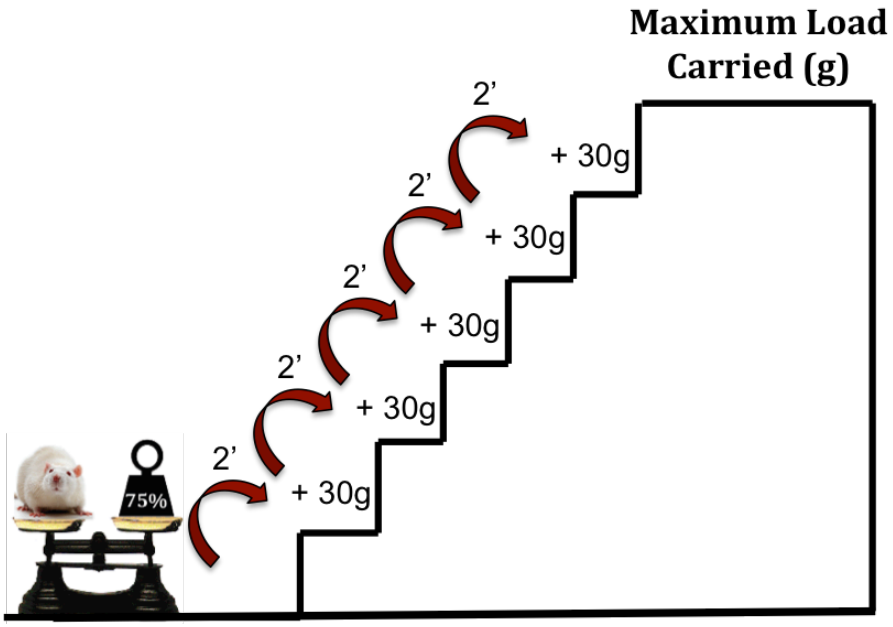


Figure 8. Maximum load determination from 75% of rat's body mass.

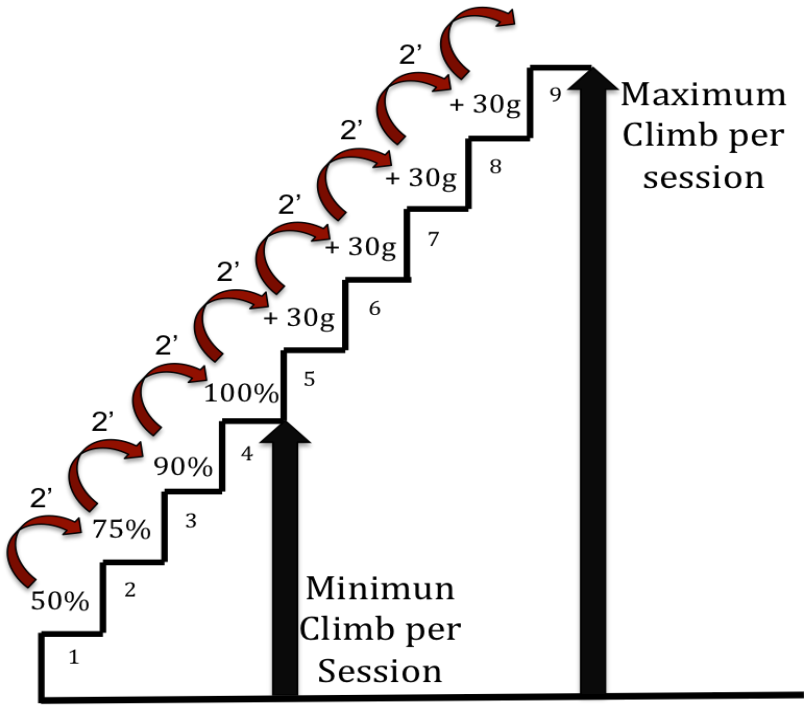


Figure 9. Carried load per training session.

4.7 Food intake and body weight control

Food intake (grams per cage) was daily monitored always at determined hour. Body mass was checked every Monday, Wednesday and Friday also at determined hour. Both procedures were conducted during all the experimental period. These controls were performed to certify the possible changes caused by the three different performed interventions.

4.8 Animals euthanasia and tissue sampling

Rats were euthanized between 9:00 AM and 12:00 PM after removal of food at 7:00 AM. Rats in the trained groups were euthanized 48 h following the last training session. The animals were euthanized by decapitation with the same period of experimental interventions. Immediately after decapitation, blood samples were collected and centrifuged at 3000 rpm for 10 minutes at 4°C, and then stored at -20°C.

The livers were weighed and then washed with saline (0.9%). The median of lobe each liver was selected by anatomical visualization. This lobe was separated from the others and divided into 3 aliquots in microtube being; 1) to genetic analysis; 2) to protein analysis and 3) to reserve for other analyzes. The other liver lobes were stored in aluminum foil in a freezer -80˚ C.

4.9 17β-estradiol plasma level

Measurement of plasma levels was performed by the ELISA technique (MA; SHIEH; LEE, 2006) using commercial kits (ADI-900-174, Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. The assay sensibility was 10 pg/mL and all

samples were tested in duplicate. Quantification was given in relation to the standard curve using logistic 4-parameter nonlinear regression.

4.10 Quantitative Real Time Polymerase Chain Reaction (RT-PCR)

The hepatic tissue homogenization was performed in liquid nitrogen in a crucible, a porcelain pestle. Without defrosting the tissue was macerated until form a powder. This powder was collected and stored in a new microtube. Approximately 20 mg of powder hepatic tissue was weighed and placed in a new microtube. The samples were lysed with *Lysis Buffer* containing 1% 2-mercaptoethanol (10 µl / 1 ml) and shaken quickly (3 min 30 s / sec) on a rotor with small metal spheres. It was centrifuged at 2600 g for 5 minutes at room temperature and 250 µl of the supernatant was transferred to a RNAse free microtube from a mini kit of Invitrogen according to the manufacturer's protocol. Then, the RNA was treated with DNAse (Invitrogen) in order to avoid genomic contamination.

All next steps, to obtain the Ct values were performed in the IRIC - Institute for Research in Immunology and Cancer at the University of Montreal - Canada. The RNA integrity analysis was performed by electrophoresis of RNA micro capillary separation. This method generates the value of RIN (RNA integrity number) by an integrity algorithm assigned the RNA value. The RIN value between 10:08 imparts integrity of the sample. A graphical simulation of agarose gel was represented figure 10. The samples with protein contamination or integrity number below 8 values were discarded (SCHROEDER et al., 2006).

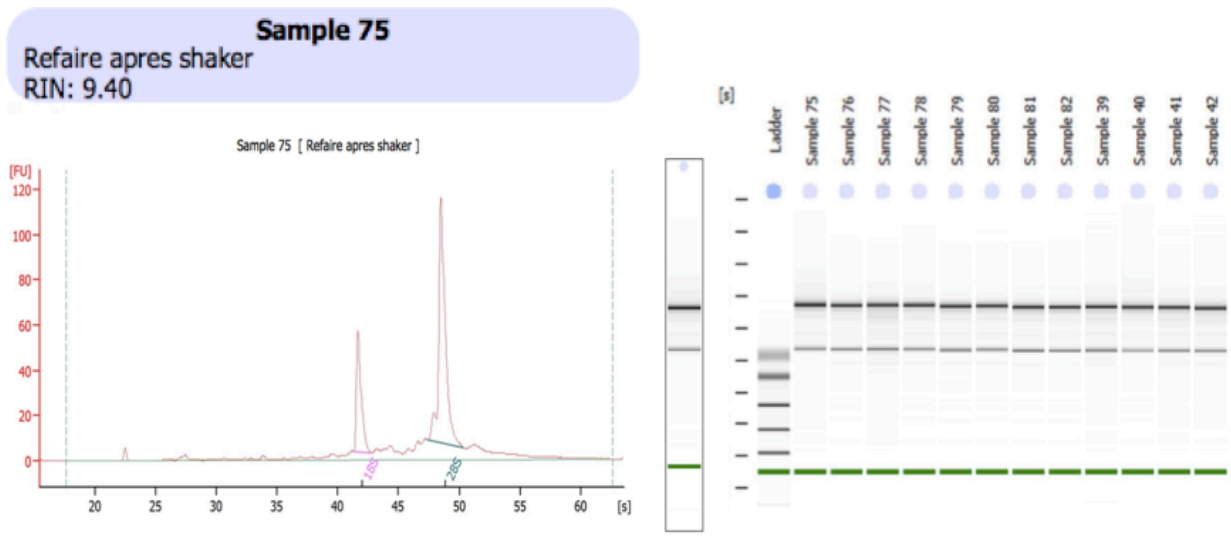


Figure 10. Graphical simulation of agarose gel and integrity number

Total RNA (2 pg) was reverse-transcribed into complementary DNA (cDNA) using high-capacity cDNA reverse transcription kits (Applied Biosystems). Reverse-transcribed samples were stored at -20°C. GAPDH gene expression was determined using a pre-validated Taqman Gene Expression Assay (Applied Biosystems, Rn01462d61, Foster City, CA). The gene expression levels of target genes were determined using assays designed with the Universal Probe Library from Roche. The primer sets and UPL probe numbers are presented in Table 2.

Table 2. Oligonucleotides primers used to RT-PCR.

Genes	Accession Nº	Forward primer (5'-3')	Reverse primer (3'-5')
GLUT2	NM_012879.2	CTGGGTCTGCAATTTCATCA	CGTAAGGCCCGAGGAAGT
PEPCK	NM_198780.3	GATGACATTGCCTGGATGAA	AACCGTTTTCTGGGTTGATG
PPARγ	NM_013124.3	TTTATAGCTGTCATTATTCTCAGTGGA	CGGGTGGTTCAGCTTCAG
GAPDH	NM_017008.3	CCCTCAAGATTGTCAGCAATG	AGTTGTCATGGATGACCTTGG
ACTB	NM_031144	CCCGCGAGTACAACCTTCT	CGTCATCCATGGCGAACT

GLUT2: Glucose transporter 2; PEPCK: Phosphoenolpyruvate carboxykinase; PPARγ: Peroxisome proliferator-activated receptor gamma GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; ACTB: Beta-actin.

To validate the efficiency of the qPCR assays, we used a mix of the samples tested in the study. The ABI PRISMÒ 7900HT (Applied Biosystems) was used to detect the

amplification level and was programmed with an initial step of 3 min at 95 °C, followed by 40 cycles of 5 sat 95° Cand 30 sat 60°C. All reactions were running duplicated, and the average values of Cts were used for quantification. GAPDH was used as endogenous control. The relative quantification of target genes was determined using the ΔΔCT method (LIVAK; SCHMITTGEN, 2001). Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control gene (GAPDH) ($\Delta CT = Ct_{target} - Ct_{GAPDH}$) and compared with a calibrator: ΔΔCT = ΔCt sample – ΔCt calibrator. Relative expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems), and the formula is $RQ = 2^{-\Delta\Delta CT}$.

4.11 Protein Assay

Fifty mg of hepatic tissue were homogenized in RIPA buffer containing protease inhibitors (aprotinin, 0.01 mg / mL, PMSF, 2mM) and phosphatase inhibitors (sodium orthovanadate, 100 mM; sodium pyrophosphate, 10mM, sodium fluoride, 10mM). ~25 µg of proteins were fractionated by SDS-PAGE (12%) and transferred to a nitrocellulose membrane by Western blotting method, described by Kurien and Scofield (2006). Before this procedure, the membrane was blocked with 5% albumin solution. After this, each membrane was incubated overnight at 4° C with appropriate dilutions of the primary antibodies; GLUT2 ~57 kDa of Sigma-Aldrich label and β-actin ~45 kDa of Cell Signaling Technology label. Each membrane was washed in TBST (3 x 5 min) and then incubated with the appropriate secondary antibody conjugated to HRP for 120 min at room temperature. Antibody binding was detected by enhanced Super Signal® West Pico Chemiluminescent Substrate (PIERCE, IL, USA), as described by the manufacturer. Blots were scanned and the densitometry of protein bands was determined by pixel intensity using Molecular Imager® ChemiDocTM XRS+ (Bio-Rad Laboratories Inc., CA, USA) with Image LabTM 2.0 Software.

4.12 Liver Triglycerides Assay

To Triglycerides assay was weighted ~ 60 mg of liver powder in a micro tube. At the same micro tube was added 500 µl of KOH and ethanol solution and was mixed for 3 minutes and allow to dry-bath for 70º C. It was added 1 ml $MgSO_4$, and centrifuged for 5 minutes and the solution A and B was prepared according to the manufacturer's specifications (Sigma 337-40 A&B). In spectrophotometric plate were pipetted 10 µl of the supernatant of the samples and 160 µl of solution A&B. The wavelength used was 540 (FRAYN; MAYCOCK, 1980).

4.13 Hepatic Glycogen Assay

To glycogen assay was used ~50 mg of liver powder. To break the membrane of the liver cells was added 500 µl of KOH 30% with Na2SO4 solution and let at the bath for 20 min. Subsequently, the samples were left on ice for 10 min and added 600µl of ethanol. The samples were centrifuged at 1900 g for 25 minutes at 4° C. The supernatant was aspirated and discarded, 10 ml of H_2O was added to the sample and shaken vigorously by vortex. In a spectrophotometric plate was added phenol solution (5%), the samples with sulfuric acid were read by spectrophotometer at 490nm and hepatic glycogen was quantified (LO; RUSSELL; TAYLOR, 1970).

4.14 Statistical Analysis

Values are expressed as means ± standard error (SE). The data were first analyzed by the Kolmogorov-Smirnov normality test (SPSS 22.1 software) and all results were considered parametric. The one-way analysis of variance (ANOVA) test was used to quantify the RT or E2 variables. To maximal load carried was applied two-way analysis of variance to

identify the initial, intermediary and final carried load in both trained groups. The Tukey *post hoc* test was used in the event of a significant ($P < 0.05$) F ratio. The relationship between variables was examined by correlation coefficient and linear regression analysis using Pearson's test.

5. RESULTS

Body mass was significantly *(P* < 0.01) higher in Ovx than in Sham rats (Table 3). Exercise training did not significantly affect these measurements, though it did significantly (*P* < 0.01) reduce body mass in Ovx-E2 rats as compared to Sham-Sed animals. On the other hand, food intake was not affected by any of the experimental conditions, while the only change in liver mass was an increase (*P* < 0.01) seen in Ovx-E2 rats.

Figure 11. Color and size of liver.

Sham in the sedentary (Sed) groups compared with ovariectomized (Ovx) rats given 17*β*-estradiol (E2) (Ovx-E2). The arrows indicate the whitest areas of tissue in Ovx-E2 group.

As expected, uterus weight and 17*β*-estradiol plasma levels were lower *(P* < 0.01) in Ovx compared to Sham animals and were increased (*P* < 0.01) in Ovx-E2 rats (Table 3). There was no significant difference between the Sham and the Ovx rats in their capacity to carrying increasing loads throughout the 12-week resistance training program.

Table 3. Anthropometric parameters and food intake.

	Sham-SED	Sham-RT	Ovx-SED	Ovx-RT	Ovx-E2
Body mass (g)	308.5 ± 10	315.7 ± 6	358.4 ± 10 ^{aa}	339.4 ± 10 ^{a}	310.5 ± 11 ^{cc}
Food intake (g/day)	21.2 ± 0.4	23.1 ± 0.8	23.7 ± 0.9	23.6 ± 0.8	23.8 ± 0.9
Liver mass (g)	8.4 ± 0.2	8.4 ± 0.3	8.3 ± 0.5	8.1 ± 0.3	10.2 ± 0.4 $^{aa\ cc}$
Uterus mass (g)	0.65 ± 0.07	0.58 ± 0.06	0.09 ± 0.01 ^{aa}	0.10 ± 0.01 ^{aa}	0.59 ± 0.07 ^{cc}
17β-estradiol (pg/ml)	34.0 ± 0.1	34.1 ± 0.1	16.8 ± 0.2 ^{aa}	17.2 ± 0.3 ^{aa}	44.2 ± 0.9 $^{aa\ cc}$

Values are mean ± SE in Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) and in Ovx-Sed rats given 17β-estradiol (E2); n = 8 rats per group. For 17β-estradiol, n = 4 rats per group.
a: Significantly different from Sham-SED (P<0.05); aa (P<0.01)
c: Significantly different from Ovx-SED (P<0.05); cc (P<0.01)

The loads carried by both groups of rats increased significantly between the 1st and the 15th session (P < 0.01), and again between the 15th and the 27th session (P < 0.05). On the whole, carried loads increased from ~ 362 g to ~ 985 g at the end of the 12-week period, thus indicating the efficiency of the resistance training program.

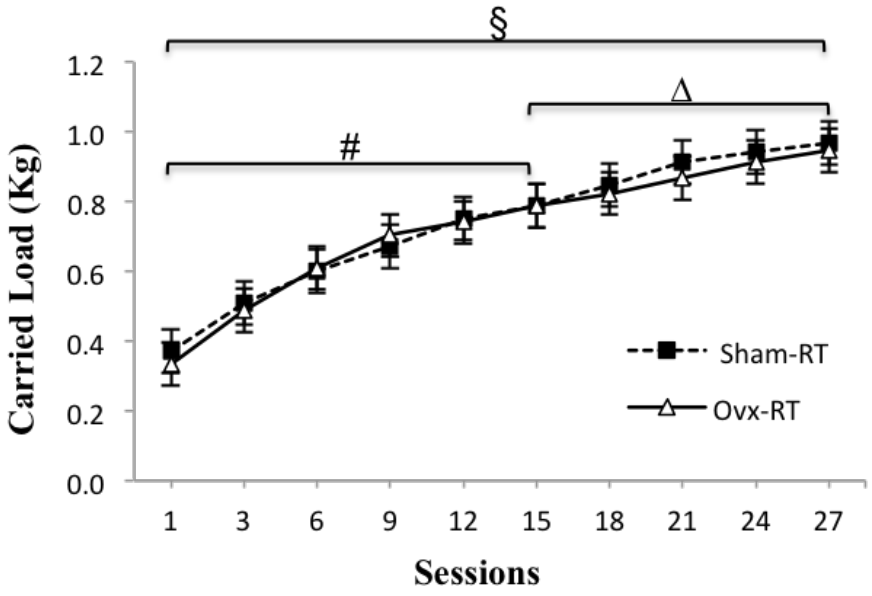


Figure 12. Carried Load on trained groups throughout training.

Sham: sham-operated; Ovx: ovariectomized; RT: Resistance Training; Values are mean ± SE; n = 8 rats per group.
#: Significantly different from 1st session P< 0.01
Δ: Significantly different from 15th session P< 0.01
§: Significantly different from 27th session P< 0.01

Hepatic GLUT2 transcript levels were not significantly affected either by Ovx or training state but were significantly (P < 0.01) lower in Ovx animals supplemented with estrogen (Fig. 13). On the other hand, protein levels of GLUT2 in the liver were significantly (P < 0.01) higher in Ovx compared to Sed animals, and the levels were back to normal in Ovx-RT and Ovx-E2 rats.

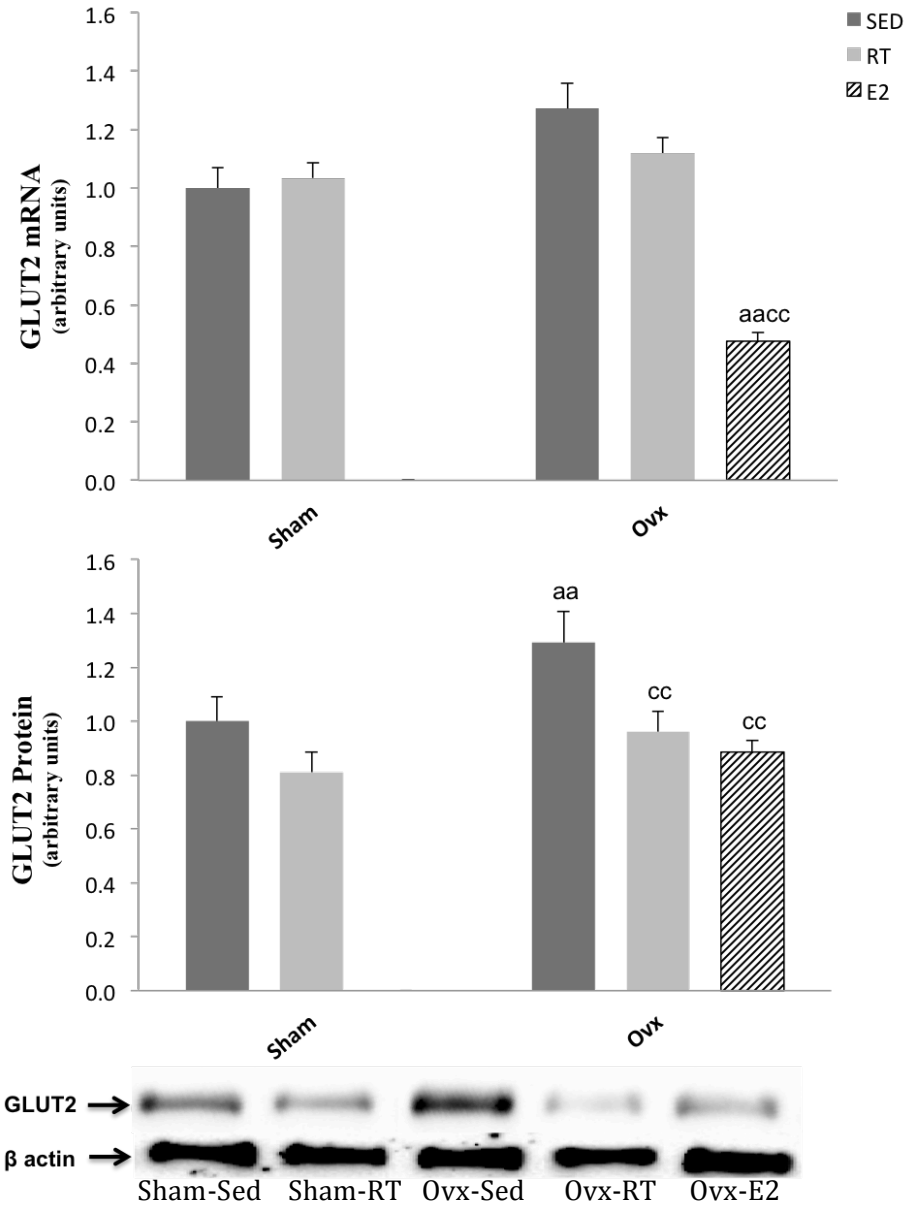


Figure 13. Hepatic mRNA and protein levels of the GLUT2.

Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) groups and in Ovx-Sed rats given 17β-estradiol (E2). Values are mean ± SE with n = 8 rats per group for mRNA expression and n = 4 per group for protein expression.
a (P < 0.05) Significantly different from Sham-SED, aa (P < 0.01)
c (P < 0.05) Significantly different from Ovx-SED, cc (P < 0.01)

Ovariectomy in the Sed state was associated with a significant ($P < 0.01$) increase in liver fat (Fig. 14). Resistance training, however, significantly ($P < 0.01$) lowered hepatic fat content in Ovx as well as in Sham rats. E2 replacement reduced ($P < 0.05$) hepatic fat content to levels even under those of Sham-Sed rats. As for GLUT2, gene expression of the transcription factor PPARγ was significantly *(*$P < 0.01$) higher in Ovx-Sed compared to Sham-Sed rats (Fig. 14). This difference was not observed in Ovx-RT animals, indicating that training reduced gene expression of PPARγ in Ovx rats. E2 replacement in Ovx rats significantly ($P < 0.01$) lowered PPARγ transcript levels in Ovx animals.

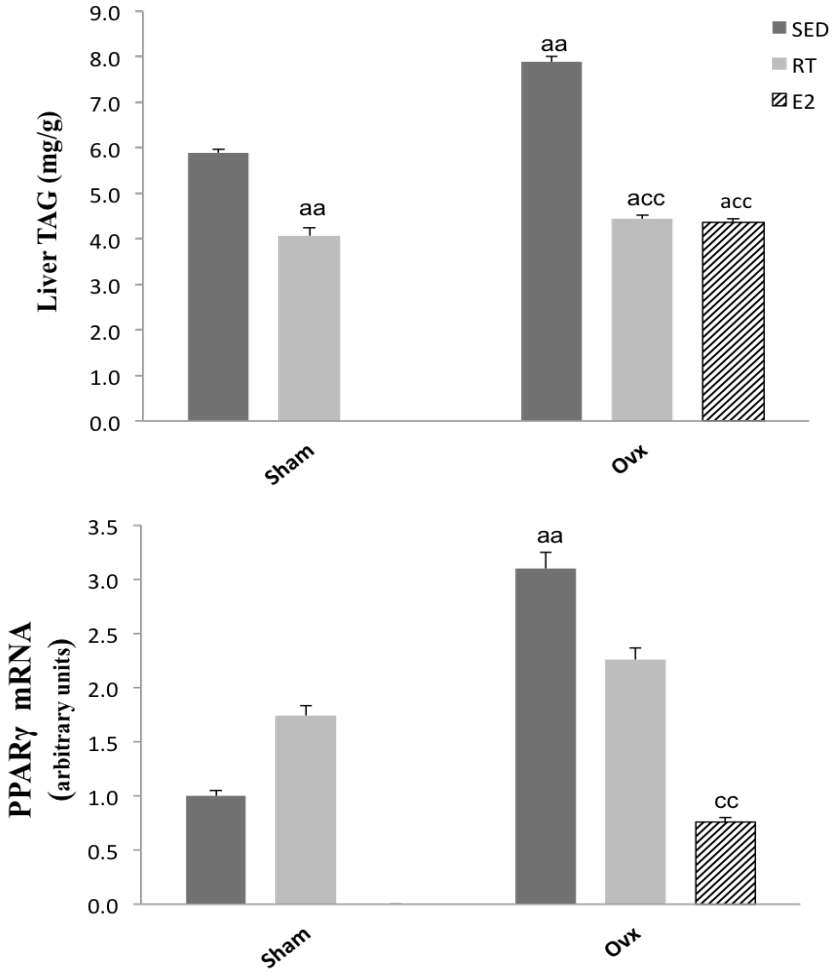


Figure 14. Liver TAG and hepatic mRNA expression of PPARγ.

Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) and in Ovx-Sed rats given 17β- estradiol (E2). Values are mean ± SE with n = 8 rats per group.
a *(*$P < 0.05$) Significantly different from Sham-SED, aa *(*$P < 0.01$)
c ($P < 0.05$) Significantly different from Ovx-SED, cc *(*$P < 0.01$)

To gain insight into liver glucose metabolism, we measured liver glycogen concentration and gene expression of phosphoenolpyruvate carboxykinase (PEPCK), the main enzyme in the gluconeogenesis pathway. Hepatic glycogen levels were not affected by ovariectomy or training state but were highly (P < 0.01) increased by E2 replacement (Fig.15). Gene expression of PEPCK was not affected by ovariectomy or training state but was significantly (P < 0.01) lower in Ovx-E2 compared to Ovx- Sed rats.

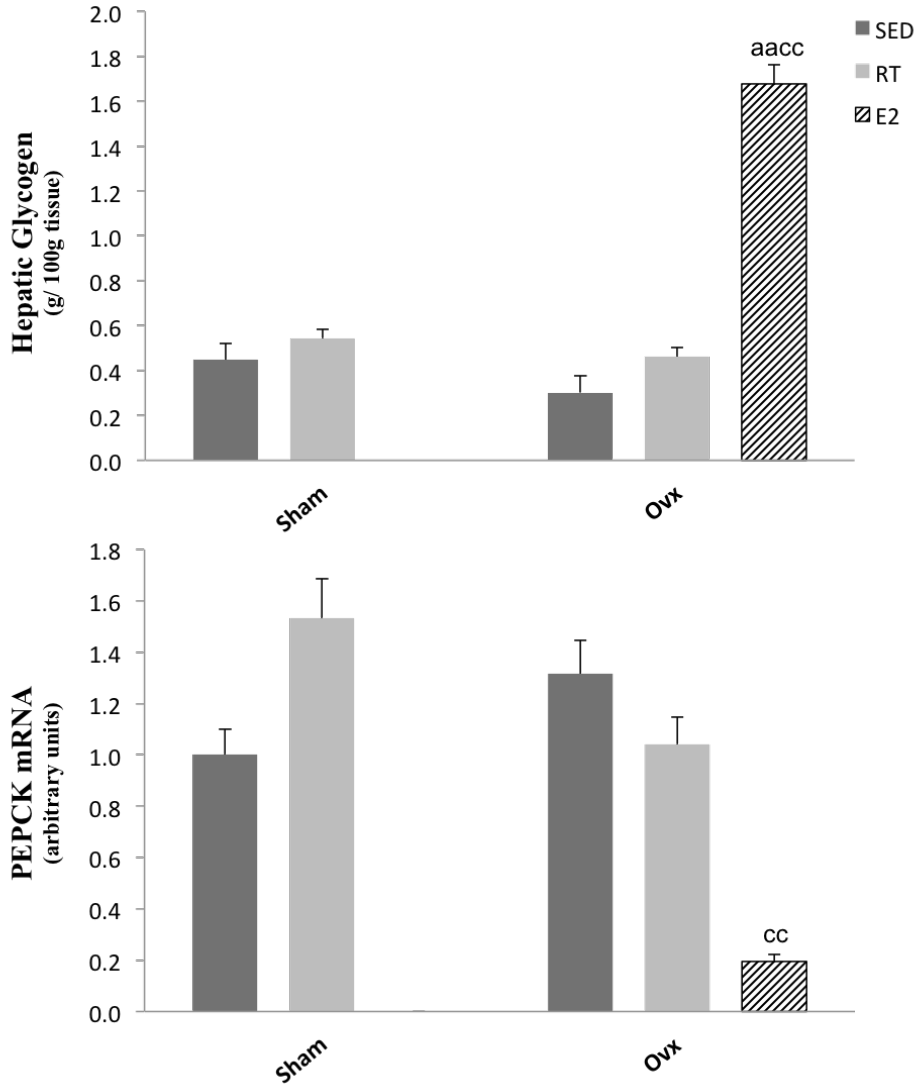


Figure 15 Glycogen levels and mRNA expression of PEPCK.

Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) and in Ovx-Sed rats treated with 17β-estradiol (E2). Values are mean ± SE with n = 8 rats per group.
a (P < 0.05) Significantly different from Sham-SED, aa (P < 0.01)
c (P < 0.05). Significantly different from Ovx-SED, cc (P < 0.01)

After obtaining gene expression values of PPARγ and GLUT2, we considered a correlation analysis between these two variables. They are not correlated in Sham-Sed group while Ovx-Sed group showed a significant correlation ($P < 0.05$) (Fig 16). RT and E2 effects in Ovx groups showed to decrease these correlations when compared to Ovx-Sed. Both RT and E2 groups were not significantly different.

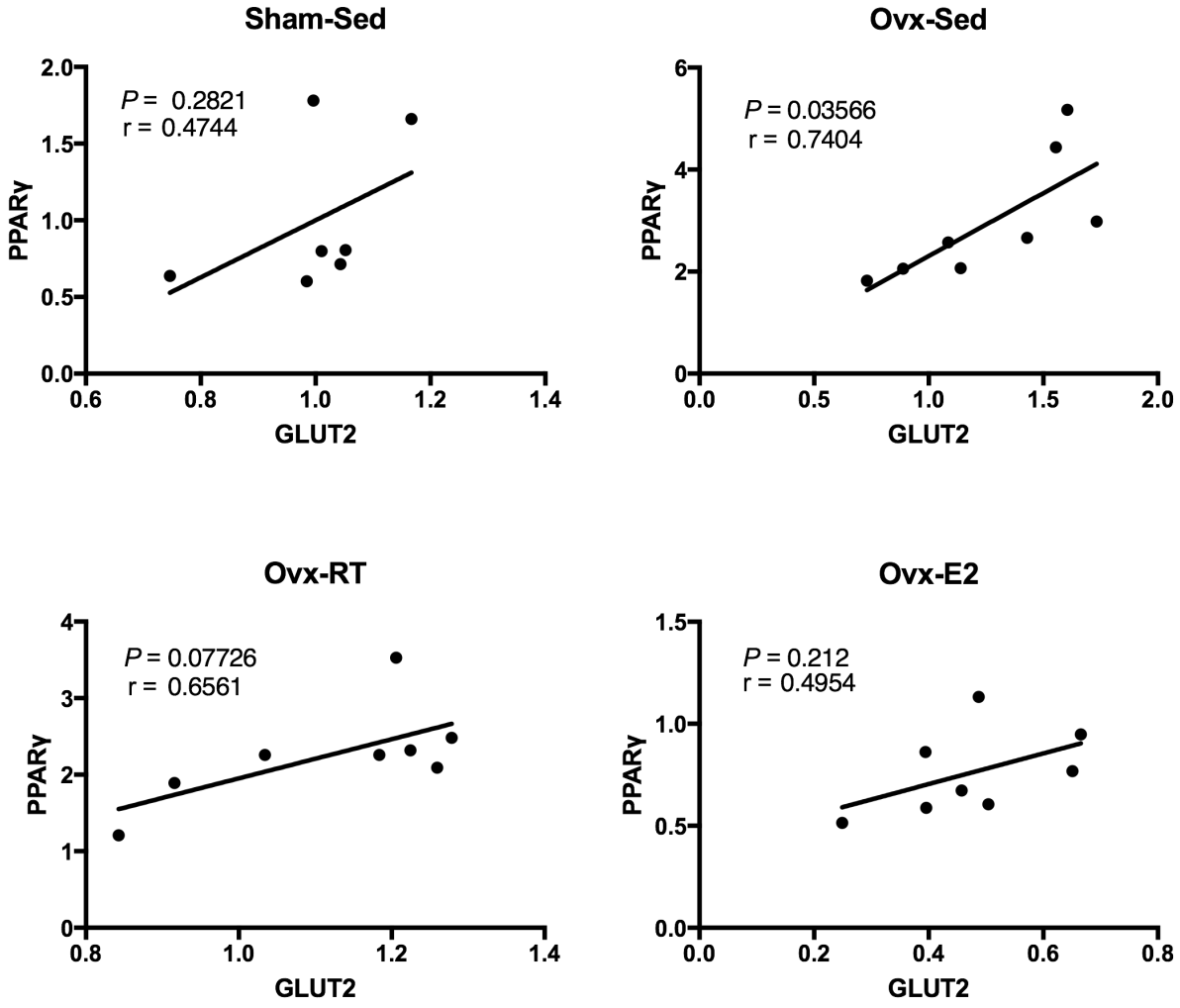


Figure 16. Coefficient correlation and linear regression of GLUT2 and PPARγ gene expression.

Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) and in Ovx-Sed rats treated with 17β-estradiol (E2). n = 8 rats per group.

6. DISCUSSION

6.1 Ovariectomy

The results of the present study first indicated that the well-documented fat accumulation in livers of Ovx rats is accompanied by an increase in the expression of GLUT2, the major glucose transporter in the plasma membranes of hepatocytes, along with an increase in gene expression of the transcription factor PPARγ, which is known to modulate GLUT2 gene expression (IM et al., 2005a). These results suggest that alterations in liver fat metabolism, known to occur in Ovx rats, such as increased *de novo* lipogenesis, also affect hepatic glucose uptake. In addition, the present data indicate that a regular resistance training program may overcome these disturbances in liver fat and glucose transport. These results reinforce the concept that exercise training may alleviate some of the metabolic consequences of low estrogenic status.

Liver fat content was increased by about 35% in Ovx rats in this study. Our hypothesis that Ovx in rats may lead to an increase in gene expression of the GLUT2 transporter was based on previous findings that fat accumulation in the livers of Ovx animals is associated with an increase in *de novo* lipogenesis (DOMINGOS; RODRIGUES; STOTZER, 2011; PAQUETTE et al., 2008). Increased lipogenesis logically requires more glucose molecules to be available as substrates, thus, hypothetically, leading to increased glucose uptake through GLUT2 transport. Studies in GLUT2 knockout mice confirm that, indeed, glucose uptake by hepatocytes is a major substrate for lipogenesis (SEYER et al., 2013). Although the basic function of GLUT2 is to catalyze the passive transport of glucose across the plasma membrane, it is required for glucose uptake, whereas it is dispensable for glucose output (THORENS, 2015). Also supporting the link between liver fat accumulation

and GLUT2 transport is the finding that glucose-induced SREBP- 1c upregulates GLUT2 mRNA in primary hepatocytes of mouse liver (IM et al., 2005b; RENCUREL et al., 1996). SREBP-1c is a well-documented regulator of lipogenesis and has been repeatedly reported to be upregulated in Ovx animals (DOMINGOS; RODRIGUES; STOTZER, 2011; STOTZER et al., 2014). Altogether, the present data are in line with the concept that an increase in fat content in livers of Ovx rats is linked to increased glucose uptake through an increased expression of GLUT2 protein.

The increase in gene expression of the PPARγ transcription factor supports the increase in GLUT2 synthesis in the livers of Ovx rats. PPARγ expression is low in liver tissue (MATSUSUE et al., 2003; ROGUE et al., 2010). Nevertheless, it has been reported that PPARγ is able to modulate gene expression of GLUT2 in liver (IM et al., 2005a). Our results presented the correlations between GLUT2 and PPARγ gene expression was significantly only in Ovx-Sed groups. The association of GLUT2 and PPARγ already was showed in some diabetics experimental model study. As LEE et al., (2012) showed that Ankaflavin, a natural PPARγ agonist, has been shown to enhance insulin sensitivity by increasing hepatic GLUT2 expression and glucose uptake in rats. Similar, SUZUKI et al. (2000) observed in diabetic mice treated with Pioglitazone (another PPARγ agonist) for 2 weeks. They did analysis of 42 genes associated with diabetes gene expression in the liver, and their results showed a higher expression of Gk, GLUT2, PPARγ, and a series of fatty acid oxidation enzymes while those of triglyceride lipase, lipoprotein lipase, apolipoprotein A-I, and insulin receptor substrate 2 were decreased. Likewise, our results suggest diabetic's modulation also in Ovx model, highlighting the importance of estrogen in the hepatic glucose metabolism.

There is little doubt that the increase in fat content in the liver cells of Ovx animals is related to a reduction in plasma estradiol levels (GLOY et al., 2011; LEITE et al., 2009). The present data, therefore, support the existence of a link between liver fat accumulation in Ovx

rats at least partially through increased *de novo* lipogenesis and GLUT2 transport and an increase in PPARγ gene expression.

From a mechanical point of view, it is possible that the present increase in GLUT2 gene expression in Ovx animals is linked to a transient decrease in intracellular glucose level secondary to increased lipogenesis. Glucose has been reported to induce a dose-dependent increase in GLUT2 mRNA levels in cultured hepatocytes, while insulin had no effects (OKA et al., 1990). To go one step further into hepatic glucose metabolism in Ovx animals, we measured glycogen levels and PEPCK mRNA levels. Both of these parameters remain unchanged following estrogen withdrawal. The absence of an effect on glycogen levels in Ovx rats indicate that if indeed glucose uptake is increased in the present Ovx rats, it was not used for glycogen synthesis. Actually, an increase in liver fat, as seen in the present Ovx rats, is associated with a reduction in glycogen storage (BERGERON et al., 2014). There is also no indication that gluconeogenesis is increased for estrogen withdrawal, indicating no shortage of glucose.

6.2 Estrogen Replacement

E2 replacement in Ovx rats resulted, as would be expected, in a reduction in liver fat levels but also in a large increase in liver glycogen levels and a reduction in gene expression of PEPCK. The 5% hormone concentration used in the present study appears to be an effective replacement over a three-week (CAMPOS et al., 2014). However, the present replacement over a 12-weeks period has led to supraphysiological plasma levels of estrogen, as judged from the high estrogen plasma concentrations and the increase in liver weight. Nevertheless, the present E2 replacement data are in line with a previous report indicating that treatment with estradiol in Ovx mice increases liver glycogen and reduces gluconeogenesis (BAILEY; AHMED-SOROUR, 1980).

6.3 Resistance training

Training has been reported to be effective in reducing liver fat accumulation in Ovx animals (CORRIVEAU et al., 2008; HALLSWORTH et al., 2011; LEITE et al., 2009). The present study extended these findings by showing that GLUT2 protein and PPARγ transcript levels were restored almost to normal levels in the livers of Ovx rats submitted to resistance training. This at first glance supports the link between liver fat accumulation through increased *de novo* lipogenesis and GLUT2 transporters. The present RT program is an interesting alternative to classical endurance training. Regarding the effect of different training protocols to reduces liver diseases in women, a recently study of SKRYPNIK et al., (2016) showed that endurance associated with strength exercise led to a greater improvement, compared to only endurance exercise in the liver function in women with abdominal obesity. Similar RT programs have been used successfully in previous studies, leading to similar reductions in liver fat accumulation (CORRIVEAU et al., 2008; HALLSWORTH et al., 2011; PIGHON et al., 2009). It is intriguing that RT and E2 replacement in the present study led to similar metabolic improvements in the livers of Ovx animals. The same phenomenon has been previously observed on the reduction of subclinical inflammation in Ovx rats (PIGHON; GUTKOWSKA; JANKOWSKI, 2010). The present data are, therefore, in line with the concept that exercise training has estrogenic-like effects. In line with this idea, it is interesting to note the recent finding that rats selectively bred for high intrinsic aerobic fitness are protected from ovariectomy-induced metabolic disorders (VIEIRA-POTTER et al., 2015).

7. CONCLUSION

The present study indicates that estrogen withdrawal-induced liver GLUT2 protein and PPARγ mRNA in Ovx rats are accompanied by an increase in fat accumulation, and that both of these responses are normalized when rats are submitted to a resistance training program, confirming our initial hypothesis.

These results suggest that, in addition to fat metabolism, the level of estrogen also affects hepatic glucose metabolism. From a clinical point of view, the present data reiterate the importance of exercise training to alleviate some of the metabolic consequences of low estrogenic status in postmenopausal period.

8. STUDY LIMITATIONS

One limitation point of this study is related to glucose and insulin curves. This analysis in some points of experimental period and at last moment should be interesting to show us a systemic alteration of glucose and insulin metabolism.

We planned that just estrogen reposition should be enough to attend our propose. We did not do estrogen release time line, thus we did not have enough information to understand the 12 weeks of estrogen effects. The time line should be great to understand the dose at the end of experimental period.

9. FUTURE PERSPECTIVES

The present study reported that Ovx is able to promote similarly effects as diabetes model on GLUT2 and PPARγ mRNA expression as shown LEE et al., 2012 and SUZUKI et al., 2000, highlighting the importance of estrogen in the hepatic glucose metabolism. However it is important to consider a deep research on others markers that modulate positive and negative the hepatic metabolism of lipid associated with glucose to prevent disease as diabetes and NAFLD. A wide research needs to be developed to discover new factors, enzymes and proteins that are influenced by estrogen and surround the hepatic metabolisms.

Regarding the effect of different training protocols to reduce liver diseases in women, as shown by the study of SKRYPNIK et al., (2016), different exercise protocol induced adaptation in the hepatic function of women with abdominal obesity. Thus, more research should be conducted to increase and include the physical exercise prescription even more as therapeutic strategies for treatment or prevention of liver diseases. Since the exercise applied by different protocols induces different systemic metabolic changes and it could be a potential therapeutic targets for metabolic dysfunction in women's physiology in menopause period.

10. ATTACHMENTS

10.1 Published Article

JENB

J Exerc Nutrition Biochem. 2016;20(2):051-057

OPEN ACCESS

http://dx.doi.org/10.20463/jenb.2016.06.20.2.7

GLUT2 proteins and PPARγ transcripts levels are increased in liver of ovariectomized rats: reversal effects of resistance training

Luciane M. Tomaz[1]*/ Marina R. Barbosa[2]/ Zahra Farahnak[3]/ Cristiani G. Lagoeiro[1]/ Natalia S.S Magosso[1]/ Jean-Marc Lavoie[3]/ Sérgio E. A. Perez[1]

1. Department of Physiological Sciences, Federal University of São Carlos, São Carlos, SP, Brazil
2. Department of Education and Health, Federal University of Sergipe, Lagarto, SE, Brazil
3. Department of Kinesiology, Université de Montréal, Montréal, QC, Canada

Received: 2015/12/22, Revised: 2016/03/30, Accepted: 2016/04/14, Published: 2016/06/30

*Corresponding author : Luciane M. Tomaz
Department of Physiological Sciences, Federal University of São Carlos, São Carlos, SP, Brazil
Email : lumagri@gmail.com

[Purpose] This study investigated the effects of ovariectomy (Ovx) and 12 weeks of resistance training (RT) on gene expression of GLUT2, the main glucose transporter in the liver, and on PPARγ, a transcription factor known to target GLUT2 expression.

[Methods] Forty Holtzman rats were divided into 5 groups: Sham-sedentary (Sed), Sham-RT, Ovx-Sed, Ovx-RT, and Ovx-Sed with hormone replacement (E2). The RT protocol consisted of sessions held every 72 h for 12 weeks, during which the animals performed 4 to 9 vertical climbs (1.1 m) at 2 min intervals with progressively heavier weights (30 g after the fourth climb) tied to the tail. The E2 silastic capsule was inserted into the rats' backs 48 hours before the first RT session.

[Results] In addition to liver fat, GLUT2 protein levels and PPARγ transcripts were increased ($P < 0.05$) in Ovx compared to Sham-Sed animals, suggesting increased hepatic glucose uptake under estrogen deficient conditions. RT and E2 in Ovx rats decreased liver fat accumulation as well as GLUT2 and PPARγ gene expression to the level of Sham-Sed animals.

[Conclusion] The results of this study suggest that liver GLUT2 as well as PPARγ expression in Ovx rats are accompanied by increased fat accumulation and glucose uptake, thus providing a substrate for increased *de novo* lipogenesis. RT appears to be an appropriate exercise model to circumvent these effects.

[Key words] Liver fat, PEPCK, Hepatic glycogen, Exercise and Glucose.

INTRODUCTION

Menopause, as well as estrogen withdrawal in rats through ovariectomy or other animal models of menopause, is associated with several metabolic changes that include increased liver fat deposition[1-3]. Liver fat accumulation is recognized as the hepatic component of metabolic syndrome[4].

The specific origin of the lipids that accumulate in rat liver under estrogen withdrawal remains unclear. Measurements of molecular markers in different studies suggest a reduction in lipid oxidation and in the production of very low density lipoprotein triglycerides (VLDL-TG)[5,6] and an increase in *de novo* lipogenesis[7,8]. Several molecular markers of *de novo* lipogenesis in the liver, including the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c) and its downstream enzyme stearoyl-CoA desaturase-1 (SCD-1), have been reported to be greatly increased in ovariectomized rats[7,8]. If, indeed, *de novo* lipogenesis is increased in the livers of Ovx rats, then extra glucose molecules must be available as substrates for lipid synthesis. Therefore, we postulated that Ovx-induced liver fat accumulation would also result in increased hepatic glucose uptake through increased expression of glucose transporters. The first purpose of the present study was to test the hypothesis that hepatic gene expression of GLUT2, the main glucose transporter in hepatocytes of rodents and humans[9], is increased in Ovx rats. In addition, we also targeted the transcription factor PPARγ. Gene expression of PPARγ is low in liver tissue[10]. Nevertheless, there is some evidence that liver GLUT2 is a direct target of PPARγ, thus contributing to glucose transport into the liver[11].

There is accumulating evidence that endurance exercise training overcomes several of the metabolic effects of ovariectomy in rats. For instance, Ovx animals can benefit from exercise training by a reduction in fat gain[12,13] and insulin resistance in skeletal muscle[14]. Endurance exercise training has also been reported to prevent liver fat accumulation in rats[15] as well as the upregulation of several genes involved in *de novo* lipogenesis[16]. There is also evidence that resistance or strength training in rats, as an alternative to endurance training, prevents or decreases liver fat content[17,18]. Furthermore, RT has been reported to promote muscle strength and hypertrophy in Ovx animals, thus providing protection

JENB
Journal of Exercise Nutrition & Biochemistry

against menopause-associated sarcopenia and osteopenia[8,17,18]. More recently, it was reported that resistance training restored the gene expression of key molecules involved in *de novo* lipogenesis in livers of ovariectomized rats[8]. We, therefore, used a resistance training program, which can be considered a model of strength training, to test the hypothesis that resistance training ameliorates the increase in GLUT2 expression in livers of Ovx rats.

METHODS

Animal care

Female Holstman rats (n = 40) from the animal facility of the University of São Paulo State (UNESP, Araraquara, Brazil) weighting ~ 220 g upon arrival were housed in collective cages. The animals had *ad libitum* access to food and tap water. All animals were fed with commercial rodent chow. Their environment was controlled in a reverse light cycle (12 h dark starting at 08:00 AM). Food intake was monitored daily over the entire experimental period. Body mass was measured 3 times/week at the same time of day. All experiments described in the present report were conducted according to the Guide for Care and Use of Laboratory Animals[19] and approved by the Ethics Committee on Animal Use from the Federal University of São Carlos (CEUA-UFSCar) Protocol n° 005/2013.

Experimental groups

Rats were randomly distributed into five experimental groups (n = 8/group). Rats first underwent a bilateral ovariectomy (n = 24) or a bilateral sham operation (Sham, n = 16). Thereafter, one group of Ovx and one group of Sham rats remained sedentary while one group of Ovx and one group of Sham rats were submitted to a 12-week resistance training program. The third group of Ovx rats remained sedentary and were given 17β-estradiol supplementation. Altogether, five groups were compared: Sham-sedentary (Sed), Sham-resistance trained (RT), Ovx-Sed, Ovx-RT, and Ovx-Sed with hormone replacement (Ovx-E2).

Surgery

Ovariectomy and sham surgeries were performed when the rats reached 250 g of body mass, according to the technique described by Kalu[20]. To perform this procedure, rats were first anesthetized with a mixture of ketamine-zylazine (61.5 - 7.6 mg/kg, ip). A bilateral incision (1.0 - 1.5 cm) was made through the skin and muscle layers. The ovaries were removed and the skin and muscles were sutured. The sham surgery was performed using the same procedure but the ovaries were not removed. The antibiotic tramadol hydrochloride (20 mg/kg, sc) was injected every 24 h for five days after surgery. The rats were allowed to recover from surgery for seven days, which also permitted us to ensure the systemic effects of Ovx. At the end of this period, rats in the Ovx-E2 group underwent a second surgical procedure to insert silastic capsules under the skin of the neck to provide hormonal replacement.

Estrogen replacement (E2)

Silastic capsules (15 mm) with internal and external diameter of 1.02 mm and 2.16 mm, respectively (Dow Corning VWR International, Buffalo Grove, IL, USA) were used for E2 replacement. Sunflower oil was used as the vehicle, with a 5% concentration of 17β-estradiol (50 mg/ml oil). Ten µL of this solution was pipetted into each capsule and then both sides were sealed with silastic glue. The capsules were stored for 24 h to allow the glue to dry. After drying, the capsules were kept in saline (0.9%) solution[21].

Resistance training protocol

The training sessions consisted of climbing a vertical wooden and metal ladder with the following dimensions: 80° of inclination, 110 cm height x 18 cm width, and 2 cm spacing between the ladder rungs. At the top of the ladder, the animals reached a 20 x 20 x 20 cm cage, which allowed them to rest between climbing sessions. The first three training sessions were used to allow the animal to adapt to the process of climbing and to determine the maximum possible weight load. The load consisted of fishing sinkers that were previously weighed, labeled, and stored in falcon tubes. The falcon tubes were tied to proximal portion of the rats' tails. The present RT protocol is an adaptation of the program described by Hornberg & Farrar[22].

Familiarization step

The animals were first familiarized with the training procedures over two consecutive days. The overload apparatus was attached to the rat's tail without any weight. The rats were placed at the bottom of the ladder and familiarized with the climb. Once they reached the cage at the top of the ladder, the rats were allowed a 2 min rest period. If necessary, a physical stimulus with fingers or tweezers was applied to the animal's tail to initiate climbing movements. The rats were considered to have adapted to the ladder protocol when voluntary climbing was performed three consecutive times without any stimulus.

Maximal load determination step

The maximal load determination was initiated with a load equivalent to 75% of the rat's body mass. After each successful climb, 30 g were added to the apparatus until the rat was unable to climb the entire length of the ladder. The highest carried load was considered the maximum carrying capacity of each rat.

Resistance training program

The RT program consisted of training sessions held every 3 days for 12 weeks, for a total of 27 sessions. The training sessions consisted of 4 to 9 climbs with progressively increasing weights. The size of the ladder required the animals to perform 8 - 12 movements per climb. The first four climbs were carried out with loads corresponding to 50, 75, 90, and 100% of the rat's maximal carrying capacity as determined in the last training session. For the 5 subsequent climbs, an additional 30 g load were progressively added until the rat was unable to climb the entire

JENB
Journal of Exercise Nutrition & Biochemistry

ladder. The new maximal load determined during the training program was used for the next session.

Blood and tissue sampling

Rats were euthanized between 9:00 AM and 12:00 PM after removal of food at 7:00 AM. Rats in the trained groups were euthanized 48 h following the last training session. Immediately after decapitation, blood samples were collected and centrifuged at 3000 rpm for 10 minutes at 4°C, and then stored at -20°C. The liver was weighed and then washed with saline (0.9%). The median lobe was used for all molecular analyses.

Biochemical analyses

Measurement of 17β-estradiol plasma levels was performed by the ELISA technique[23] using commercial kits (ADI-900-174, Enzo Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Liver triglyceride concentrations were determined from glycerol released after KOH hydrolysis according to the technique described by Frayn and Maycock[24]. Liver glycogen content was determined using phenol-sulfuric acid reactions as described by Lo et al.[25].

Quantitative real time polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen livers with the use of the RNA extraction Mini kit (Invitrogen) according to the manufacturer's protocol. Then, the RNA was treated with DNase (Invitrogen) in order to avoid genomic contamination. Total RNA (2 pg) was reverse-transcribed into complementary DNA (cDNA) using high-capacity cDNA reverse transcription kits (Applied Biosystems). Reverse-transcribed samples were stored at -20°C. GAPDH gene expression was determined using a pre-validated Taqman Gene Expression Assay (Applied Biosystems, Rn01462d61, Foster City, CA). The gene expression levels of target genes were determined using assays designed with the Universal Probe Library from Roche. The primer sets and UPL probe numbers are presented in Table 1. To validate the efficiency of the qPCR assays, we used a mix of the samples tested in the study. An ABI PRISM® 7900HT (Applied Biosystems) was used to detect amplification level and was programmed with an initial step of 3 min at 95°C, followed by 40 cycles for 5 s at 95°C and 30 s at 60°C. All reactions were run in triplicate and the average values of threshold cycle (C_T) were used for quantification. *GAPDH* was used as endogenous control. The relative quantification of target genes was determined using the ΔΔCT method[26]. Briefly, the CT values of target genes were normalized to an endogenous control gene (*GAPDH*) ($\Delta C_T = C_{T\ Target} - C_{T\ GAPDH}$) and compared with a calibrator: ($\Delta\Delta C_T = \Delta C_{T\ Sample} - \Delta C_{T\ Calibrator}$). Relative expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) with the formula $RQ = 2^{-\Delta\Delta C_T}$.

Protein assay

Protein analyses were performed by the Western blotting method, described by Kurien and Scofield[27]. Aliquots of hepatic lysate (~ 25 mg) were separated by SDS- PAGE gel and transferred to a nitrocellulose membrane. Before the procedure, the membrane was blocked with 5% albumin solution. After this, each membrane was incubated overnight at 4°C with appropriate dilutions of the primary antibodies, including GLUT2 (Sigma-aldrich) and β-actin (Cell Signaling Technology). Each membrane was washed in TBST (3 x 5 min) and then incubated with the appropriate secondary antibody conjugated to HRP for 120 min at room temperature. Antibody binding was detected by enhanced SuperSignal® West Pico Chemiluminescent Substrate (PIERCE, IL, USA), as described by the manufacturer. Blots were scanned and the densitometry of protein bands was determined by pixel intensity using Molecular Imager® ChemiDoc™ XRS+ (Bio-Rad Laboratories Inc., CA, USA) with Image Lab™ 2.0 Software.

Statistical analysis

Values are expressed as means ± standard error (SE). The data were first analyzed by the Kolmogorov-Smirnov normality test (SPSS 22.1 software) and all results were considered parametric. The one-way analysis of variance (ANOVA) test was used to quantify the RT or E2 variables. The Tukey *post hoc* test was used in the event of a significant ($P < 0.05$) F ratio.

RESULTS

Body mass was significantly ($P < 0.01$) higher in Ovx than in Sham rats (Table 2). Exercise training did not significantly affect these measurements, though it did significantly ($P < 0.01$) reduce body mass in Ovx-E2 rats as compared to Sham-Sed animals. On the other hand, food intake was not affected by any of the experimental conditions, while the only change in liver mass was an increase ($P < 0.01$) seen in Ovx-E2 rats. As expected, uterus weight and 17β-estradiol plasma levels were lower ($P < 0.01$) in Ovx

Table 1. Oligonucleotide primers used for PRC-RT

Genes	Accession N"	Sence primer(5'-3')	Antisense primer(5'-3')
GLUT2	NM_012879.2	CTGGGTCTGCAATTTCATCA	CGTAAGGCCCGAGGAAGT
PEPCK	NM_198780.3	GATGACATTGCCTGGATGAA	AACCGTTTTCTGGGTTGATG
PPARγ	NM_013124.3	TTTATAGCTGTCATTATTCTCAGTGGA	CGGGTGGTTCAGCTTCAG
GAPDH	NM_017008.3	CCCTCAAGATTGTCAGCAATG	AGTTGTCATGGATGACCTTGG

JENB
Journal of Exercise Nutrition & Biochemistry

Table 2. Anthropometric parameters and food intake

Parameters	Sham-Sed	Sham-RT	Ovx-Sed	Ovx-RT	Ovx-E2
Body mass (g)	308.5 ± 10	315.7 ± 6	358.4 ± 10[aa]	339.4 ± 10[a]	310.5 ± 11[cc]
Food intakte (g/day)	21.2 ± 0.4	23.1 ± 0.8	23.7 ± 0.9	23.6 ± 0.8	23.8 ± 0.9
Liver mass (g)	8.4 ± 0.2	8.4 ± 0.3	8.3 ± 0.5	8.1 ± 0.3	10.2 ± 0.4[aa cc]
Uterus mass (g)	0.65 ± 0.07	0.58 ± 0.06	0.09 ± 0.01[aa]	0.10 ± 0.01[aa]	0.59 ± 0.07[cc]
17β-estradiol (pg/ml)	34.0 ± 0.1	34.1 ± 0.1	16.8 ± 0.2[aa]	17.2 ± 0.3[aa]	44.2 ± 0.9[aa cc]

Values are mean ± SE in Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) and in Ovx-Sed rats given 17β-estradiol (E2); n = 8 rats per group. For 17β-estradiol, *n* = 4 rats per group. a: Significantly different from Sham-SED (*P*<0.05); aa (*P*<0.01) c: Significantly different from Ovx-SED (*P*<0.05); cc (*P*<0.01)

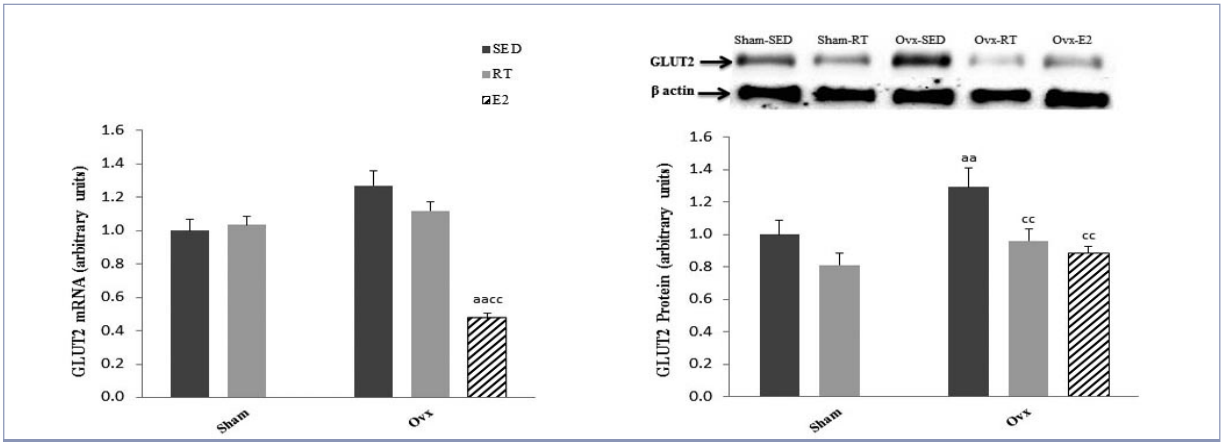


Figure 1. Hepatic mRNA and protein levels of the GLUT2 glucose transporter in Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) groups and in Ovx-Sed rats given 17β-estradiol (E2). Values are mean ± SE with *n* = 8 rats per group for mRNA expression and *n* = 4 per group for protein expression. a (*P* < 0.05) Significantly different from Sham-SED, aa (*P* < 0.01), c (*P* < 0.05) Significantly different from Ovx-SED, cc (*P* < 0.01)

compared to Sham animals and were increased ($P < 0.01$) in Ovx-E2 rats (Table 2). There was no significant difference between the Sham and the Ovx rats in their capacity to carrying increasing loads throughout the 12-week resistance training program. The loads carried by both groups of rats increased significantly between the 1st and the 15th session ($P < 0.01$), and again between the 15th and the 27th session ($P < 0.05$). On the whole, carried loads increased from ~ 362 g to ~ 985 g at the end of the 12-week period, thus indicating the efficiency of the resistance training program.

Hepatic GLUT2 transcript levels were not significantly affected either by Ovx or training state but were significantly ($P < 0.01$) lower in Ovx animals supplemented with estrogen (Fig. 1). On the other hand, protein levels of GLUT2 in the liver were significantly ($P < 0.01$) higher in Ovx compared to Sed animals, and the levels were back to normal in Ovx-RT and Ovx-E2 rats.

Ovariectomy in the Sed state was associated with a significant ($P < 0.01$) increase in liver fat (Fig. 2). Exercise training, however, significantly ($P < 0.01$) lowered hepatic fat content in Ovx as well as in Sham rats. E2 replacement reduced ($P < 0.05$) hepatic fat content to levels even under those of Sham-Sed rats. As for GLUT2, gene expression of the transcription factor PPARγ was significantly ($P < 0.01$) higher in Ovx-Sed compared to Sham-Sed rats (Fig. 1). This difference was not observed in Ovx-RT animals,

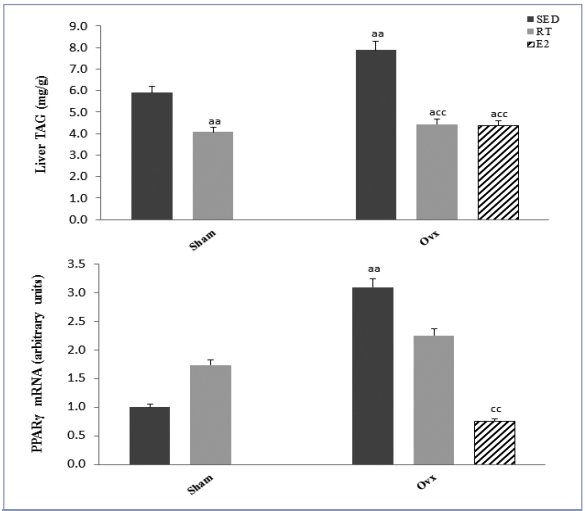


Figure 2. Liver triacylglycerol and hepatic mRNA expression of peroxysome proliferator-activated receptor γ (PPARγ) in Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) and in Ovx-Sed rats given 17β-estradiol (E2). Values are mean ± SE with n = 8 rats per group. a (*P* < 0.05) Significantly different from Sham-SED, aa (*P* < 0.01), c (*P* < 0.05) Significantly different from Ovx-SED, cc (*P* < 0.01)

JENB
Journal of Exercise Nutrition & Biochemistry

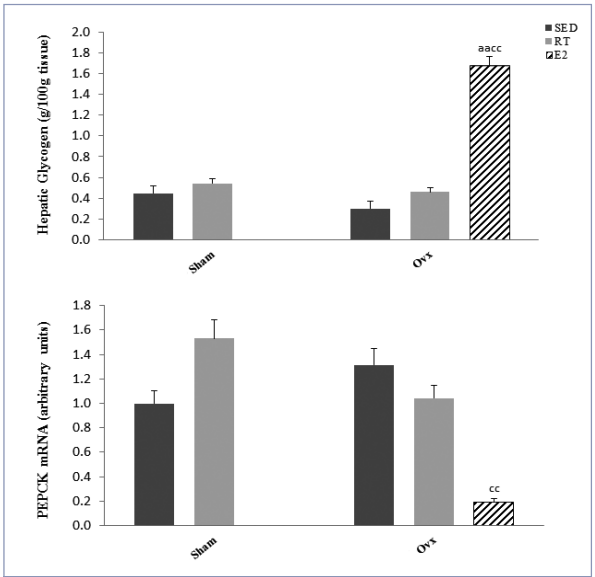


Figure 3. Glycogen levels and mRNA expression of phosphoenolpyruvate carboxykinase (PEPCK) in Sham and ovariectomized (Ovx) rats in the sedentary (Sed) and resistance training (RT) and in Ovx-Sed rats treated with 17β-estradiol (E2). Values are mean ± SE with n = 8 rats per group. a ($P < 0.05$) Significantly different from Sham-SED, aa ($P < 0.01$) c ($P < 0.05$). Significantly different from Ovx-SED, cc ($P < 0.01$)

indicating that training reduced gene expression of PPARγ in Ovx rats. E2 replacement in Ovx rats significantly ($P < 0.01$) lowered PPARγ transcript levels in Ovx animals.

To gain insight into liver glucose metabolism, we measured liver glycogen concentration and gene expression of phosphoenolpyruvate carboxykinase (PEPCK), the main enzyme in the gluconeogenesis pathway. Hepatic glycogen levels were not affected by ovariectomy or training state but were highly ($P < 0.01$) increased by E2 replacement (Fig. 3). Gene expression of PEPCK was not affected by ovariectomy or training state but was significantly ($P < 0.01$) lower in Ovx-E2 compared to Ovx- Sed rats (Fig. 3).

DISCUSSION

Ovariectomy

The results of the present study first indicated that the well-documented fat accumulation in livers of Ovx rats is accompanied by an increase in the expression of GLUT2, the major glucose transporter in the plasma membranes of hepatocytes, along with an increase in gene expression of the transcription factor PPARγ, which is known to modulate GLUT2 gene expression[11]. These results suggest that alterations in liver fat metabolism, known to ocur in Ovx rats, such as increased *de novo* lipogenesis, also affect hepatic glucose uptake. In addition, the present data indicate that these disturbances in liver fat and glucose transport may be overcome by a regular resistance training program. These results reinforce the concept that exercise training may alleviate some of the metabolic consequences of low estrogenic status.

Liver fat content was increased by about 35% in Ovx rats in this study. Our hypothesis that Ovx in rats may lead to an increase in gene expression of the GLUT2 transporter was based on previous findings that fat accumulation in the livers of Ovx animals is associated with an increase in *de novo* lipogenesis[7,8]. Increased lipogenesis logically requires more glucose molecules to be available as substrates, thus, hypothetically, leading to increased glucose uptake through GLUT2 transport. Studies in GLUT2 knockout mice confirm that, indeed, glucose uptake by hepatocytes is a major substrate for lipogenesis[28]. Although the basic function of GLUT2 is to catalyze the passive transport of glucose across the plasma membrane, it is required for glucose uptake, whereas it is dispensable for glucose output[29]. Also supporting the link between liver fat accumulation and GLUT2 transport is the finding that glucose-induced SREBP- 1c upregulates GLUT2 mRNA in primary hepatocytes of mouse liver[30,31]. SREBP-1c is a well-documented regulator of lipogenesis and has been repeatedly reported to be upregulated in Ovx animals[8,32]. Altogether, the present data are in line with the concept that an increase in fat content in livers of Ovx rats is linked to increased glucose uptake through an increased expression of GLUT2 protein.

The increase in gene expression of the PPARγ transcription factor supports the increase in GLUT2 synthesis in the livers of Ovx rats. PPARγ expression is low in liver tissue[10]. Nevertheless, it has been reported that PPARγ is able to modulate gene expression of GLUT2 in liver[11]. Ankaflavin, a natural PPARγ agonist, has been shown to enhance insulin sensitivity by increasing hepatic GLUT2 expression and glucose uptake in rats[33]. There is little doubt that the increase in fat content in the liver cells of Ovx animals is related to a reduction in plasma estradiol levels[18,34]. The present data, therefore, support the existence of a link between liver fat accumulation in Ovx rats at least partially through increased *de novo* lipogenesis and GLUT2 transport and an increase in PPARγ gene expression.

From a mechanical point of view, it is possible that the present increase in GLUT2 gene expression in Ovx animals is linked to a transient decrease in intracellular glucose level secondary to increased lipogenesis. Glucose has been reported to induce a dose-dependent increase in GLUT2 mRNA levels in cultured hepatocytes, while insulin had no effects[35]. To go one step further into hepatic glucose metabolism in Ovx animals, we measured glycogen levels and PEPCK mRNA levels. Both of these parameters remain unchanged following estrogen withdrawal. The absence of an effect on glycogen levels in Ovx rats indicate that if indeed glucose uptake is increased in the present Ovx rats, it was not used for glycogen synthesis. Actually, an increase in liver fat, as seen in the present Ovx rats, is associated with a reduction in glycogen storage[36]. There is also no indication that gluconeogenesis is increased for estrogen withdrawal, indicating no shortage of glucose.

JENB
Journal of Exercise Nutrition & Biochemistry

Estrogen Replacement

E2 replacement in Ovx rats resulted, as would be expected, in a reduction in liver fat levels but also in a large increase in liver glycogen levels and a reduction in gene expression of PEPCK. The 5% hormone concentration used in the present study appears to be an effective replacement over a three-week period[21]. However, the present replacement over a 12-weeks period has led to supraphysiological plasma levels of estrogen, as judged from the high estrogen plasma concentrations and the increase in liver weight. Nevertheless, the present E2 replacement data are in line with a previous report indicating that treatment with estradiol in Ovx mice increases liver glycogen and reduces gluconeogenesis[37].

Resistance training

Training has been reported to be effective in reducing liver fat accumulation in Ovx animals[17,18,38]. The present study extended these findings by showing that GLUT2 protein and PPARγ transcript levels were restored almost to normal levels in the livers of Ovx rats submitted to resistance training. This at first glance supports the link between liver fat accumulation through increased *de novo* lipogenesis and GLUT2 transporters. The present RT program is an interesting alternative to classical endurance training. Similar RT programs have been used successfully in previous studies, leading to similar reductions in liver fat accumulation[17,38,39]. It is intriguing that RT and E2 replacement in the present study led to similar metabolic improvements in the livers of Ovx animals. The same phenomenon has been previously observed on the reduction of subclinical inflammation in Ovx rats[16]. The present data are, therefore, in line with the concept that exercise training has estrogenic-like effects.

CONCLUSION

The present study indicates that estrogen withdrawal-induced liver GLUT2 protein and PPARγ mRNA in Ovx rats are accompanied by an increase in fat accumulation, and that both of these responses are normalized when rats are submitted to a resistance training program. These results suggest that, in addition to fat metabolism, the level of estrogens also affects hepatic glucose metabolism. From a clinical point of view, the present data reiterate the importance of exercise training to alleviate some of the metabolic consequences of low estrogenic status in postmenopausal women. In line with this idea, it is interesting to note the recent finding that rats selectively bred for high intrinsic aerobic fitness are protected from ovariectomy-induced metabolic disorders[40].

ACKNOWLEDGEMENTS

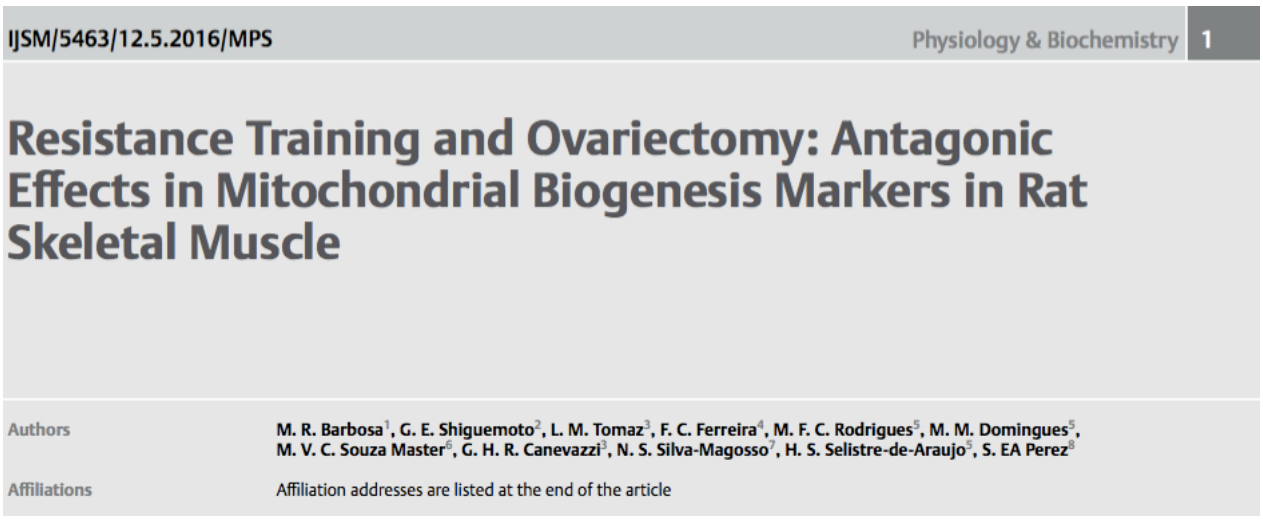
This study was funded by FAPESP (São Paulo Research Foundation) (project number 2011/21607-5) and by the Natural Sciences and Engineering Research Council of Canada (NSERC: 7594).

REFERENCES

1. Volzke H, Schwarz S, Baumeister SE, Wallaschofski H, Schwahn C, Grabe HJ, Kohlmann T, John U, Doren M. Menopausal status and hepatic steatosis in a general female population. *Gut*. 2007;56:593-4.
2. Paquette A, Shinoda M, Lhoret RR, Prud'homme D, Lavoie JM. Time course of liver lipid infiltration in ovariectomized rats: Impact of a high-fat diet. *Maturitas*. 2007;58:182-90.
3. Nemoto Y, Toda K, Ono M, Fujikawa-Adachi K, Saibara T, Onishi S, Enzan H, Okada T, Shizuta Y. Altered expression of fatty acid-metabolizing enzymes in aromatase-deficient mice. *J Clin Invest*. 2000;105:1819-25.
4. Marchesini G, Marzocchi R, Agostini F, Bugianesi E. Nonalcoholic fatty liver disease and the metabolic syndrome. *Curr Opin Lipidol*. 2005;16:421-7.
5. Paquette A, Chapados N a, Bergeron R, Lavoie J-M. Fatty acid oxidation is decreased in the liver of ovariectomized rats. *Horm Metab Res*. 2009;41:511-5.
6. Barsalani R, Chapados J-ML. Hepatic VLDL-TG Production and MTP gene expression are decreased in ovariectomized rats : effects of exercise training. *Horm Metab Res*. 2010;42:860-7.
7. Paquette A, Wang D, Jankowski M, Gutkowska J, Lavoie J-M. Effects of ovariectomy on PPAR alpha, SREBP-1c, and SCD-1 gene expression in the rat liver. *Menopause*. 2008;15:1169-75.
8. Domingos M, Rodrigues MFC, Stotzer US. Resistance training restores the gene expression of molecules related to fat oxidation and lipogenesis in the liver of ovariectomized rats. *Eur J Appl Physiol*. 2012;112:1437–1444
9. Guillam MT, Burcelin R, Thorens B. Normal hepatic glucose production in the absence of GLUT2 reveals an alternative pathway for glucose release from hepatocytes. *Proc Natl Acad Sci USA*. 1998;95:12317-21.
10. Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, Brewer, Bryan R, Marc L, Gonzalez, Frank J. Liver-specific disruption of PPARgamma in leptin- deficient mice improves fatty liver but aggravates diabetic phenotypes. *J Clin Invest*. 2003;111:737-47.
11. Im S-S, Kim J-W, Kim T-H, Song X-L, Kim S-Y, Kim H, Ahn, Y-H. Identification and characterization of peroxisome proliferator response element in the mouse GLUT2 promoter. *Exp Mol Med*. 2005;37:101-10.
12. Richard D, Rochon L, Deshaies Y. Effects of exercise training on energy balance of ovariectomized rats. *Am J Physiol*. 1987;253:R740-5.
13. Shinoda M, Latour MG, Lavoie J-M. Effects of physical training on body composition and organ weights in ovariectomized and hyperestrogenic rats. *Int J Obes Relat Metab Disord*. 2002;26:335-43.
14. Saengsirisuwan V, Pongseeda S, Prasannarong M, Vichaiwong K, Toskulkao C. Modulation of insulin resistance in ovariectomized rats by endurance exercise training and estrogen replacement. *Metabolism*. 2009;58:38-47.

15. Pighon A, Barsalani R, Yasari S, Prud'homme D, Lavoie J-M. Does exercise training prior to ovariectomy protect against liver and adipocyte fat accumulation in rats. *Climacteric*. 2010; 13:238-48.
16. Pighon A, Gutkowska J, Jankowski M. Exercise training in ovariectomized rats stimulates estrogenic-like effects on expression of genes involved in lipid accumulation and subclinical inflammation in liver. Metabolism. *Elsevier Inc*.;2010;60:629-39.
17. Corriveau P, Paquette A, Brochu M, Prud'homme D, Rabasa-Lhoret R, Lavoie J- M. Resistance training prevents liver fat accumulation in ovariectomized rats. *Maturitas*. 2008;59:259-67.
18. Leite RD, Prestes J, Bernardes CF, Shiguemoto GE, Pereira GB, Duarte JO, Domingos, MM, Baldissera V, Perez SEA. Effects of ovariectomy and resistance training on lipid content in skeletal muscle, liver, and heart;fat depots;and lipid profile. *Appl Physiol Nutr Metab*. 2009;34:1079-86.
19. National Research Council (US) Institute for Laboratory. *Guide for the Care and Use of Laboratory Animals*. 1996. p.1-137
20. Kalu DN. Ovarietomized Rat Model of Postmenopausal Bone Loss. *Bone Miner*. 1991;15:175-92.
21. Campos C, Casali KR, Baraldi D, Conzatti A, Khaper N, Llesuy S, Rigatto K, Bell-Klein A. Efficacy of a low dose of estrogen on antioxidant defenses and heart rate variability. *Oxid Med Cell Longev*. 2014;2014:218749-56.
22. Hornberger TA, Farrar RP. Physiological hypertrophy of the FHL muscle following 8 weeks of progressive resistance exercise in the rat. *Can J Appl Physiol*. 2004;29:16-31.
23. Ma H, Shieh K-J, Lee S. Study of ELISA technique. *Nature*. 2006;4:36-7.
24. Frayn KN, Maycock PF. Skeletal muscle triacylglycerol in the rat: methods for sampling and measurement, and studies of biological variability. *J Lipid Res*. 1980;21:139-44.
25. Lo S, Russell JC, Taylor AW. Determination of glycogen in small tissue samples. *J Appl Physiol*. 1970;28:234-6.
26. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-8.
27. Kurien BT, Scofield RH. Western blotting. *Methods*. 2006;38: 283-93.
28. Seyer P, Vallois D, Poitry-Yamate C, Schutz F, Metref S, Tarussio D, et al. Hepatic glucose sensing is required to preserve P cell glucose competence. *J Clin Invest*. 2013;123:1662-76.
29. Thorens B. GLUT2, glucose sensing and glucose homeostasis. *Diabetologia*. 2015;58:221-32.
30. Im S.S, Kang SY, Kim SY, Kim H Il, Kim JW, Kim KS, Ahn YH. Glucose- stimulated upregulation of GLUT2 gene is mediated by sterol response element-binding protein-1c in the hepatocytes. *Diabetes*. 2005;54:1684-91.
31. Rencurel F, Waeber G, Antoine B, Rocchiccioli F, Maulard P, Girard J, Leturque A. Requirement of glucose metabolism for regulation of glucose transporter type 2 (GLUT2) gene expression in liver. *Biochem J*. 1996;314:903-9.
32. Stotzer U, Rodrigues MF, Domingos MM, Silva G, Duarte FO, Gatto C, Shiguemoto G, Perez S, Selistre-de-Araujo H. Resistance Training Suppresses Intra-abdominal Fatty Acid Synthesis in Ovariectomized Rats. *Int J Sports Med*. 2014;36:226-33.
33. Lee B-H, Hsu W-H, Chang Y-Y, Kuo H-F, Hsu Y-W, Pan T-M. Ankaflavin: a natural novel PPARγ agonist upregulates Nrf2 to attenuate methylglyoxal-induced diabetes in vivo. *Free Radic Biol Med*. 2012;53:2008-16.
34. Gloy V, Langhans W, Hillebrand JJ, Geary N, Asarian L. Ovariectomy and overeating palatable, energy-dense food increase subcutaneous adipose tissue more than intra-abdominal adipose tissue in rats. *Biol Sex Differ*. 2011;2:6-19.
35. Oka Y, Asano T, Shibasaki Y, Lin JL, Tsukuda K, Akanuma Y, et al. Increased liver glucose-transporter protein and mRNA in streptozocin-induced diabetic rats. *Diabetes*. 1990;39:441-6.
36. Bergeron R, Mentor JS, Cote I, Ngo Sock ET, Rabasa-Lhoret R, Lavoie JM. Loss of Ovarian Estrogens Causes Only Mild Deterioration of Glucose Homeostasis in Female ZDF Rats Preventable by Voluntary Running Exercise. *Horm Metab Res*. 2014;46:774-81.
37. Bailey CJ, Ahmed-Sorour H. Role of ovarian hormones in the long-term control of glucose homeostasis. Effects of insulin secretion. *Diabetologia*. 1980;19:475-81.
38. Hallsworth K, Fattakhova G, Hollingsworth KG, Thoma C, Moore S, Taylor R, Day CP, Trenell MI. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. *Gut*. 2011;60:1278-83.
39. Pighon A, Paquette A, Barsalani R, Chapados N a, Yasari S, Doucet E, Lavoie J- M. Substituting food restriction by resistance training prevents liver and body fat regain in ovariectomized rats. *Climacteric*. 2009;12:153-64.
40. Vieira-Potter VJ, Padilla J, Park Y-M, Welly RJ, Scroggins RJ, Britton SL, Koch LG, Jenkins NT, Crissey JM, Zidon T, Morris ME, Meers GM, Thyfault JP. Female rats selectively bred for high intrinsic aerobic fitness are protected from ovariectomy-associated metabolic dysfunction. *Am J Physiol Regul Integr Comp Physiol*. 2015;308:R530-42.

10.2 Published Articles and in submission process related to this Project



Resistance Training and Ovariectomy: Antagonic Effects in Mitochondrial Biogenesis Markers in Rat Skeletal Muscle

Authors M. R. Barbosa[1], G. E. Shiguemoto[2], L. M. Tomaz[3], F. C. Ferreira[4], M. F. C. Rodrigues[5], M. M. Domingues[5], M. V. C. Souza Master[6], G. H. R. Canevazzi[3], N. S. Silva-Magosso[7], H. S. Selistre-de-Araujo[5], S. EA Perez[8]

Affiliations Affiliation addresses are listed at the end of the article

Farahnak, Zahra; **Tomaz, Luciane M.**; Bergeron, Raynald; Chapados, Natalie Ann; Lavoie, Jean-Marc. Upregulation of small heterodimer partner transcript by exercise training in liver of Ovx rats. **Submitted in Climacteric, 2016.**

Markus V.C. Souza, Anderson D. S. Lino, Leandro G. D. Ruffoni, Mateus M. Domingos, Marina R. Barbosa, Maria F. C. Rodrigues, Fabiano C. Ferreira, **Luciane M. Tomaz**, Gustavo H. R. Canevazzi, Natalia S. S. Magosso, Jeferson A. A. Teixeira, Richard D. Leite, Gilberto E. Shiguemoto and Sérgio E. A. Perez. Resistance training and hormone replacement increase MMP-2 activity, quality and quantity of bone in ovariectomized rats – **Submitted in Connective Tissue Research, 2016.**

Natalia S. Silva-Magosso, Marina R. Barbosa, **Luciane M. Tomaz**, Maria F. C. Rodrigues, Gustavo H. R. Canevazzi, Markus V.C. Souza, Sabrina P. Messa, Guilherme B. Pereira, Heloisa S. Selistre-Araujo, Sérgio E. A. Perez. Irisin signaling is upregulated by resistance training in sham and ovariectomized rats. **- Submitted in Metabolism, 2016**

10.3 Abstracts Published in International Conferences

Tomaz L.M., Barbosa M.R., Ferreira F.C., Domingos M.M., Souza M.V.C., Silva N.S., Lagoeiro C.G., Rodrigues M.F.C., Perez S.E.A., Canevazzi G.H.R. 12-weeks Resistance Training is better than hormone replacement to prevent skeletal muscle mass reduction induced by Ovariectomy. ***Canadian Society for Exercise Physiology*** - *CSEP*, 2015, Hamilton, Canada.

Tomaz L.M., Barbosa M.R., Ferreira F.C., Domingos M.M., Canevazzi G.H.R. , Souza M.V.C., Silva N.S., Lagoeiro C.G., Rodrigues M.F.C., Farahnak Z., Lavoie J.M., Perez S.E.A. Effects of ovariectomy in rats submitted or not to a 12-week resistance training programme on hepatic GLUT2 gene expression. ***Canadian Society for Exercise Physiology*** - *CSEP*, 2014, St. Johns, Canada.

Barbosa M.R., **Tomaz L.M.,** Ferreira F.C., Domingos M.M., Canevazzi G.H.R. , Souza M.V.C., Silva N.S., Lagoeiro C.G., Rodrigues M.F.C. Perez S.E.A. Effects of Resistance training on Skeletal Muscle of Ovariectomized Rats. ***American College of Sports Medicine***, 2014, Orlando, United States of America.

Domingos M.M., Rodrigues M.F.C. Barbosa M.R., **Tomaz L.M.,** Ferreira F.C., Canevazzi G.H.R., Souza M.V.C., Silva N.S., Lagoeiro C.G., Perez S.E.A. Resistance training increase gene expression of biomarkers to mitochondrial biogenesis in brain on ovariectomized rats. ***Integrative Physiology of Exercise*** 2014 Miami, Florida USA.

Souza M.V.C., Domingos M.M., Rodrigues M.F.C. Barbosa M.R., **Tomaz L.M.,** Ferreira F.C., Canevazzi G.H.R. , Silva N.S., Lagoeiro C.G., Perez S.E.A. Resistance Training And Hormone Replacement Increase MMP-2 Activity And Improve Bone Properties In Ovariectomized Rats. ***American College of Sports Medicine*** 2015, San Diego CA

10.4 Ethics Committee's opinion on the Animal's Use

UNIVERSIDADE FEDERAL DE SÃO CARLOS
PRÓ-REITORIA DE PESQUISA
Comissão de Ética no Uso de Animais
Via Washington Luís, km. 235 - Caixa Postal 676
Fones: (016) 3351.8025 / 3351.9679
Fax: (016) 3351.8025
CEP 13560-970 - São Carlos - SP - Brasil
ceua@ufscar.br - www.propg.ufscar.br

Parecer da Comissão de Ética no Uso de Animais

nº 005/2013

Protocolo nº. 005/13

A Comissão de Ética no Uso de Animais da Universidade Federal de São Carlos - CEUA/UFSCar **APROVOU** o projeto de pesquisa intitulado *"Estudo dos efeitos do treinamento resistido na sinalização molecular e remodelamento tecidual em ratas ovariectomizadas"* submetido pelo pesquisador *Sergio Eduardo de Andrade Perez.*

São Carlos, 21 de fevereiro de 2013.

Profa. Dra. Azair Liane Matos do Canto de Souza

Presidente da Comissão de Ética no Uso de Animais

11. REFERENCE

BAILEY, C. J.; AHMED-SOROUR, H. Role of ovarian hormones in the long-term control of glucose homeostasis. Effects of insulin secretion. **Diabetologia**, v. 19, n. 5, p. 475–481, 1980.

BARBOSA, M. R. et al. Resistance Training and Ovariectomy: Antagonic Effects in Mitochondrial Biogenesis Markers in Rat Skeletal Muscle. **Int J Sports Med**, v. 37, n. 11, p. 841–848, 2016.

BARROS, R. P. A.; MACHADO, U. F.; GUSTAFSSON, J.-A. Estrogen receptors: new players in diabetes mellitus. v. 12, n. 9, p. Trends in Molecular Medicine, 2006.

BARSALANI, R. et al. Liver of ovariectomized rats is resistant to resorption of lipids. **Physiology and Behavior**, v. 95, n. 1–2, p. 216–221, 2008.

BENATTI, F. B.; PEDERSEN, B. K. Exercise as an anti-inflammatory therapy for rheumatic diseases[mdash]myokine regulation. **Nat Rev Rheumatol**, v. 11, n. 2, p. 86–97, fev. 2015.

BERGERON, R. et al. Loss of Ovarian Estrogens Causes Only Mild Deterioration of Glucose Homeostasis in Female ZDF Rats Preventable by Voluntary Running Exercise. **Hormone and metabolic research**, v. 46, n. 11, p. 774–81, 2014.

BURCELIN, R. et al. Evidence that GLUT-2 mRNA and protein concentrations are decreased by hyperinsulinaemia and increased by hyperglycaemia in liver of diabetic rats. **Biochemical Journal**, v. 288 (Pt 2, p. 675–679, 1992.

CAMPOS, C. et al. Efficacy of a low dose of estrogen on antioxidant defenses and heart rate variability. **Oxidative Medicine and Cellular Longevity**, v. 2014, 2014.

CHOUKEM, S.; GAUTIER, J. How to measure hepatic insulin resistance ? **Diabetes Metabolism**, v. 34, n. 6, p. 664–673, 2008.

CLARK, J.; BRANCATI, F.; DIEHL, A. Non-alcoholic fatty liver disease. **Journal of gastroenterology and hepatology**, v. 17 Suppl, p. S186–S190, 2002.

COLLINS, P. et al. Randomized trial of effects of continuous combined HRT on markers of lipids and coagulation in women with acute coronary syndromes: WHISP Pilot Study. **European Heart Journal**, v. 27, n. 17, p. 2046–2053, 2006.

CORRIVEAU, P. et al. Resistance training prevents liver fat accumulation in ovariectomized rats. **Maturitas**, v. 59, n. 3, p. 259–267, 2008.

DOMINGOS, M.; RODRIGUES, M. F. C.; STOTZER, U. S. Resistance training restores the gene expression of molecules related to fat oxidation and lipogenesis in the liver of ovariectomized rats. **European Journal of Applied Physiology**, 2011.

FRAYN, K. N.; MAYCOCK, P. F. Skeletal muscle triacylglycerol in the rat: methods for sampling and measurement, and studies of biological variability. **Journal of lipid research**, v. 21, n. 1, p. 139–144, 1980.

GLOY, V. et al. Ovariectomy and overeating palatable, energy-dense food increase

subcutaneous adipose tissue more than intra-abdominal adipose tissue in rats. **Biology of sex differences**, v. 2, n. 1, p. 6, 2011.

HALLSWORTH, K. et al. Resistance exercise reduces liver fat and its mediators in non-alcoholic fatty liver disease independent of weight loss. **Gut**, v. 60, n. 9, p. 1278–1283, 2011.

HORNBERGER, T. A.; FARRAR, R. P. Physiological hypertrophy of the FHL muscle following 8 weeks of progressive resistance exercise in the rat. **Canadian journal of applied physiology**, v. 29, n. 1, p. 16–31, 2004.

IM, S.-S. et al. Identification and characterization of peroxisome proliferator response element in the mouse GLUT2 promoter. **Experimental & molecular medicine**, v. 37, n. 2, p. 101–10, 2005a.

IM, S. S. et al. Glucose-stimulated upregulation of GLUT2 gene is mediated by sterol response element-binding protein-1c in the hepatocytes. **Diabetes**, v. 54, n. 6, p. 1684–1691, 2005b.

JELENIK, T.; RODEN, M. How estrogens prevent from lipid-induced insulin resistance. **Endocrinology**, v. 154, n. 3, p. 989–992, 2013.

KALU, D. N. Ovarietomized Rat Model of Postmenopausal Bone Loss. **Bone and Mineral**, v. 15, p. 175–192, 1991.

KURIEN, B. T.; SCOFIELD, R. H. Western blotting. **Methods**, v. 38, n. 4, p. 283–293, 2006.

LAVOIE, J. M.; PIGHON, A. NAFLD, estrogens, and physical exercise: The animal model. **Journal of Nutrition and Metabolism**, v. 2012, 2012.

LEE, B.-H. et al. Ankaflavin: a natural novel PPARγ agonist upregulates Nrf2 to attenuate methylglyoxal-induced diabetes in vivo. **Free radical biology & medicine**, v. 53, n. 11, p. 2008–16, 2012.

LEITE, R. D. et al. Effects of ovariectomy and resistance training on lipid content in skeletal muscle, liver, and heart; fat depots; and lipid profile. **Applied physiology, nutrition, and metabolism,** v. 34, n. 6, p. 1079–1086, 2009.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods (San Diego, Calif.)**, v. 25, n. 4, p. 402–408, 2001.

LO, S.; RUSSELL, J. C.; TAYLOR, A. W. Determination of glycogen in small tissue samples. **Journal of applied physiology**, v. 28, n. 2, p. 234–236, 1970.

MA, H.; SHIEH, K.-J.; LEE, S. Study of ELISA technique. **Nature**, v. 4, n. 2, p. 36–37, 2006.

MALTAIS, M. L.; DESROCHES, J.; DIONNE, I. J. Changes in muscle mass and strength after menopause. **Journal of musculoskeletal & neuronal interactions**, v. 9, n. October, p. 186–197, 2009.

MARCHESINI, G. et al. Nonalcoholic fatty liver disease and the metabolic syndrome. **Curr Opin Lipidol**, v. 16, n. 4, p. 421–427, 2005.

MATSUSUE, K. et al. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. **Journal of Clinical Investigation**, v. 111, n. 5, p. 737–747, 2003.

MESSIER, V. et al. Menopause and sarcopenia: A potential role for sex hormones. **Maturitas**, v. 68, n. 4, p. 331–336, 2011.

MUSSO, G.; GAMBINO, R.; CASSADER, M. **Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD)Progress in Lipid Research**, 2009.

NAKAMURA, M. T.; YUDELL, B. E.; LOOR, J. J. Regulation of energy metabolism by long-chain fatty acids. **Progress in Lipid Research**, v. 53, n. 1, p. 124–144, 2014.

NEMOTO, Y. et al. Altered expression of fatty acid-metabolizing enzymes in aromatase-deficient mice. **The Journal of clinical investigation**, v. 105, n. 12, p. 1819–25, 2000.

OKA, Y. et al. Increased liver glucose-transporter protein and mRNA in streptozocin-induced diabetic rats. **Diabetes**, v. 39, n. 4, p. 441–446, 1990.

PAQUETTE, A. et al. Time course of liver lipid infiltration in ovariectomized rats: Impact of a high-fat diet. **Maturitas**, v. 58, n. 2, p. 182–190, 2007.

PAQUETTE, A. et al. Effects of ovariectomy on PPAR alpha, SREBP-1c, and SCD-1 gene expression in the rat liver. **Menopause (New York, N.Y.)**, v. 15, n. 6, p. 1169–1175, 2008.

PAQUETTE, A et al. Fatty acid oxidation is decreased in the liver of ovariectomized rats. **Hormone and metabolic research**, v. 41, n. 7, p. 511–5, 2009.

PETERSEN, K. F. et al. Mechanism by which glucose and insulin inhibit net hepatic glycogenolysis in humans. **Journal of Clinical Investigation**, v. 101, n. 6, p. 1203–1209, 1998.

PIGHON, A. et al. Substituting food restriction by resistance training prevents liver and body fat regain in ovariectomized rats. **Climacteric**, v. 12, n. 2, p. 153–64, 2009.

PIGHON, A. et al. Does exercise training prior to ovariectomy protect against liver and adipocyte fat accumulation in rats? **Climacteric : the journal of the International Menopause Society**, v. 13, n. 3, p. 238–48, 2010.

PIGHON, A.; GUTKOWSKA, J.; JANKOWSKI, M. Exercise training in ovariectomized rats stimulates estrogenic-like effects on expression of genes involved in lipid accumulation and subclinical inflammation in liver. **Metabolism**, v. 60, n. 5, p. 629–639, 2010.

RENCUREL, F. et al. Requirement of glucose metabolism for regulation of glucose transporter type 2 (GLUT2) gene expression in liver. **The Biochemical journal**, v. 314 (Pt 3, p. 903–9, 1996.

RICHARD, D.; ROCHON, L.; DESHAIES, Y. Effects of exercise training on energy balance of ovariectomized rats. **The American Journal of Physiology**, v. 253, n. 5 Pt 2, p. R740-5, 1987.

ROGUE, A. et al. Gene Expression Changes Induced by PPAR Gamma Agonists in Animal and

Human Liver. **PPAR research**, v. 2010, p. 325183, 2010.

SAENGSIRISUWAN, V. et al. Modulation of insulin resistance in ovariectomized rats by endurance exercise training and estrogen replacement. **Metabolism: Clinical and Experimental**, v. 58, n. 1, p. 38–47, 2009.

SCARABIN, P. Y.; OGER, E.; PLU-BUREAU, G. Differential association of oral and transdermal oestrogen-replacement therapy with venous thromboembolism risk. **Lancet**, v. 362, n. 9382, p. 428–432, 2003.

SCHROEDER, A. et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. **BMC molecular biology**, v. 7, p. 3, 2006.

SEYER, P. et al. Hepatic glucose sensing is required to preserve β cell glucose competence. **Journal of Clinical Investigation**, v. 123, n. 4, p. 1662–1676, 2013.

SHINODA, M.; LATOUR, M. G.; LAVOIE, J.-M. Effects of physical training on body composition and organ weights in ovariectomized and hyperestrogenic rats. **International journal of obesity and metabolic disorders** , v. 26, n. 3, p. 335–43, 2002.

SKOUBY, S. O. et al. A 1-year randomized study to evaluate the effects of a dose reduction in oral contraceptives on lipids and carbohydrate metabolism: 20 microg ethinyl estradiol combined with 100 microg levonorgestrel. **Contraception**, v. 71, n. 2, p. 111–117, 1 ago. 2005.

SKRYPNIK, D. et al. Effects of endurance and endurance-strength exercise on biochemical parameters of liver function in women with abdominal obesity. **Biomedicine and Pharmacotherapy**, v. 80, n. May, p. 1–7, 2016.

STAELS, B.; FRUCHART, J.-C. Therapeutic Roles of Peroxisome Proliferator-Activated Receptor Agonists. **Diabetes**, v. 54, n. 8, p. 2460–2470, 2005.

STEVENSON, J. C. Justification for the use of HRT in the long-term prevention of osteoporosis. **Maturitas**, v. 51, n. 2, p. 113–126, 1 ago. 2005.

STOTZER, U. et al. Resistance Training Suppresses Intra-abdominal Fatty Acid Synthesis in Ovariectomized Rats. **International Journal of Sports Medicine**, v. 36, n. 3, p. 226–233, 2014.

SUZUKI, A. et al. Alteration in expression profiles of a series of diabetes-related genes in db/dbmice following treatment with thiazolidinediones. **Japanese Journal of Pharmacology**, v. 84, n. 2, p. 113–123, 2000.

THORENS, B. GLUT2, glucose sensing and glucose homeostasis. **Diabetologia**, v. 58, n. 2, p. 221–232, 2015.

THORENS, B.; CHARRON, M. J.; LODISH, H. F. Molecular physiology of glucose transporters. **Diabetes care**, v. 13, n. 3, p. 209–18, 1990.

VIEIRA-POTTER, V. J. et al. Female rats selectively bred for high intrinsic aerobic fitness are protected from ovariectomy-associated metabolic dysfunction. **American Journal of Physiology - Regulatory, Integrative and Comparative Physiology**, v. 308, n. 6, p. R530–R542, 2015.

VÖLZKE, H. et al. Menopausal status and hepatic steatosis in a general female population. **Gut**, v. 56, n. 4, p. 593–594, 2007.

WOOD, I. S.; TRAYHURN, P. Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. **The British journal of nutrition**, v. 89, n. 1, p. 3–9, 2003.

YANG, J.; KALHAN, S. C.; HANSON, R. W. What is the metabolic role of phosphoenolpyruvate carboxykinase? **Journal of Biological Chemistry**, v. 284, n. 40, p. 27025–27029, 2009.

ŽEGURA, B. et al. The effect of various menopausal hormone therapies on markers of inflammation, coagulation, fibrinolysis, lipids, and lipoproteins in healthy postmenopausal women. **Menopause**, v. 13, n. 4, 2006.

ZHU, L. et al. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. **Diabetes**, v. 62, n. 2, p. 424–434, 2013.