UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Ciências Aplicadas

RODRIGO MARTINS PEREIRA

EFEITOS DE DIFERENTES PROTOCOLOS DE TREINAMENTO FÍSICO EM VIAS BIOMOLECULARES DE CONTROLE DE PRODUÇÃO HEPÁTICA DE GLICOSE E METABOLISMO LIPÍDICO NO FÍGADO DE ROEDORES OBESOS

EFFECTS OF DIFFERENT PHYSICAL TRAINING PROTOCOLS IN BIOMOLECULAR PATHWAYS OF HEPATIC GLUCOSE PRODUCTION CONTROL AND LIPID METABOLISM IN THE LIVER OF OBESE RODENTS

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Tese de doutorado apresentada à Faculdade de Ciências Aplicadas da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutor em Ciências da Nutrição e do Esporte e Metabolismo na área de Metabolismo e Biologia Molecular

Orientador: Prof. Dr. Leandro Pereira de Moura.

ESTE EXEMPLAR CORRESPONDE À VERSÃO DA TESE DEFENDIDA PELO ALUNO RODRIGO MARTINS PEREIRA, E ORIENTADA PELO PROF. DR LEANDRO PEREIRA DE MOURA

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"Frequentemente é necessário mais coragem para ousar fazer certo do que temer fazer errado."

Abraham Lincoln.

RESUMO

Doença hepática gordurosa não alcoólica (DHGNA) é caracterizada pelo acúmulo excessivo de gordura no fígado e tem íntima relação com a obesidade e diabetes *mellitus* do tipo 2 (DM2). Sabe-se que o fígado é um dos principais órgãos responsáveis pela manutenção da normoglicemia, e o combate à DHGNA para o aumento da ação da insulina no fígado é uma das estratégias primárias no manuseio da DM2. Apesar dos avanços da medicina, o treinamento físico é considerado uma das principais estratégias para o aumento da ação hepática da insulina e redução da DHGNA em obesos. Porém, os mecanismos envolvidos nesses processos não são completamente compreendidos. Portanto, o nosso objetivo foi investigar os mecanismos biomoleculares pelos quais diferentes protocolos de treinamento físico atuam no combate à DHGNA e melhoram o controle da produção hepática de glicose (PHG) em camundongos obesos. Após aprovação do Comitê de Ética, camundongos Swiss machos foram alimentados com dieta rica em gordura saturada para a indução da obesidade e em seguida foram submetidos a diferentes protocolos de treinamento, sendo: treinamento de força em escada, treinamento aeróbio em esteira e treinamento combinado (força e aeróbio na mesma sessão). O treinamento de força consistiu de 1 sessão diária durante 15 dias; o treinamento aeróbio em 1 sessão diária durante 7 dias; e o treinamento combinado em 1 sessão diária durante 7 dias. O treinamento de força reduziu a DHGNA, inibindo a atividade da proteína lipogênica *Acetyl-CoA carboxylase* (ACC), reduzindo o conteúdo proteico de *Fatty Acid Synthase* (FAS) e ACC e os níveis de RNA mensageiro de *Fasn* e *Scd1*. Observamos também redução da atividade de *Forkhead box protein O1* (FOXO1), reduzindo assim o conteúdo de *Glucose 6 Phosphatase* (G6Pase) e *Phosphoenolpyruvate Carboxykinase* (PEPCK). Os níveis da proteína gliconeogênica *Pyruvate Carboxylase* (PC) e da proteína induzida por inflamação *Protein tyrosine phosphatase 1B* (PTP1B) também foram reduzidos. Quando analisamos o treinamento combinado, observamos que essa modalidade também reduziu a DHGNA e o estímulo lipogênico. Por fim, observamos que tanto o treinamento de força quanto aeróbio aumentaram o conteúdo hepático de clusterina nos animais obesos. Juntos, esses resultados nos permitem concluir que os 3 protocolos de treinamento apresentam interessante potencial contra a DHGNA, reduzindo o conteúdo de lipídios no fígado, inibindo a maquinaria de lipogênese hepática e melhorando o controle da PHG.

Palavras-chave: DHGNA, obesidade, DM2, PHG, treinamento físico.

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive liver fat accumulation and is closely related to obesity and type 2 diabetes *mellitus* (T2DM). The liver is one of the main organs responsible for normoglycemia, and the fight against NAFLD to increase hepatic insulin action is one of the main strategies in T2DM management. Despite medical advances, the plysical training is the primary interventions for increasing the hepatic insulin sensivity and reducing NAFLD in obese individuals. However, the mechanisms involved in these processes are not fully understood. The our aim was to investigate biomolecular mechanisms by which different physical training protocols act against NAFLD and increase insulin sensivity and hepatic glucose production (HGP) control in obese mice. After Ethics Committee approval, male Swiss mice were fed a high saturated fat diet for obesity induction and then subjected to different training protocols: ladder climb strength training, treadmill aerobic training and combined training (strength and aerobic in the same session). Strength training consisted of 1 daily session for 15 days; aerobic training in 1 daily session for 7 days; and the combined training in 1 daily session for 7 days. Strength training reduced NAFLD, inhibiting the activity of the lipogenic protein Acetyl-CoA carboxylase (ACC), reducing the protein content of Fatty Acid Synthase (FAS) and ACC and the messenger RNA levels of *Fasn* and *Scd1*. We also observed a reduction in Forkhead box protein O1 (FOXO1) activity, reducing Glucose 6 Phosphatase (G6Pase) and Phosphoenolpyruvate Carboxykinase (PEPCK). The gluconeogenic protein levels of Pyruvate Carboxylase (PC) and inflammation-induced protein Protein tyrosine phosphatase 1B (PTP1B) were also reduced. When we analyzed combined training, we found that this modality also reduced NAFLD and lipogenic stimulation. Finally, we observed that both strength and aerobic training increased clusterin hepatic content in obese animals. Together, these results allow us to conclude that the 3 exercise training protocols have interesting potential against NAFLD, reducing liver lipids, inhibiting the hepatic lipogenesis machinery and improving the control of HGP.

Keywords: NAFLD, obesity, T2DM, HGP, physical training.

SUMÁRIO

1. INTRODUÇÃO

De acordo com a Organização Mundial de Saúde (OMS), a obesidade pode ser definida como um estado de excessiva quantidade de massa adiposa, sendo essa uma condição crônica e multifatorial (WORLD HEALTH ORGANIZATION, 2020a). Trata-se de uma doença envolvida com tanto com fatores genéticos quanto ambientais (GLUCKMAN et al., 2011), com custos globais relacionados ao tratamento de suas complicações que irão ultrapassar a cifra de U$1,2 trilhão ao ano a partir de 2025, quando estima-se que mais de um terço da população mundial será obesa (mais de 2,7 bilhões de pessoas) (WORLD OBESITY FEDERATION, 2021).

No Brasil o cenário é igualmente preocupante. No período compreendido entre 2003 e 2019, o número de de homens com excesso de peso se elevou de 43,3% para 60%, e de obesos de 9,6% para 22,8% (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2020). Atualmente, segundo os dados mais recentes da Vigitel (órgão que compõe o sistema de Vigilância de Fatores de Risco para doenças crônicas não transmissíveis do Ministério da Saúde no Brasil), 55,4% da população brasileira apresenta excesso de peso e 20,3% apresenta obesidade (VIGITEL BRASIL 2019, 2020). Um recente estudo constatou que em 2018 mais de 1,8 milhões de internações no Sistema Único de Saúde (SUS) foram relacionadas à obesidade e suas comorbidades, o que corresponde a aproximadamente 16% das internações (NILSON et al., 2020). O mesmo estudo ainda mostrou que os custos totais com hospitalizações e gastos com medicamentos somam R$ 669 milhões e R$ 722 milhões, respectivamente, totalizando R$ 1,39 bilhão aos cofres públicos (NILSON et al., 2020).

Já é bem descrito que a obesidade apresenta uma íntima relação com diversas outras doenças de cunho metabólico como doenças cardiovasculares, aterosclerose, alguns tipos de câncer e a diabetes *mellitus* do tipo 2 (DM2), sendo essa última a doença destacada na presente tese. A DM2 é uma doença caracterizada pela hiperglicemia de jejum, em um estado em que o corpo não é capaz de produzir insulina suficiente para a captação de glicose ou a insulina não é capaz de exercer devidamente suas funções (PETERSEN; SHULMAN, 2017; SOCIEDADE BRASILEIRA DE DIABETES, 2021). A OMS estimou que em 2014 aproximadamente

8,5% da população adulta apresentavam DM2 (WORLD HEALTH ORGANIZATION, 2016) e que em 2012 a DM2 foi responsável por 1,5 milhão de mortes (WORLD HEALTH ORGANIZATION, 2016). No Brasil, 15,9% da população é diabética (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2020) e estima-se que aproximadamente 50% dos acometidos por essa doença não sabem de sua condição (SCHMIDT et al., 2014).

Apesar dos inúmeros avanços da ciência sobre o entendimento da relação causal entre a obesidade e a gênese da DM2, esse é um assunto ainda não totalmente compreendido. Entretanto, alguns possíveis mecanismos podem ser apontados:

1) A hipertrofia do tecido adiposo que culmina em uma hiperativação do sistema imune e maior recrutamento de monócitos por esse tecido e diferenciação à macrófagos do tipo M1, com maior potencial inflamatório e maior produção de citocinas-pró inflamatórias (HOTAMISLIGIL, 2006; MARZULLO et al., 2021);

2) O aumento nos níveis circulantes de ácidos graxos livres que sabidamente comprometem tanto a secreção quanto a ação da insulina de modo dose-dependente tanto em diabéticos quanto não-diabéticos (GOLAY; YBARRA, 2005);

3) O estresse de retículo endoplasmático (ERE) devido a uma saturação do sistema de degradação de proteínas mal enoveladas, induzindo aumento na ativação da maquinaria biomolecular inflamatória que contraregula a ação da insulina (OZCAN et al., 2004; ZHANG et al., 2013);

4) Alterações nas populações da microbiota intestinal, aumentando a razão *Firmicutes:Bacteroidites* e favorecendo a absorção de lipopolissacarídeos, estimulando a condição próinflamatória (LIU et al., 2021; MARZULLO et al., 2021; ZWARTJES; GERDES; NIEUWDORP, 2021).

Porém, todos esses fenômenos parecem se encontrar em um ponto em comum para o desencadeamento do DM2 induzido pela obesidade: a resistência à insulina. A insulina após ser secretada em condições de disponibilidade de nutrientes se liga ao seu receptor tirosina quinase *Insulin Receptor* (IR), uma proteína transmembrana composta por uma porção extracelular com um domínio de ligação para a insulina e que regula sua atividade intrínseca de se auto-fosforilar em resíduos de tirosina. Uma vez ligada à subunidade α extracelular do IR, a insulina promove a fosforilação em tirosina da porção β transmembrana, recrutando assim uma famílias

de proteínas *downstream* a sua via conhecidas como *scaffold*, fundamentais para o início da transdução do seu sinal biomolecular. Dentre elas, as mais responsivas ao sinal insulínico parecem ser os substratos do receptor de insulina 1 e 2 (IRS1 e IRS2, respectivamente), apesar de outras proteínas como IRS3, IRS4, SHC, CBL, APS, SH2B, GAB1, GAB2, DOCK1, e DOCK2 também serem recrutadas por IR. As proteínas IRSs se associam aos *motifs* NPXY no IR e também são fosforiladas principalmente em sítios de tirosina, que vão proporcionar a ligação a proteínas efetoras como *phosphatidylinositol 3-kinase* (PI3K). Por sua vez, PI3K converte o fosfolipídio de membrana PIP2 para PIP3, que recruta as proteínas serina/treonina quinase B / Akt (PERRY et al., 2014; SALTIEL, 2021). As proteínas Akt compõem uma família de proteínas composta por 3 isoformas altamente homólogas, sendo Akt1 (PKBα), Akt2 (PKBβ) e Akt3 (PKBγ), que embora codificadas por diferentes genes em diferentes cromossomos, apresentam mais 80% de similaridade (TOKER; MARMIROLI, 2014). Trata-se de uma proteína de atividade nodal, com ações tecido-específicas relacionadas ao controle metabólico e energético. Dentre elas, 4 podem ser destacadas: 1) a atuaçao no núcleo arqueado do hipotálamo potencializando a ação da leptina e promovendo saciedade, progando seus sinais anorexígenos por meio dos seus receptores *Leptin Receptor* b (LepR-b) e IR, respectivamente, principalmente nos neurônios *agouti-related protein* (AgRP) e *proopiomelanocortin* (POMC) (VARELA; HORVATH, 2012); 2) a captação de glicose, síntese de glicogênio e a síntese proteica no músculo esquelético, por mecanismos relacionados, respectivamente, à translocação do *glucose transporter 4* (GLUT4) para a membrana plasmática mediada pelo substrato da Akt de 160kDa (AS160), que permite a entrada da glicose por meio de difusão facilitada, à fosforilação e inibição da proteína *glycogen synthase kinase 3* (GSK3) que permite a ação da *glycogen synthase* (GS), e à ativação de *mammalian target of rapamycin complex 1* (mTORC1) e seus efetores *downstream p70 ribosomal S6 protein kinase-1* (S6K1) e *eukaryotic translation initiation factor-4E* (eIF4E)-*binding protein-1* (4E-BP1) (HUANG et al., 2018); 3) o estímulo lipogênico no tecido adiposo, regulando principalmente a síntese de ácidos graxos e de colesterol pela ativação de *sterol regulatory element-binding proteins* (SREBP) e inibição da lipólise pela inativação da *Forkhead box O1* (FOXO1), proteína que regula a lipólise controlando a expressão de *adipose triglyceride lipase* (ATGL) (HUANG et al., 2018) e 4) a síntese de glicogênio e de lipídios no fígado por mecanismos semelhantes aos descritos no músculo e tacido

adiposo previamente, além da inibição da produção hepática de glicose (PHG) principalmente por meio da migração para o núcleo e fosforilação do fator de transcrição FOXO1, induzindo sua extrução nuclear e a impossibilitando de transcrever os genes gliconeogênicos de *Phosphoenolpyruvate Carboxykinase* (PEPCK) e *Glucose- 6-Phosphatase* (G6Pase) (PERRY et al., 2014). Entretanto, na condição de obesidade a insulina começa a ter sua efetividade reduzida, principalmente devido aos fenômenos previamente citados, induzindo assim hiperfagia, redução da captação de glicose e perda do controle da PHG (HOTAMISLIGIL, 2006; MAGNUSSON et al., 1992; VAN DE SANDE-LEE et al., 2011). Uma vez instaurada a resistência insulina, um mecanismo compensatório frente a redução da ação do hormônio é o aumento de sua produção, gerando assim o fenótipo de hiperinsulinemia que, de modo crônico, induz falência e morte das células beta pancreáticas, sendo esse o fenômeno tido como maior contribuidor para o desenvolvimento e progressão da DM2 (KHIN; LEE; JUN, 2021; SHANIK et al., 2008).

A ciência tem apresentado importantes avanços no tratamento das condições obesidade e DM2. Tratando-se da obesidade, diversas estratégias farmacológicas e intervenções como cirurgias bariátricas e o uso de balões intragástricos têm apresentado considerada eficiência. Drogas como orlistate, fentermina/topiramato, naltrexona/bupropiona, e liraglutide são exemplos de substâncias já aprovadas pela agência federal do departamento de Saúde e Serviços Humanos dos Estados Unidos, a *Food and Drug Administration* (FDA) por seus promissores efeitos gastrointestinais de redução na absorção de lipídios, anorexigênicos e de aumento de gasto energético. Porém, podem apresentar alto custo e seus efeitos ainda são limitados e/ou seus colaterais requerem cautela no uso (TAK; LEE, 2021). Já o uso a cirurgia bariátrica e os balões intragástricos ainda tem sua aplicabilidade limitada, apesar do crescente número de casos em seus usos (KIM et al., 2016; KUSHNER; EAGON, 2021). Em relação à DM2 associada à obesidade, drogas como agonistas de GLP-1 (Exenatide BID, Liraglutide, Exenatide QW, Albiglutide, Dulaglutide e Lixisenatide), inibidores dos *sodium-glucose cotransporter*-2 (SGLT-2), análogos da amilina, inibidores de alfa-glicosidase e metformina são largamente utilizados, ainda que possam promover efeitos adversos como náuseas, vômitos, infecções urinárias, hipoglicemias, flatulências, diarreias e deficiências de vitaminas (ESQUIVEL; LANSANG, 2017). Portanto, mudanças no

estilo de vida como alterações comportamentais, intervenções dietéticas e a prática de exercícios são apontadas como as mais eficientes estratégias para a redução da adiposidade e redução dos níveis glicêmicos, além de ser a mais sustentável a longo prazo para a manutenção do peso (FOSTER et al., 2018; JEVTOVIC, 2021; OLATEJU et al., 2021).

Entretanto, um órgão merece destaque para a perpetuação da hiperglicemia de jejum que caracteriza a DM2: o fígado. Dentre suas funções, 3 podem ser destacadas: 1) metabolismo glicídico, atuando na manutenção dos níveis glicêmicos seguros por meios dos mecanismos de gliconeogênese, glicogenólise e glicogênese; 2) função secretória e excretória pela formação da bile e 3) função de síntese proteica como albumina e fatores de coagulação (WORLD HEALTH ORGANIZATION, 2020b). Tratando-se do metabolismo glicídico, esse órgão é responsável pela produção de aproximadamente 90% da glicose endógena, e acredita-se que o aumento da gliconeogênese e perda do controle da PHG são os fenômenos proximais para a hiperglicemia na DM2 devido à falha da insulina em controlar esses processos, em um estado conhecido como resistência hepática à insulina (PETERSEN; SHULMAN, 2017).

A resistência hepática à insulina parece ser um fenômeno reversível. Novamente, a redução da adiposidade e a prática de exercícios ainda são as estratégias mais eficientes para esse fim (BACCHI et al., 2013; PETERSEN et al., 2005; TILG; MOSCHEN; RODEN, 2017). Um elegante estudo, revelou que a perda de aproximadamente 8 kg foi eficiente para obesos diabéticos normalizarem os níveis de glicose sérica em jejum e a supressão da PHG induzida por insulina (PETERSEN et al., 2005). Já uma importante revisão sistemática revelou que mesmo sem a redução de peso o exercício é capaz de melhorar a sensibilidade hepática à insulina por proporcionar redução nos triglicerídeos intra-hepáticos, embora seus benefícios sejam substancialmente maiores quando a redução de peso é observada (SARGEANT et al., 2018a). Entretanto, os mecanismos pelos quais o exercício promove benefícios ao metabolismo hepático, refletindo em um melhor controle glicêmico na condição da obesidade, são pouco conhecidos. Portanto, a presente tese foi desenvolvida com o objetivo de investigar mecanismos biomoleculares regulados pela obesidade e por diferentes modalidades de treinamento físico no fígado de camundongos obesos, avaliando o impacto dessas intervenções no metabolismo hepático e no controle glicêmico desses animais.

2. REVISÃO DA LITERATURA

2.1 CAPÍTULO 1: O FÍGADO COMO TECIDO-CHAVE PARA A HIPERGLICEMIA INDUZIDA PELA OBESIDADE

O fígado é o principal órgão responsável pela produção endógena de glicose, e a falha da ação da insulina em inibir essa produção em períodos de abundancia de nutrientes é tida como o principal evento para a indução da hiperglicemia característica da DM2 (PETERSEN; SHULMAN, 2017). Os mecanismos de controle da PHG mediados por insulina ocorrem por dois processos: 1) pela inibição da quebra do glicogênio hepático, processo chamado de glicogenólise e 2) pela inibição da produção de uma nova molécula de glicose a partir de corpos não-glicídicos, processo esse chamado de gliconeogênese (PAREDES-FLORES; MOHIUDDIN, 2021; PERRY et al., 2014). E para que possamos compreender a influência negativa da obesidade sobre esses mecanismos, é necessário antes compreender como esse controle é realizado nas condições em que a insulina exerce efetivamente suas funções:

Glicogenólise: trata-se do processo bioquímico pelo qual o glicogênio é quebrado para a liberação de glicose. O glicogênio trata-se de um polissacarídeo ramificado constituído por unidades de glicose, servindo como o principal estoque de glicose para situações de privação de nutrientes (ELLINGWOOD; CHENG, 2018). Tanto o músculo esquelético quanto o fígado têm a capacidade de armazenar glicose na forma de glicogênio, entretanto, enquanto o fígado o utiliza para a manutenção da normoglicemia, o músculo esquelético o utiliza para o fornecimento de energia para a contração muscular (PAREDES-FLORES; MOHIUDDIN, 2021). A principal enzima responsável por regular esse processo é a *glycogen phosphorylase*, predominantemente expressa no músculo esquelético, cérebro e fígado (NADEAU; FONTES; CARLSON, 2018), que catalisa a liberação de glicose-1-fosfato do fim da cadeira do glicogênio, pela clivagem das ligações do tipo α-1,4 (ELLINGWOOD; CHENG, 2018). Pode ser estimulada por hormônios como glucagon e catecolaminas, além do aumento dos níveis de AMP cíclico (cAMP) e pelo influxo de Ca+2 (PAREDES-FLORES; MOHIUDDIN, 2021).

Gliconeogênese: conforme comentado anteriormente, esse é o nome dado ao grupo de reações metabólicas responsável pela produção de uma nova molécula de glicose, sendo esse um sistema fundamental para a manutenção dos níveis minimamente seguros de glicose circulante em situações de privação de nutrientes (CHOURPILIADIS; MOHIUDDIN, 2021). De fato, durante as primeiras horas de jejum o processo de gliconególise é o principal mecanismo para a manutenção da normoglicemia, porém, estimasse que após 14 horas de jejum 54% da glicose circulante seja oriunda da gliconeogênese hepática, podendo chegar a 84% após 42 horas (CHANDRAMOULI et al., 1997). O primeiro passo no processo de gliconeogênese é mediado pela enzima *Pyruvate Carboxylase* (PC), responsável pela adição de um grupo carboxil oriundo do dióxido de carbono (CO2) ao piruvato, formando oxaloacetato no interior da mitocôndria, para que possa ser enviado ao citosol por meio do transporte de malato. Uma vez no citosol, o oxaloacetato será convertido novamente à fosfoenolpiruvato pela ação da enzima *Phosphoenolpyruvate Carboxykinase* (PEPCK), usando agora GTP como doador de fosfato. As próximas reações são reversíveis e comuns à glicólise, até a formação de frutose-1,6-bifosfatase, que sofrerá a ação irreversível de conversão à frutose-6-fosfato pela ação da enzima *Fructose-1,6 bisphosphatase* (FBPase), sendo esse um passo determinante para todo o processo, finamente regulado pelos níveis de ATP, citrato e glucagon. Finalmente, frutose-1,6-bifosfatase será convertida a frutose-6-fosfato e em seguida a glicose-6-fosfato, para então sofrer a última reação irreversível no processo de gliconeogênese, a hidrólise mediada pela proteína *Glucose-6 Phosphatase* (G6Pase) para a formação da molécula de glicose (CHOURPILIADIS; MOHIUDDIN, 2021).

Um estudo clássico conduzido ainda na década de 90 foi um dos pioneiros a demonstrar a participação fundamental da perda do controle da PHG na hiperglicemia durante a DM2 (MAGNUSSON et al., 1992). Utilizando a técnica de ressonância magnética nuclear de carbono 13 os autores mensuraram o volume de glicogênio hepático e as taxas de glicogenólise e gliconeogênese em sujeitos saudáveis e com DM2. Conforme esperado, sujeitos diabéticos apresentaram níveis reduzidos de glicogênio hepático quando comparados aos não-diabéticos, mesmo os participantes submetidos à refeições padronizadas durantes 3 dias antes das avaliações. Dessa forma, a participação líquida da glicogenólise mostrou-se inferior para os sujeitos com DM2 para a manutenção dos níveis glicêmicos constantes em

períodos de jejum, porém, a taxa de gliconeogênese hepática mostrou-se elevada, responsável por 88±2% da PHG em diabéticos contra 70±6% nos saudáveis (MAGNUSSON et al., 1992). Coerentemente, estudos posteriores trouxeram mais informações e nos proporcionaram uma maior compreensão a respeito dos mecanismos biomoleculares de controle de PHG afetados pela obesidade.

Ainda na década de 90, um estudo com roedores demonstrou que um mecanismo pelo qual indivíduos diabéticos tem menores estoques de glicogênio no estado de resistência hepática à insulina se dá por meio da maior ativação de GSK3 (ELDAR-FINKELMAN et al., 1999), uma vez que, conforme já descrito, essa proteína tem a habilidade de inibir a atividade de GS, proporcionando assim menor estímulo para a síntese de glicogênio. Sabendo que GSK3 é diretamente fosforilada e inativada por Akt após estímulo de insulina (HERMIDA; DINESH KUMAR; LESLIE, 2017), é esperado uma maior atividade dessa proteína em sujeitos diabéticos. Coerentemente, intervenções que proporcionam aumento da sensibilidade hepática à insulina e aumento da atividade de Akt resultam em maior fosforilação e inbição de GSK3, com concomitante aumento no conteúdo hepático de glicogênio em modelo animal (MARINHO et al., 2012a). Quanto aos processos de gliconeogênese, foi demonstrado que camundongos obesos apresentam menor fosforilação de FOXO1 hepática (WANG et al., 2018), com muitos estudos mostrando aumento tanto de PEPCK quanto de G6Pase na condição de obesidade (HONMA et al., 2018; MARINHO et al., 2012a). Ainda, foi demonstrado que o acúmulo de triglicerídeos intra-hepáticos tem uma grande participação na perda do controle da PHG, sendo que pessoas obesas com essa condição apresentam a gliconeogênse aumentada em 25%, com aumento também no estresse oxidativo e dano hepático (SUNNY et al., 2011).

Não obstante, a inibição farmacológicas dos mecanismos de PHG são alvos terapêuticos no tratamento da obesidade e DM2, focando principalmente na inibição da atividade de FOXO1 hepática (CHOI et al., 2021; LANGLET et al., 2017). Recentemente, o composto JY-2, foi proposto como uma promissora droga para o tratamento da DM2, inibindo a atividade transcricional de FOXO1 hepática e melhorando a tolerância à glicose de animais com obesidade induzida tanto por alterações genéticas (*db/db*) quanto por dieta hiperlipídica (CHOI et al., 2021). Em seu estudo, Langlet e colaboradores (2017) também identificaram vários inibidores de FOXO1, que foram efetivas em sensibilizar o fígado de roedores à insulina e

reduzir a PHG, reduzindo a hiperglicemia (LANGLET et al., 2017). Entretanto, a aplicabilidade desses inibidores ainda apresenta algumas limitações: 1) o uso de inibidores tem um efeito colateral indesejável de aumentar a síntese de triglicerídeos e 2) as propriedades farmacocinéticas de alguns compostos impedem sua aplicação in vivo.

Outro modelo de medicamentos propostos para o combate da hiperglicemia focando na PHG envolve a inibição dos receptores de glucagon (CHO; MERCHANT; KIEFFER, 2012). Conforme descrito, o glucagon tem um efeito hiperglicemiante por estimular a glicogenólise, porém ele também tem a função de estimular a gliconeogênese hepática (KALANT, 1956). Esse hormônio atua por seu receptor *glucagon receptor* (GCGR), aumentando os níveis de cAMP e estimulando a ativação da proteína quinase A (PKA) (AUTHIER; DESBUQUOIS, 2008). No fígado, a proteína PKA tem a função de regular a expressão das já descritas aqui proteínas gliconeogênicas como PEPCK, G6Pase e FBPase (JIANG; ZHANG, 2003), além de aumentar a atividade de *Peroxisome proliferator-activated receptor γ coactivator-1* (PGC1α), conhecida por se associar à FOXO1 hepática e aumentar a sua atividade transcricional dos genes de PEPCK e G6Pase (ROPELLE et al., 2009). Coerentemente, a inibição transiente pelo uso de antisense oligonucleotídeos (ASOs) de GCGR em modelo animal de ratos diabéticos reverteu a hiperglicemia e aumentou a tolerância à glicose (LIANG et al., 2004; SLOOP et al., 2004). Em humanos, estratégias para a inibição do glucagon incluem antagonistas e anticorpos monoclonais, porém poucos produtos já foram testados. Um fármaco oral desenvolvido pela Marck® que tem se mostrado promissor trata-se de um antagonista de GCGR contendo uma cadeia lateral de β-alanina, chamado de MK-0893. Em seus primeiros testes clínicos ele tem se mostrado eficiente na redução dos níveis glicêmicos quando combinado a metformina em pacientes diabéticos (CHO; MERCHANT; KIEFFER, 2012; FILIPSKI et al., 2012). Outra promissora molécula é conhecida como Bay 27-9955, e que demonstrou promissores resultados, reduzindo a PHG induzida por glucagon em sujeitos saudáveis (PETERSEN; SULLIVAN, 2001). Já o agonista de GCGR LY 2409021 foi eficiente em reduzir a hiperglicemia de jejum e os níveis de HbA1c em diabéticos do tipo 2, com aumento nos níveis de GLP-1 (KELLY et al., 2015). Já se tratando das terapias com anticorpos monoclonais, essa é uma estratégia que aboliu a ação hiperglicimiante em modelo experimental de ratos diabéticos (BRAND et al., 1994). Em camundongo *ob/ob*,

apenas uma dose de anticorpo monoclonal melhorou a tolerância à glicose por reduzir a PHG, e de modo crônico o tratamento reduziu também os níveis circulantes de glicose, triglicerídeos e HbA1c (SORENSEN et al., 2006). Resultados semelhantes também foram observados em primatas, sem ocorrência de hipoglicemia (YAN et al., 2009). Porém, ainda carecemos de estudos que investigaram os efeitos dessa terapia em humanos. Entretanto, todas essas estratégias farmacológicas ainda são limitadas para a manutenção de um controle glicêmico duradouro, de modo que a perda progressiva de peso continua sendo a principal estratégia para o tratamento da DM2 associada à obesidade. Ainda, as estratégias de inibição de GCGR requerem grande cuidado, pois proporciona predisposição à hipoglicemia, compromete a recuperação de quadros hipoglicêmicos pela bloqueio de mecanismos contra-regulatórios, podem induzir danos hepáticos ou ainda apresentam custos inacessíveis (CHO; MERCHANT; KIEFFER, 2012).

2.2 CAPÍTULO 2: DOENÇA HEPÁTICA GORDUROSA NÃO ALCOÓLICA (DHGNA): UM LINK ENTRE A OBESIDADE E A DM2

De modo intimamente próximo à crescente nos números de obesidade e DM2 ao redor do mundo, uma terceira condição diretamente relacionada ao metabolismo hepático e às complicações metabólicas também apresenta um grande aumento nas últimas décadas, a DHGNA (PAIS; MAUREL, 2021). A DHGNA é caracterizada pelo acúmulo de gordura no fígado com quantidade superior a 5% do perênquima, sendo conhecida como uma “condição guarda-chuva” que abrange um spectro de fenótipos incluindo a esteatose hepática sem dano celular, a esteatohepatíte não alcoólica (do inglês *non-alcoholic steatohepatitis* – NASH) com um processo necroinflamatório com dano celular, a fibrose, a cirrose e o hepatocarcinoma (BENEDICT; ZHANG, 2017). Essa é a doença hepática crônica mais comum. Dentre a população geral, estima-se que até 25% da população geral apresenta DGHNA (LAZARUS et al., 2021), e devido à sua íntima relação com a síndrome metabólica, a DHGNA é encontrada em 63,7% das pessoas com DM2 e 80% das pessoas com obesidade ao redor do mundo, porém podendo ocorrer também em pessoas eutróficas (POWELL; WONG; RINELLA, 2021). Embora pouco compreendidas as relações de causalidade, condições como obesidade, DM2, dislipidemias e resistência à insulina são conhecidas como associadas ao desenvolvimento da DHGNA (MARUŠIĆ et al., 2021). Nessas condições, são observados acúmulos de lipídios tóxicos, como ceramidas e diacilgliceróis, conhecidos lipídios bioativos capazes de inibir a ativação da via da insulina por mecanismos envolvendo a ativação de isoformas alternativas do proteína kinase C (PKC), como a isoforma épsilon (PKCε) (PETERSEN; SHULMAN, 2017). Esses lipídios sabidamente também provocam eventos como ERE, estresse oxidativo e disfunção mitrocondrial, induzindo inflamação e morte celular (FERGUSON; FINCK, 2021).

Ainda, não somente a presença de obesidade, mas também a distribuição da gordura corporal é aceita como um fator de predisposição para a ocorrência de DHGNA (PAIS; MAUREL, 2021). É sabido que o tecido adiposo visceral trata-se de um tecido mais metabolicamente ativo quando comparado ao tecido adiposo subcutâneo, com grande produção de citocinas próinflamatórias (ALVEHUS et al.,

2010). Recentemente foi demonstrado que metabólitos próinflamatóris liberados pelo tecido adiposo visceral são diretamente relacionados à progreção da DHGNA para NASH (MUSSO et al., 2018). E, coerentemente, uma importante revisão sistemática demonstrou que a razão da taxa de acúmulo de gordura no tronco/membros aumenta conforme o aumento da severidade da doença hepática (BEDOSSA et al., 2017)

E conforme descrito acima, a DHGNA também comumente coexiste com a DM2 e a resistência à insulina. Já foi demonstrado que indivíduos diabéticos com DHGNA apresentam maiores níveis de insulina circulante e apresentam resitência à insulina mais severa quando comparados aos diabéticos sem DHGNA (GASTALDELLI et al., 2007), e sujeitos com DM2 apresentam até 5 vezes mais chances de serem hospitalizados devido a complicações do acúmulo excessivo de gordura (WILD et al., 2016). E de fato, a insulina sabidamente apresenta um papel fundamental no controle das vias moleculares de síntese de lipídios no fígado, estimulando principalmente um processo conhecido lipogênese *de novo* (SANDERS; GRIFFIN, 2016). Como descrito no tópico anterior, uma vez ligada ao seu receptor transmembrânico, a insulina apresenta a habilidade de inibir a produção hepática de glicose e estimular a síntese de glicogênio, principalmente por mecanismos envolvendo as proteínas FOXO1 e GSK3, ambas com atividade regulada pela proteína Akt. Por outro lado, a Akt também tem a habilidade de estimular a síntese de lipídios no fígado (SANDERS; GRIFFIN, 2016). Uma vez ativada, a Akt promove a ativação do fator de transcrição *Sterol Regulatory Element Binding Protein 1c* (SREBP1c), que irá transcrever o genes lipogênicos de *Fatty Acid Synthase* (FAS), *Acetyl-CoA Carboxylase* (ACC) e *Stearoyl-CoA Desaturase 1* (SCD-1), proteínas fundamentais nos processos bioquímicos de síntese de lipídeos (SANDERS; GRIFFIN, 2016), conforme melhor descrito a seguir. Entretanto, paradoxalmente ao observado com os mecanismos de controle da PHG, no estado de obesidade tanto a atividade de SREBP1c quando os níveis de FAS, ACC e SCD-1 estão aumentados, mesmo com a insulina tendo sua função reduzida (NTANDJA WANDJI et al., 2020; PEREIRA et al., 2019). Recentemente, foi demonstrado que no estado de resistência hepática à insulina, esse hormônio realmente perde a capacidade de fosforilar e inibir FOXO1, entretanto as vias lipogências envolvendo SREBP1c continuam ativas (BROWN; GOLDSTEIN, 2008). Esse fenômeno passou a ser chamado como resistência seletiva à insulina, em que esse hormônio falha em inibir a PHG, porém continua estimulando vias de síntese de lipídios. Embora esse conceito já tenha mais

de uma década, ainda hoje os mecanismos envolvidos nesse processo continuam desconhecidos.

A lipogênese *de novo* hepática trata-se de uma fina cadeia de reações bioquímicas fundamental para a síntese, estoque e secreção de lipídios pelo fígado, sugerida como a principal anormalidade para a gênese da DHGNA e comumente aumentada na condição de DM2 e resistência à insulina (AMEER et al., 2014; DONNELLY et al., 2005). Tem como objetivo a síntese de cadeias de ácido graxo a partir das moléculas de acetil-CoA oriundas da glicólise, associando-as a esqueletos de glicerol (COLEMAN, 2004; SMITH; TSAI, 2007). O processo inicia-se pela conversão de acetil-CoA em malonil-CoA em uma reação catalisada pela enzima ACC. O malonil-CoA então será processado pelo complexo proteico FAS, passando por processos de condensação, desidratação e alongamento repetidas vezes para a formação de corpo de 16 carbonos chamado de ácido palmítico (PAGLIALUNGA; DEHN, 2016; SANDERS; GRIFFIN, 2016).

Apesar da DHGNA ser tão presente, ainda não existem drogas licenciadas para o seu tratamento. Entretanto, algumas drogas utilizadas no tratamento da DM2 apresentam alguns benefícios contra a DHGNA e na redução de marcadores de NASH, mais uma vez expondo a intima inter-relação entre essas duas doenças. Interessantemente, a principal droga utilizada no tratamento da DM2, a metformina, parece não apresentar efeitos benéficos contra a NASH, entretanto moduladores de GLP-1, tiazolidinedionas (pioglitazona e rosiglitazona) e inibidores de SGLT-2 apresentam resultados interessantes (FERGUSON; FINCK, 2021). Os tratamentos de diabéticos com agonistas dos receptores de GLP-1 liragluteide e semaglutide foram eficientes em reduzir os níveis de gordura no fígado dos participantes, com redução também em marcadores de inflamação e dano hepático (ARMSTRONG et al., 2016; NEWSOME et al., 2019). Tiazolidinedionas parecem aumentar a captação de triglicerídeos pelo tecido adiposo e potencializar a ação antilipolítica da insulina, reduzindo assim os níveis de ácidos graxos livres circulantes e a oferta de substrato para o acúmulo de lipídios hepáticos, atuando no tecido adiposo aumentando a ação da proteína lipogênica *Peroxisome proliferator-activated receptor γ* (PPARγ) (GROSS et al., 2017; MAYERSON et al., 2002). Coerentemente, uma importante meta-análise incluindo somente estudos clínicos randomizados e controlados com grupo placebo concluiu que as tiazolidinedionas reduzem a inflamação e a esteatose hepática no tratamento da NASH (BOETTCHER et al., 2012). O tratamento de

diabéticos com inibidores de SGLT-2 os lipídios hepáticos foram reduzidos, aumentando a sensibilizade hepática à inslina, a secreção de insulina e reduzindo o peso corporal (CUSI et al., 2019).

Ainda sobre o tratamento farmacológico contra a DHGNA, alguns inibidores dos intermediários da lipogênese *de novo* hepática também já foram desenvolvidos, e embora alguns deles já sejam estudados em humanos, ainda são considerados como experimentais. A inibição farmacológica da ACC reduziu a lipogênese hepática em roedores de modo dose-dependente, porém aumentou os níveis de triglicerídeos plasmáticos em 200% (KIM et al., 2017). Já a inibição de FAS apresenta resultados promissores, reduzindo a lipogênese *de novo* em até 90% e reduzindo marcadores de dano hepático em obesos após 10 dias de tratamento, sem alterar a trigliceridemia (SYED-ABDUL et al., 2020). Similarmente, a inibição de SCD-1 também reduziu a gordura no fígado de modo dose-dependente após 3 meses de uso (SAFADI et al., 2014). Nesse estudo, os pacientes que receberam 300mg/dia do composto Aramchol apresentaram 12,57% de redução dos lipídios hepáticos e sem eventos adversos, enquanto o grupo placebo apresentou aumento de 6,39%. Por fim, o uso de ASO para a inibição de DGAT-2 hepática também reduziu os níveis hepáticos em pacientes com DM2 e DHGNA, mesmo sem alterar o perfil lipídico e glicêmico, e também sem sérios efeitos adversos (LOOMBA et al., 2020).

Entretanto, diversos efeitos adversos ainda impedem a expansão do uso desses fármacos. Quanto aos agonistas do receptor de GLP-1, apesar dos importantes resultados apresentados na redução dos marcadores de NASH, parece que esses benefícios são alcançados por mecanismos indiretos relacionados à redução da adiposidade e melhora metabólica sistêmica, uma vez que os receptores de GLP-1 são pouco expressos no fígado (MÜLLER et al., 2019). Já uma meta-análise concluiu que a rosiglitazona está associada ao aumento do risco de morte por complicações cardiovasculares e risco de infarto do miocárdio, sendo assim pouco usada em pacientes diabéticos (NISSEN; WOLSKI, 2007). E assim como os receptores de GLP-1, SLGT-2 não é expresso no fígado (HEERSPINK et al., 2016), com seus efeitos hepáticos também acontecendo de modo indireto. Porém, os inibidores de SLGT-2 parecem apresentar maior risco de amputação de membros e de ocorrência de cetoacidose em diabéticos, em comparação ao tratamento com os agonistas dos receptores de GLP-1 (UEDA et al., 2018). Dessa forma, estratégias relacionadas a alterações no estilo de vida que promovem redução da adiposidade

e aumento do gasto energético diário continuam sendo consideradas as estratégias primárias no combate à DHGNA (BACCHI et al., 2013; FRANCO et al., 2019; TILG; MOSCHEN; RODEN, 2017).

2.3 CAPÍTULO 3: O EXERCÍCIO FÍSICO COMO ALIADO NO COMBATE À DHGNA E A PERDA DO CONTROLE DA PHG

Apesar dos importantes avanços na área da saúde, alterações no estilo de vida continuam sendo a intervenção primaria no tratamento da DHGNA e no aumento da sensibilidade hepática à insulina e melhor controle da PHG. Dessa forma, a aderência a um programa de exercícios mostra-se como uma excelente estratégia, uma vez que é largamente demonstrado que o exercício físico é eficiente em proporcionar tanto redução da adiposidade corporal (SARGEANT et al., 2018b) quanto do consumo energético (RODRIGUES et al., 2018; VATANSEVER-OZEN et al., 2011), proporcionando também redução do acúmulo excessivo de gordura hepática (PEREIRA et al., 2019; SARGEANT et al., 2018b), melhor controle da PHG (PEREIRA et al., 2017b, 2019) e melhora na homeostase glicêmica (PEREIRA et al., 2020; SARGEANT et al., 2018a).

Tratando-se do exercício aeróbio, é relativamente maior o número de evidências mostrando seus benefícios contra a DHGNA e resistência hepática à insulina. Recentemente, duas meta-análises demonstraram que o treinamento aeróbio é eficiente em reduzir diversos marcadores de DHGNA e de dano hepático induzidos pela obesidade em humanos (SARGEANT et al., 2018b; ZOU et al., 2018). Similarmente, Abdelbasset e colaboradores demonstraram que 8 semanas de treinamento aeróbio de alta intensidade combinado ao tratamento farmacológico contra DHGNA em obesos diabéticos proporcionou benefícios mais robustos quando comparados ao tratamento farmacológico realizado de modo isolado (ABDELBASSET et al., 2019). Logo, toda a maquinaria biomolecular de lipogênese hepática também é reduzida com a prática dessa modalidade de exercícios (CHO et al., 2014; VAN DER WINDT et al., 2017; YU et al., 2019). Em seu estudo, Yasari e colaboradores observaram que ratos tanto magros quanto obesos reduzem o conteúdo proteico de SCD-1 após 8 semanas de treinamento aeróbio em esteira (YASARI et al., 2010). Camundongos obesos submetidos a um protocolo de treinamento de natação por 10 semanas apresentaram o mesmo resultado, com redução também na expressão tanto *Scd1* quanto *Fasn* (WU et al., 2015). Kalaki-Jouybari e colaboradores também observaram consistentes reduções na expressão de *Fasn* e *Acc* no fígado de ratos obesos que realizaram treinamento aeróbio de alta

intensidade durante 8 semanas (KALAKI-JOUYBARI et al., 2018). Por fim, 8 semanas de treinamento em esteira proporcionaram aumento na inibição da atividade da proteína lipogênica ACC no fígado de camundongos obesos (CHO et al., 2014). Consequentemente, estudos prévios também demonstram que o exercício e o treinamento aeróbio são eficientes em aumentar a ação hepática da insulina e mitigar as disfunções hepática tanto em modelo animal (MARCINKO et al., 2015; MUÑOZ et al., 2018a) quanto em humanos (DONG et al., 2016; SHAH et al., 2009). Camundongos obesos apresentaram redução da PHG após 6 semanas de treinamento aeróbio de alta intensidade (MARCINKO et al., 2015). Similarmente, 6 meses de um programa de treinamento aeróbio aliado a orientações dietéticas proporcionou redução no perfil lipídico e marcadores de dano hepático em obesos, com aumento da sensibilidade hepática à insulina (SHAH et al., 2009). Um dos mecanismos propostos pelo qual o treinamento aeróbio contribui para esse melhor controle da PHG no estado obeso está relacionado à redução do conteúdo proteico de PC, inibindo assim os passos iniciais da gliconeogênese (MUÑOZ et al., 2018b). Porém, demais estudos com roedores também demonstram que o treinamento aeróbio atua em pontos mais distais no processo de gliconeogênese. Camundongos obesos que realizaram natação por 8 semanas apresentaram aumento da fosforilação de FOXO1 hepática, reduzindo assim sua capacidade de transcrever genes gliconeogênicos e culminando em reduções no conteúdo proteico de PEPCK e G6Pase (MARINHO et al., 2012b). Resultados semelhantes foram encontrados por Chang e colaboradores, que também observaram redução de PEPCK no fígado de ratos Zucker obesos treinados, com redução também da hiperglicemia induzida pela obesidade (CHANG et al., 2006).

Embora seja mais recente o interesse da comunidade científica sobre os efeitos metabólicos do exercício de força, já existem importantes achados sobre os benefícios hepáticos proporcionados por essa modalidade. Em 2013, o primeiro estudo randomizado controlado mostrou que indivíduos diabéticos apresentavam redução do acúmulo de gordura hepática após 4 meses de treinamento de força, de modo similar à redução apresentada pelo grupo que realizou treinamento aeróbio (BACCHI et al., 2013). Posteriormente, Shamsoddini e colaboradores observaram que 2 meses de treinamento de força eram capazes de reduzir os níveis circulates de alanina aminotransferase e aspartate aminotransferase (SHAMSODDINI et al., 2015). Botezelli e colaboradores demonstraram que o treinamento de força é mais

eficiente do que o treinamento aeróbio em reduzir o acúmulo de gordura hepática e inflamação tecidual induzidas por dieta rica em frutose em roedores (BOTEZELLI et al., 2016). Por fim, uma meta-análise concluiu que o treinamento de força pode ser também uma eficiente estratégia na redução do excesso de gordura hepática (MEDRANO et al., 2018). Recentemente, Pereira e colaboradores mostraram que camundongos obesos que realizaram somente 15 sessões de treinamento de força já apresentavam redução na maquinaria de lipogênese hepática, com redução no conteúdo de mRNA de genes lipogênicos *Fasn* e *Scd1*, aumento do mRNA de genes oxidativos *Cpt1* e *Ppara*, redução do conteúdo proteico de FAS e ACC e redução da inflamação hepática (PEREIRA et al., 2019). Dessa forma, os animais apresentaram aumento da fosforilação da Akt após estímulo intraperitoneal de insulina e melhor controle da PHG durante o teste de tolerância ao piruvato. Interessantemente, esse protocolo de treinamento de curta duração não proporcionou redução na massa corporal e na adiposidade dos animais, concluindo assim que os efeitos proporcionados pelo treinamento de força no metabolismo hepático são proporcionados efetivamente pela prática da modalidade, e não como um efeito secundário à redução da massa adiposa. Em outro estudo, animais obesos submetidos ao mesmo protocolo de treinamento também apresentaram redução do conteúdo hepático de PC, trazendo mais evidências de que o treinamento de força pode também ser uma eficiente estratégia para o controle da PHG por meio da inibição da maquinaria de gliconeogênese hepática (PEREIRA et al., 2020). Similarmente, mulheres idosas que realizaram treinamento de força por 4 meses apresentaram redução da produção endógena de glicose após estímulo com insulina (HONKA et al., 2016), evidenciando assim o treinamento de força como uma eficiente estratégia no combate tanto à DHGNA quanto à perda do controle da PHG.

Entretanto, é crescente o número de estudos propondo a combinação de exercícios tanto de caráter aeróbio quanto de força para a promoção de saúde (AMERICAN COLLEGE OF SPORTS MEDICINE, 2009; GARBER et al., 2011; PEREIRA et al., 2017b), dando assim origem à modalidade de treinamento conhecida como treinamento combinado. Apesar de sabido que essa modalidade de treinamento proporciona diversos benefícios metabólicos na condição da obesidade como redução da hiperglicemia e hiperinsulinemia de jejum (MEDEIROS et al., 2015), redução dos níveis de leptina e aumento dos níveis de adiponectina (BHARATH et al., 2018), os efeitos relacionados ao metabolismo hepático continuam pouco

explorados. Em um estudo pioneiro, Antunes e colaboradores observaram que 20 semanas de treinamento combinado reduziu a adiposidade corporal, esteatose hepática, colesterol total e LDL-c em adolescentes obesos (ANTUNES et al., 2013). Posteriormente, foi demonstrado que 10 semanas de treinamento combinado é eficiente em reduzir a gordura hepática em mulheres diabéticas, culminando assim em reduções nos níveis de glicemia e insulinemia de jejum (BANITALEBI et al., 2019). No já citado estudo com roedores conduzido por Botezelli e colaboradores, foi demonstrado que 8 semanas de treinamento combinado são eficientes para prevenir as complicações metabólicas induzidas por dieta rica em frutose, reduzindo a hiperglicemia, hiperinsulinemia, resistência à insulina e intolerância à glicose, além de proteger os animais de ganhos excessivos de gordura hepática e reduzir a inflamação nesse tecido (BOTEZELLI et al., 2016). Recentemente, foi demonstrado que o treinamento combinado de curta duração reverte a resistência hepática à insulina em camundongos obesos, aumentando assim a fosforilação da Akt após estímulo de insulina e reduzindo a hiperglicemia de jejum (CAMPOS et al., 2020). Entretanto, o conhecimento detalhado de como as diferentes modalidades de treinamento proporcionam seus efeitos metabólicos reduzindo o acúmulo excessivo de gordura hepática, aumentando a ação hepática à insulina e auxiliando no controle da PHG continuam limitados.

2.4 CAPÍTULO 4: REDUÇÃO DA AÇÃO HEPÁTICA DA INSULINA MEDIADA POR PTP1B INDUZIDA POR INFLAMAÇÃO: MAIS UMA PROTEÍNA SENSÍVEL AO EXERCÍCIO FÍSICO

A fosforilação proteica em resíduos de tirosina é um mecanismo biomolecular pós-traducional indispensável para o controle da homeostase glicêmica e metabólica, sendo esse um processo minuciosamente regulado por Proteínas Tirosina Quinase (PTKs) e Proteínas Tirosina Fosfatase (PTPs) (TONKS, 2006). Após o sequenciamento do genoma humano aproximadamente 100 proteínas foram identificadas como pertencentes à grande família de PTPs (CHEN et al., 2015), e dentre elas, destaca-se a *Protein-tyrosine phosphatase* 1B (PTP1B), que dentre outras funções tem a habilidade de regular negativamente a ação da insulina em tecidos que regulam o metabolismo da glicose como fígado, músculo esquelético e tecido adiposo (BAKKE; HAJ, 2015; TIGANIS, 2013). Trata-se de uma abundante fosfatase não-receptora intracelular e codificada pelo gene *PTPN1,* inicialmente purificada na placenta humana ainda no fim da década de 80 (CHARBONNEAU et al., 1989). Conforme já descrito, a insulina exerce seus efeitos no fígado promovendo a fosforilação principalmente em sítios de tirosina do seu receptor IR e dos seus substratos, os IRSs. Nesse contexto, a contra regulação da via da insulina mediada por PTP1B dá-se justamente por sua função de fosfatase sobre IR e IRS1/2 (KOREN; FANTUS, 2007; TIGANIS, 2013). Portanto, a remoção dos fosfatos nos resíduos de tirosina dessas proteínas envolvidas com a transdução do sinal da insulina pela PTP1B a coloca como um importante alvo terapêutico para o combate a resistência à insulina e desenvolvimento do diabetes do tipo 2 (T2DM) (CHO, 2013; FERBER, 1999).

Recentemente, foi demonstrado que a administração oral crônica de inibidores de PTP1B aumenta a sensibilidade hepática à insulina e reduz a hiperglicemia em diabetic BKS db mice (LI et al., 2019). Similarmente, liver-specific PTP1B$^{-/-}$ mice apresentaram increased hepatic insulin signaling e maior supressão da HGP pela insulina, mesmo sem alterações de adiposidade (DELIBEGOVIC et al., 2009b). O knockdown de PTP1B induzido por tamoxifeno no fígado de camundongos obesos adultos também melhorou não só a tolerância à glicose como também promoveu melhor controle da HGP durante o teste intraperitoneal de tolerância ao

piruvato (ipPTT), proporcionando melhora no perfil lipídico e redução dos níveis de triglicérides hepáticos (OWEN et al., 2013). E quanto à DHGNA, a PTP1B parece também ter crucial importância no desenvolvimento da doença, regulando não só a resistência à insulina mas também a lipogênese (CHEN et al., 2015; SHIMIZU et al., 2003). De modo geral, PTP1B vem sendo descrita como uma proteína capaz de ativar o processo de lipogênese hepática. Em roedores, diferentes modelos de indução de DHGNA apresentam aumento nos níveis hepáticos de PTP1B, tanto alimentados com dieta rica em gordura saturada e rica em frutose (TAGHIBIGLOU et al., 2002; ZABOLOTNY et al., 2008) quanto em modelos com obesidade induzida por alterações genéticas (ZABOLOTNY et al., 2008). E um dos mecanismos propostos pelos quais a PTP1B pode regular o acúmulo de gordura hepática está relacionado ao controle do conteúdo de SREBP-1c. Em seu estudo, Waring e colaboradores observaram que o tratamento de roedores diabéticos *ob/ob* e *db/db* com PTP1B oligonucleotídio antisense reduziu a expressão de SREBP-1c, culminando em ocorrência de esteatose hepática (WARING et al., 2003). Similarmente, a deleção de PTP1B especificamente no fígado de roedores também culminou em níveis reduzidos de SREBP-1c no fígado dos animais após a exposição à dieta rica em gordura saturada, com redução também nos níveis das proteínas lipogênicas ACC e FAS, resultados ainda acompanhados de reduções nos níveis de triglicerídeos e colesterol hepático (AGOUNI et al., 2011; DELIBEGOVIC et al., 2009a; OWEN et al., 2013). Por outro lado, a superexpressão do gene de PTP1B culminou em aumento também na maior expressão de SREBP-1c, tornando os animais hipertrigliceridêmicos e hiperglicêmicos (UGI et al., 2009). Assim, é proposto que PTP1B pode se associar a região *promoter* do gene de SREBP-1c, potencializando sua atividade transcricional, que vai culminar em um aumento no conteúdo proteico de proteínas lipogênicas e proporcionando mais acúmulo de gordura hepática (CHEN et al., 2015).

É também sabido que os níveis hepáticos de PTP1B são afetados pela prática de exercício físico. Somente uma sessão de natação foi capaz de reduzir os níveis de PTP1B hepática de ratos idosos, aumentando assim os níveis de ativação da Akt em resposta à insulina e redução da gliconeogênese (DE MOURA et al., 2013). Similarmente, o treinamento de 8 semanas em esteira reduziu PTP1B no fígado de ratos obesos (PASSOS et al., 2015). Por fim, mostramos em nosso laboratório que o treinamento de força de curta duração reduz o conteúdo total de

PTP1B no fígado de camundongos obesos, mesmo sem alteração na adiposidade (DA CRUZ RODRIGUES et al., 2021). E recentemente, foi demonstrado que 12 semanas de treinamento de força reduz também a atividade de PTP1B hepática de ratos obesos (VIVERO et al., 2020). Dessa forma, o melhor entendimento sobre os efeitos de diferentes modalidades de treinamento sobre a expressão e atividade de PTP1B hepática pode fornecer importantes informações para a proposta de estratégias para o tratamento da perda da PHG e gênese da DM2 e DHGNA associadas à obesidade.

2.5 CAPÍTULO 5: CLUSTERINA: UM POSSÍVEL LINK ENTRE O EXERCÍCIO E A REDUÇÃO DA RESISTÊNCIA HEPÁTICA SELETIVA À INSULINA

Inicialmente descrita por Blaschuk e colaboradores (BLASCHUK; BURDZY; FRITZ, 1983), uma proteína conhecido como apolipoproteína J ou clusterina vem chamando a atenção por sua capacidade de regular diversas funções metabólicas (ARONIS; KIM; MANTZOROS, 2011; KWON et al., 2014). Presente na maioria dos fluídos corporais, sua isoforma predominante trata-se de uma glicoproteína secretada na forma de um heterodímero de 75-80 kDa de peso molecular, composta por duas cadeias de monômeros nomeados de alpha e beta unidas por cinco pontes de dissulfeto (RIZZI; COLETTA; BETTUZZI, 2009). Seu gene codificador mostra-se altamente conservado entre os mamíferos, sugerindo a proteína como fator fundamental para a evolução, sendo sintetizada na maioria dos tecidos (PARK; MATHIS; LEE, 2014). Em humanos, um gene de cópia simples de nove éxons com mais de 16 kb e localizado no cromossomo 8p21–p12 codifica seu RNA mensageiro (mRNA) de aproximadamente 2kb, que por sua vez sintetizará sua cadeia primária polipeptídica de 449 aminoácidos (JONES; JOMARY, 2002). A clusterina é conhecida por participar de diversos processos, como transporte de lipídeos, maturação de esperma, apoptose, processo inflamatório, aterosclerose e câncer e diabetes mellitus (PARK; MATHIS; LEE, 2014; TROUGAKOS; GONOS, 2002). Porém, por mecanismos ainda desconhecidos, a clusterina pode escapar de sua via de secreção e ser encontrada no citosol, originando sua isoforma citoplasmática (TROUGAKOS, 2013). E nos últimos anos, diversos estudos surgem trazendo a ideia de que a clusterina secretada pode estar relacionada à resistência à insulina e DM2 (ARONIS; KIM; MANTZOROS, 2011; SEO et al., 2018). Porém, os mecanismos biomoleculares pelos quais ela atua nesse contexto ainda são muito pouco explorados.

É crescente o número de evidências sugerindo a clusterina secretada como um importante componente no controle da resistência à insulina e no desenvolvimento de doenças metabólicas. Inicialmente, Trougakos e colaboradores em 2002 observaram que os níveis séricos de clusterina são aumentados com o avançar da idade, e que indivíduos com DM2 também apresentam esse aumento em relação à indivíduos saudáveis (TROUGAKOS et al., 2002). Posteriormente, esses

resultados foram confirmados por Kujiraoka e colaboradores, que observaram que o aumento de clusterina secretada associado ao DM2 ocorre em ambos os sexos, sendo esse um mecanismo compensatório frente à uma condição metabolicamente adversa (KUJIRAOKA et al., 2006). Ainda, Won e colaboradores em 2014 encontraram uma correlação positiva entre os níveis séricos de clusterina e diversos marcadores de inflamação sistêmica (WON et al., 2014). E recentemente, Seo e colaboradores observaram uma correlação positiva dos níveis de clusterina circulante com insulinemia, glicemia de jejum, resistência à insulina e índice de massa corporal (IMC) (SEO et al., 2018). Por outro lado, em 2010 foi observada uma correlação negativa entre a associação da clusterina ao HDL e IMC e resistência à insulina (HOOFNAGLE et al., 2010). Dessa forma, apesar de os níveis séricos de clusterina estarem aumentados na obesidade e no DM2, algumas de suas funções parecem estar comprometidas, de modo muito semelhante ao que acontece com os hormônios insulina e leptina (MYERS et al., 2010; TEMPLEMAN et al., 2017), originando um estado que podemos chamar de “resistência à clusterina”. Por fim, um estudo investigando polimorfismos no gene da clusterina observou que mutações em apenas um nucleotídeo já apresentava uma forte correlação com a presença de DM2 em japoneses (DAIMON et al., 2011), exaltando o potencial envolvimento da clusterina na manutenção dos níveis glicêmicos saudáveis.

Entretanto, pouco se sabe ainda sobre os mecanismos pelos quais a clusterina está envolvida com o controle metabólico e com a homeostase glicêmica. Um dos principais estudos nesse contexto foi conduzido em 2014 por Kwon e colaboradores, em que os autores mostraram que animais obesos que não expressam clusterina possuem resistência à insulina mais severa frente aos seus controles selvagens, mesmo sem alteração na composição corporal (KWON et al., 2014). Após 8 semanas de exposição à dieta rica em gordura saturada (HFD) , os animais que não expressavam clusterina apresentaram elevação do estresse oxidativo e da inflamação no músculo esquelético e no fígado (KWON et al., 2014). Coerentemente, hepatócitos e miotubos que superexpressavam clusterina ficaram protegidos do danos proporcionados pelo tratamento com palmitato (KWON et al., 2014). Os animais selvagem tiveram ainda a expressão de clusterina aumentada nesses dois tecidos quando alimentados com HFD, reforçando a hipótese do papel protetor da clusterina sobre a resistência à insulina induzida por dieta (KWON et al., 2014). Resultados semelhantes foram encontrados no tecido adiposo subcutâneo,

com os níveis de mRNA de clusterina aumentados em indivíduos obesos e com DM2 (KLOUČKOVÁ et al., 2016). Interessantemente, a expressão de clusterina nesse tecido foi reduzida após cirurgia bariátrica. Todavia, esse estudo não observou diferença nos níveis circulantes de clusterina entre indivíduos obesos e magros, porém seus níveis foram reduzidos após 2 semanas de dieta com alto déficit calórico (KLOUČKOVÁ et al., 2016).

Entretanto, a ideia de que a clusterina citoplasmática hepática pode apresentar um papel importante no controle das funções metabólicas do fígado é bastante recente. Esta teoria foi iniciada ainda nessa década, quando Kim e colaboradores em 2011 observaram que o tratamento de hepatócitos primários de camundongos com altas doses de glicose proporcionava o aumento tanto da expressão quanto dos níveis proteicos de diferentes formas de clusterina (KIM et al., 2011). Analisando o sequenciamento de seu gene, foi encontrado um *Glucose response element* (GlRE) constituído por dois *E-box motifs* em sua primeira região intrônica que se assemelham a um *Carbohydrate response element* (ChoRE). Entretanto, curiosamente, esses *E-box motifs* foram ativados somente pelo fator de transcrição SREBP-1c e não por *ChoRE binding protein* (ChREBP) *e Liver X receptor* (LXR), conhecidos por mediar sinalizações biomoleculares em resposta a nutrientes. Dois anos depois, em 2013, Seo e colaboradores observaram que a clusterina podia exercer um papel regulador sobre a atividade da SREBP-1c em resposta à insulina, uma vez que hepatócitos AML-12 que superexpressavam clusterina apresentavam uma menor quantidade de SREBP-1c em resposta à insulina (SEO et al., 2013). Consequentemente, proteínas envolvidas com o processo de lipogênese FAS, ACC e SCD1 apresentaram o mesmo comportamento. Esse fenômeno também foi observado em roedores que superexpressavam clusterina no tecido hepático, ficando protegidos do excessivo acúmulo de gordura no fígado após a exposição crônica à dieta rica em gordura saturada (SEO et al., 2013). Por fim, foi visto que clusterina inibia a expressão de SREBP-1c reprimindo a atividade de LXR e *Specificity Protein 1* (SP-1) (SEO et al., 2013), proteínas conhecidas por associarem-se à sequência promotora no gene de SREBP-1c e proporcionarem a plena ativação de sua transcrição em resposta à insulina (CAGEN et al., 2005). Juntos, esses dados nos permitem acreditar que em uma situação de abundância de nutrientes, SREBP-1c tem sua atividade aumentada, induzindo assim tanto a expressão de *CLU* quanto de genes lipogênicos. Entretanto, por um mecanismo de feedback negativo,

clusterina inibe a atividade de LXR e SP-1, culminando em um menor estímulo lipogênico mediado por SREBP-1c. Este trata-se de um novo mecanismo pelo qual a clusterina hepática apresenta um importante papel na redução da lipogênese hepática e, consequentemente, da resistência à insulina nesse tecido, sendo assim um importante componente no controle metabólico geral.

Adiante, em 2015, foi constatado que o tratamento com insulina era capaz de promover o aumento dos transcritos de clusterina tanto em células HepG2 quanto em hepatócitos primários de camundongos (OH et al., 2015), dado este que corrobora achados mais antigos que revelam que sujeitos diabéticos do tipo 2 apresentam níveis séricos de clusterina mais elevados (KUJIRAOKA et al., 2006). Esse controle da transcrição do gene da clusterina regulado por insulina parece ainda ser mediado por SREBP-1c, que associa-se a um *non-canonical E-box* no promotor de *CLU* em resposta à insulina (OH et al., 2015). Portanto, uma vez observado que um dos mais importantes hormônios para o controle do metabolismo de macronutrientes também era capaz de modular a expressão de clusterina no tecido hepático, foi reforçada a hipótese de que essa nova proteína era capaz de exercer diversas funções relacionadas ao controle da homeostase glicêmica por influenciar as funções metabólicas do fígado.

Nesse mesmo sentido, recentemente Park e colaboradores publicaram um estudo em que foram utilizados roedores que superexpressam clusterina especificamente em hepatócitos (PARK et al., 2018). Após exposição crônica à dieta pobre em metionina e colina, conhecida por induzir doença hepática gordurosa não-alcoólica (DHGNA) e esteato-hepatite não alcoólica (NASH), foi visto que os animais modificados apresentaram reduções nos níveis de triglicerídeos hepáticos e marcadores de inflamação, como infiltração de macrófagos, expressão de *Toll-like Receptor-4* (TLR-4) e níveis de *Tumor Necrosis Factor-α* (TNF-α). Esses dados confirmam achados prévios do já comentado estudo de Kwon e colaboradores, em que os animais *knockout* de clusterina apresentam uma inflamação hepática mais severa após serem alimentados com HFD por 8 semanas, com níveis elevados da isoforma induzida da proteína *Nitric oxide synthase* (iNOS) e de Interleukin-6 (IL-6) (KWON et al., 2014). Da mesma forma, células de cultura primária de hepatócitos desses animais apresentavam aumento também na expressão de *Interleukin-1β* (IL-1β) e TNF-α quando tratadas com palmitato, refletindo em uma menor fosforilação da Akt em resposta à insulina, em comparação às células controle submetidas ao

mesmo tratamento. Apesar desses animais apresentarem maior expressão dos genes relacionados à gliconeogênese *Fructose-1,6-bisphosphatase* (FBPase) e *Glucose 6-phosphatase* (G6Pase) e demonstrarem uma menor supressão da produção hepática de glicose durante o clamp euglicêmico-hiperinsulinêmico, os mecanismos biomoleculares envolvidos nesse processo não foram explorados. Assim, ainda não é claro na literatura se esses resultados estão relacionados à inflamação gerada pela ausência da clusterina, que conduziria uma menor ação da insulina, ou se a ausência da clusterina per se compromete diretamente a inibição da produção hepática de glicose mediada pela insulina.

Ainda, um importante achado do estudo de Park e colaboradores é que apesar de a constitutiva expressão de clusterina em hepatócitos proteger os animais da NASH induzida por dieta, um pequeno aumento na infiltração de macrófagos e inflamação é visto no fígado dos animais alimentados com dieta comercial (PARK et al., 2018). Esse é um resultado que vai ao encontro de dados prévios encontrados pelo mesmo grupo de pesquisadores que, apesar das já bem-descritas funções protetoras da clusterina, viram que macrófagos Raw264.7 apresentavam um aumento na expressão de diversos genes relacionados com quimiotaxia como *monocyte chemotactic protein-1* (MCP-1), *macrophage inflammatory protein-1β* (MIP-1β) e TNF-α após estímulo com clusterina (SHIM et al., 2012). Ainda, recentemente também foi demonstrado que a ativação do sistema imune proporcionado pela clusterina e a indução do processo inflamatório dá-se por mecanismos semelhantes a lipopolissacarídeos (LPS), dependentes de TLR-4 (SHIM et al., 2017). Todavia, como a clusterina mostrou uma consistente proteção hepática, com efeitos anti-oxidantes e anti-inflamatórios, podemos considerá-la como um regulador de pré-condicionamento imunológico (PARK et al., 2018). Esse processo de pré-condicionamento já é um conhecido fenômeno desde a década de 40, quando foi observado que coelhos desenvolviam uma resistência à infecções bacterianas após constantes injeções de vacinas contendo *E. typhosa* inativa (BEESON, 1946). E recentemente, Nakasone e colaboradores mostraram que o tratamento de camundongos com baixas doses de LPS protegeu o fígado desses animais de danos proporcionados por uma posterior injeção de alta dose de LPS (NAKASONE et al., 2016).

Ademais, como já comentado anteriormente, diversas evidências mostram que a clusterina parece ter a capacidade de promover a ativação da proteína Akt,

sendo assim uma importante proteína sinalizadora para a sobrevivência celular (LIU et al., 2015; WANG et al., 2015a; XIU et al., 2013). Em 2013, Xiu e colaboradores (XIU et al., 2013) observaram que após a superexpressão de clusterina em células de hepatocarcinoma (HCC) os níveis de Akt em sua forma ativa foram elevados, proporcionando uma diminuição na apoptose induzida pelo tratamento anticâncer. Da mesma forma, o mesmo estudo revelou que a depleção da clusterina diminuiu a fosforilação da Akt e aumentou os níveis de caspase 9 clivada, conhecido marcador da ativação da via mitocondrial de apoptose (CZABOTAR et al., 2014). Entretanto, apesar de os estudos supracitados não deixarem claro que a clusterina pode promover diretamente a ativação de proteínas da via da insulina em hepatócitos, um recente estudo publicado por Liu e colaboradores revelou que células HPASMCs, quando tratadas com clusterina recombinante, apresentavam um aumento na fosforilação tanto de Akt quanto de *Extracellular signal–regulated kinases 1/2* (ERK 1/2), de modo tempo-dependente (LIU et al., 2015). Portanto, acreditamos que a clusterina pode estar diretamente envolvida com a ativação de proteínas-chave para o controle da produção hepática de glicose por mecanismos independentes da insulina.

Já é bem estabelecido pela literatura científica que o fígado é um órgão de grande importância para o controle dos níveis glicêmicos considerados saudáveis (TILG; MOSCHEN; RODEN, 2017). Para que isso ocorra, a insulina secretada, em períodos de abundância de nutrientes, precisa agir nesse tecido suprimindo a produção hepática de glicose (WANG et al., 2015b). Como já discutido, em uma situação em que a resistência hepática à insulina não está presente, a insulina irá promover a ativação da Akt que, dentre outras funções, promoverá a fosforilação de FOXO para a inibição da transcrição de genes da gliconeogênese e a ativação de SREBP-1c, promovendo a transcrição de genes lipogênicos como FAS e ACC (SANDERS; GRIFFIN, 2016). Entretanto, indivíduos resistentes à insulina apresentam redução na supressão da produção hepática de glicose estimulada por insulina, mas também mantém as vias lipogênicas ativas, apresnetando assim a resistência hepática seletiva à insulina previamente descrita. Dessa forma, sabendo que o treinamento físico é uma eficiente estratégia tanto contra a DHGNA (BACCHI et al., 2013; PEREIRA et al., 2017a) quanto para a resistência hepática à insulina (SARGEANT et al., 2018a), aventa-se a hipótese que um dos mecanismos pelos quais o exercício físico pode ser um eficiente meio para o combate à esse fenômeno

está envolvido com a modulação da clusterina hepática citoplasmática. Recentemente, Jeon e colaboradores publicaram um importante estudo com informações que reforçam essa hipótese. Nele, mulheres menopausadas com DM2 participaram de um programa de exercícios durante 12 semanas. Após o protocolo de treinamento, os autores observaram que a elevação dos níveis circulantes de clusterina foi reduzida em 19,4%, concomitantemente a reduções da adiposidade e da resistência à insulina (JEON et al., 2020). Essa foi a primeira evidência de que o treinamento físico é capaz de modular os níveis de clusterina secretada, porém os efeitos do treinamento sobre a isoforma citoplasmática de clusterina hepática continuam inexplorados.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Uma vez que a resistência hepática à insulina e a DHGNA estão intimamente ligadas às complicações metabólicas relacionadas à obesidade e a DM2, o presente trabalho teve como objetivo investigar novos mecanismos pelos quais diferentes modalidades de treinamento físico proporciona aumento da ação da insulina e redução do acúmulo de gordura hepática em roedores.

3.2 OBJETIVOS ESPECÍFICOS

1. Investigar os efeitos do treinamento de força de curta duração sobre o controle da produção hepática de glicose e sensibilidade hepática à insulina de camundongos obesosm, avaliando a resposta glicêmica dos animais após administração intraperitoneal de piruvato e a ativação da Akt após estímulo de insulina;
2. Avaliar os efeitos do treinamento de força de curta duração sobre as vias de lipogênese e oxidação lipídica no fígado de camundongos obesos, analisando o conteúdo e atividade de proteínas lipogênicas e o conteúdo de transcritos de genes envolvidos com o metabolismo lipídico;
3. Investigar se o maior controle da produção hepática de glicose proporcionando pelo treinamento de força de curta duração está relacionado à redução da atividade da FOXO1 hepática, avaliando a sua sublocalização celular e o conteúdo proteico de seus transcritos PEPCK e G6Pase;
4. Investigar os efeitos do treinamento de força sobre os níveis hepáticos da proteína PC;
5. Investigar se o aumento da sensibilidade hepática à insulina proporcionado pelo treinamento de força de curta duração está relacionado a alterações no conteúdo hepático de PTP1B;
6. Investigar os efeitos do treinamento combinado de curta duração sobre o controle da produção hepática de glicose e sensibilidade hepática à insulina de camundongos obesos;

7. Avaliar os efeitos do treinamento combinado de curta duração sobre as vias de lipogênese e oxidação lipídica no fígado de camundongos obesos;

8. Investigar se o aumento da sensibilidade hepática à insulina e redução da DHGNA proporcionados pelo treinamento de força de curta duração em camundongos obesos estão relacionados a alterações no conteúdo hepático de clusterina;

9. Investigar se o aumento da sensibilidade hepática à insulina e redução da DHGNA proporcionados pelo treinamento aeróbio de curta duração em camundongos obesos estão relacionados a alterações no conteúdo hepático de clusterina.

4. MATERIAIS, MÉTODOS, RESULTADOS E DISCUSSÕES

Os materiais, métodos, resultados e discussões da presente tese estão apresentados sob forma de artigos.

4.1 ARTIGO 1

Uma vez que a literatura científica ainda carecia de um protocolo de treinamento de força sistematizado e controlado para camundongos obesos, nosso primeiro trabalhou focou-se em estabelecer um protocolo de treinamento de força para camundongos alimentados com dieta rica em gordura saturada e avaliar se este protocolo é eficiente em proporcionar redução do acúmulo de gordura hepática e influenciar as vias biomoleculares de síntese e oxidação de gordura no fígado dos camundongos.

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RESEARCH

Short-term strength training reduces gluconeogenesis and NAFLD in obese mice

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has a positive correlation with obesity, insulin resistance and type 2 diabetes mellitus (T2D). The aerobic training is an important tool in combating NAFLD. However, no studies have demonstrated the molecular effects of short-term strength training on the accumulation of hepatic fat in obese mice. This study aimed to investigate the effects of short-term strength training on the mechanisms of oxidation and lipid synthesis in the liver of obese mice. The short duration protocol was used to avoid changing the amount of adipose tissue. Swiss mice were separated into three groups: lean control (CTL), sedentary obese (OB) and strength training obese (STO). The obese groups were fed a high-fat diet (HFD) and the STO group performed the strength training protocol 1 session/day for 15 days. The short-term strength training reduced hepatic fat accumulation, increasing hepatic insulin sensitivity and controlling hepatic glucose production. The obese animals increased the mRNA of lipogenic genes *Fasn* and *Scd1* and reduced the oxidative genes *Cpt1a* and *Ppara*. On the other hand, the STO group presented the opposite results. Finally, the obese animals presented higher levels of lipogenic proteins (ACC and FAS) and proinflammatory cytokines (TNF-α and IL-1β), but the short-term strength training was efficient in reducing this condition, regardless of body weight loss. In conclusion, there was a reduction of obesity-related hepatic lipogenesis and inflammation after short-term strength training, independent of weight loss, leading to improvements in hepatic insulin sensitivity and glycemic homeostasis in obese mice. Key points: (1) Short-term strength training (STST) reduced fat accumulation and inflammation in the liver; (2) Hepatic insulin sensitivity and HPG control were increased with STST; (3) The content and activity of ACC and content of FAS were reduced with STST; (4) STST improved hepatic fat accumulation and glycemic homeostasis; (5) STST effects were observed independently of body weight change.

Key Words

- strength training
- NAFLD
- obesity
- liver
- insulin sensitivity
- T2D

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a precondition for most common liver diseases, and it can be developed from hepatic steatosis, non-alcoholic steatohepatitis (NASH), cirrhosis, to hepatocellular carcinoma (HCC) (Tilg *et al.* 2017). Insulin resistance, hyperinsulinemia and excess of circulating lipids may contribute to both increased liver lipid synthesis and hepatic insulin resistance (Marchesini *et al.* 2001, Roden 2006). Considering the liver is one of the primary organs responsible for glycemic control (Magnusson *et al.* 1992, Basu *et al.* 2005), NAFLD development may be linked to obesity and type 2 diabetes (T2D). Therefore, the discovery of strategies for the prevention and treatment of excessive accumulation of lipids in the liver is of great importance.

Although some therapeutic interventions such as the use of peroxisome proliferator-activated receptor (PPAR)γ agonists, vitamin E or liraglutide showed some efficacy in the treatment of NAFLD (Tilg *et al.* 2017), body fat reduction is still considered the primary form of treatment of this condition (Bacchi *et al.* 2013). Thus, aerobic physical exercise is considered an effective strategy in controlling NAFLD due to the reduction of both adiposity and hepatic lipids (Pereira *et al.* 2017, Muñoz *et al.* 2018, Sargeant *et al.* 2018). On the other hand, the effects of strength exercise in the fatty liver accumulation and its consequences have not yet been deeply investigated.

In 2013, the first randomized controlled study showed that diabetic subjects who underwent four months of aerobic or strength training reduced their hepatic fat accumulation (Bacchi *et al.* 2013). In 2015, Shamsoddini *et al.* showed 2 months of aerobic and resistance exercise training were enough to find a reduction in the circulating levels of alanine aminotransferase and aspartate aminotransferase (Shamsoddini *et al.* 2015). Recently, Botezelli *et al.* showed strength training was more efficient than aerobic training in reducing the content and activation of several proteins of pro-inflammatory activity in the liver of animals with hepatic steatosis (Botezelli *et al.* 2016). Moreover, the expression of lipogenic genes such as sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase (ACC) and stearoyl CoA desaturase-1 (SCD1) was reduced in the liver of ovariectomized rats after 10 weeks of strength training (Domingos *et al.* 2012). Finally, a recent meta-analysis showed that strength training could also be an important strategy for the reduction of hepatic fat content (Medrano *et al.* 2018).

However, many studies investigating the effects of aerobic or strength training on the reduction of NAFLD were accompanied by a reduction of body fat. Therefore, it has not yet been possible to determine the direct effects of exercise on NAFLD, independently of weight loss. Thus, this study aimed to investigate the influence of short-term strength training on the hepatic mechanisms of oxidation and lipid synthesis in obesity. The short-term training protocol was prescribed in order to avoid a reduction in the volume of adipose tissue.

Methods

Experimental animals

Male Swiss mice from the Unicamp Central Animal Facility (CEMIB) at 8 weeks old were used in the present study. The animal experiments were carried out respecting the Brazilian legislation on the scientific use of animals (Law No. 11.794, of October 8, 2008). The Ethics Committee on Animal Use (CEUA) of Biological Sciences (UNICAMP-Campinas-SP, number 4406-1) accepted all experiments. Four-week-old animals were maintained individually in polyethylene cages with the enriched environment (PVC pipes were sawed in the middle generating a shelter of 10 × 10 cm of the base and 5 cm of height) and under controlled conditions of the light–darkness cycle (12/12 h). The light switched on at 06:00 and off at 18:00 h, temperature controlled at 22 ± 2°C, relative humidity maintained at 45–55% and on-site noises below 85 decibels. 100 W lamps were used during the clear period of the day (Phillips soft white light; 2700 K; 565–590 nm; 60 lux). Mice had free access to water and conventional feed.

Initially, the animals were divided into two groups: the control lean group (CTL) that were fed a chow diet and the Obesity group that were fed a high-fat diet (HFD). The high-fat diet was prepared according to the American Institute of Nutrition (AIN-93G) guidelines (Reeves *et al.* 1993), modified to contain 35% of fat (4% soy oil and 31% of lard) (Oliveira *et al.* 2015). After 14 weeks of exposure to HFD, the obese animals were equally distributed according to body weight and fasting glycemia into two groups: 1 – sedentary obese (OB), animals which remained sedentary throughout the experiment; 2 – strength training obese (STO), animals which underwent a short-term strength training.

Description of apparatus for performing the strength training for mice

A ladder of iron feet and stainless steel steps with 10 cm wide, 1.5 cm distance between the steps and an angle of

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80 degrees to the ground was used (AVS projects). The ladder is 70 cm high, and the animals performed 12 ± 1 dynamic climbing movements with each of the hind legs, as proposed by Frajacomo *et al.* (2015). At the top of the ladder, there is a 30 cm^2 chamber that serves as a shelter for the animals during the rest period, between attempts to climb during the adaptation. A loading apparatus (i.e., a conical plastic tube with approximately 7.5 cm of height and 2.5 cm of diameter) was fixed with adhesive tape across the length of the tail of the animal, where the loads were coupled.

Adaptation of animals to the apparatus

The adaptation of animals to the apparatus, proposed by Cassilhas *et al.*, was performed (Cassilhas *et al.* 2013). The procedures were carried out for five consecutive days. Before the start of the first attempt to climb and with the loading apparatus empty on its tail, the animal was kept inside the chamber at the top of the ladder for 60 s. For the first attempt at climbing, the animal was positioned on the ladder at 15 cm from the entrance of the chamber. For the second attempt, the animal was positioned 25 cm away from the chamber. For the third attempt onward, the animal was positioned at the base of the ladder, 70 cm away from the chamber. When the animal reached the chamber, an interval of 60 s was given. The attempts starting from the ladder base continued until the animal performed three successful attempts without the need for any stimulus.

Maximal voluntary carrying capacity (MVCC) determination

A test to determine the maximal voluntary carrying capacity (MVCC) proposed for rats (Hornberger & Farrar 2004, Speretta *et al.* 2016) was adapted for mice in the present study. An incremental test to identify the individual maximum load in which the animal can perform one attempt of 70 cm climbing was performed. After the fifth day of adaptation to the apparatus, the animals remained at rest for one more day until the beginning of the test. During the test, the animals started from the base of the stairs, and the attempt was considered successful when the animals climbed a distance of 70 cm. The initial series was performed with an overload of 75% of the animal's body weight, and an incremental load of 5 g was added at each further attempt to climb until the animal could no longer complete the entire course. At each successful attempt, the animal was removed from the ladder and placed in an individual cage, where it rested for 5 min until the start of the next attempt. The heaviest load in which the animal performed was considered the MVCC and was used for the prescription of the individual loads in the experiment.

Short-term strength training

Forty-eight hours after the MVCC determination, the strength training protocol was initiated. The exercise sessions consisted of 20 climbing series with an overload of 70% of the MVCC and with a rest interval of 60–90 s between sets. After completing a series, the animal was removed from the ladder and placed in an individual cage during the resting time of 60 s. The animals were exercised for five consecutive days per week, followed by 2 days of rest, until they completed 13 sessions of physical exercise. Subsequently, mice were submitted to the pyruvate tolerance test. After 24 h, the animals performed two more sessions of exercise, totaling 15 sessions, as summarized in Fig. 1 and more detailed in Supplementary Fig. 1 (see section on supplementary data given at the end of this article).

Intraperitoneal pyruvate tolerance test (ipPTT)

After an 8-hour fasting period and after the 13th exercise training session, the animals were submitted to an ipPTT (2.0 g of pyruvate/kg body weight) to estimate the hepatic glucose production (HGP). The pyruvate was injected intraperitoneally (i.p.), and the blood samples were collected at 30, 60 and 120 min from the tail for blood glucose determination. Glucose levels were determined using a glucometer (Accu-Chek; Roche Diagnostics). The results were evaluated determining the areas under the serum glucose curves (AUC) during the test by the trapezoidal method (Matthews *et al.* 1990), using Microsoft Excel.

Tissue extraction and immunoblotting analysis

After the ipPTT, all animals were submitted to other two sessions of strength exercise and were anesthetized i.p. by the injection of chloral hydrate of ketamine (50 mg/kg, Parke-Davis, Ann Arbor, MI, USA) and xylazine (20 mg/kg, Rompun, Bayer, Leverkusen) respecting an 8-h fasting period and 8 h after the last exercise session. After the verification and assurance of the corneal reflexes, mice were injected i.p. with human insulin (8 U/kg body wt Humulin-R; Lilly, Indianapolis, IN, USA) or saline.

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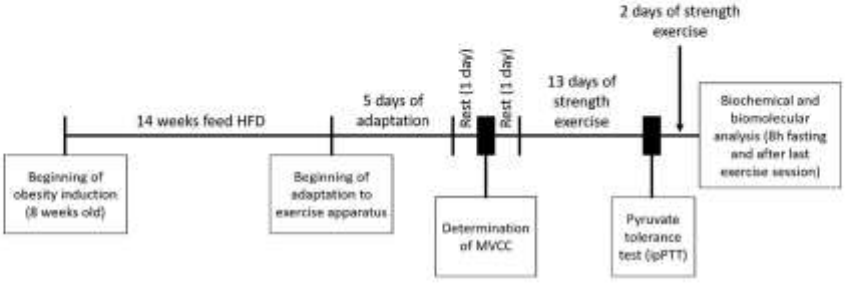

Figure 1
Experimental design. Summarized representation of the experiments during the short-term strength training protocol. The tests were performed 8 h after the exercise session respecting a period of 8-h fasting. MVCC, maximum voluntary carrying capacity.

After 10 min, the liver was rapidly removed and snap-frozen in liquid nitrogen and stored at −80°C until analysis and adipose tissue (right side) was removed and weighted. The liver was homogenized in an extraction buffer (1% Triton-X 100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/mL) at 4°C with a TissueLyser II (QIAGEN) operated at maximum speed for 120 s. The lysates were centrifuged (Eppendorf 5804R) at 12.851 ***g*** at 4°C for 15 min to remove insoluble material, and the supernatant was used for the assay. The protein content was determined by the bicinchoninic acid method (Walker 1994). The samples containing 60 µg of total protein were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk at room temperature for 1 h and incubated with primary antibodies against the protein of interest. After that, a specific secondary antibody was used. The specific bands were labeled by chemiluminescence and visualization was performed by photo documentation system in G: box (Syngene). The bands were quantified using the software UN-SCAN-IT gel 6.1. The primary antibodies used were anti-Phospho-Akt ser473 (4060), anti-Akt (4685), anti-phospho-Acetyl-CoA Carboxylase ser79 (3661), anti-acetyl-coA carboxylase (3662), anti-α-tubulin (2144) from Cell Signaling Technology, anti-fatty acid synthase (sc-48357) from Santa Cruz Biotechnology and anti-TNF- α (Cat # 506101) and IL-1β (Cat # 503501) from BioLegend. The secondary antibodies used were anti-rabbit IgG, HRP-linked antibody (7074) and anti-mouse IgG, HRP-linked antibody (7076) from Cell Signaling Technology.

Liver hematoxylin-eosin histology and oil red O staining

Liver samples were collected and fixed in isopentane for cryopreservation at −80°C. The tissue was sliced in a Leica Cryostat cryostat (CM1850) to a thickness of 10µm and placed on identified adhesion slides. The slices were subjected to the hematoxylin-eosin (H&E) and oil red O staining methods. The slices were stained with hematoxylin for 10 min or with oil red O solution (Sigma-Aldrich) for 25 min, washed and stained with eosin (5 min). The slices stained with oil red O were used to analyses of lipid droplets area and red stained area using ImageJ (Schneider *et al.* 2012) program using the 40× image, in agreement with previous studies (Botezelli *et al.* 2016, da Rocha *et al.* 2017, Muñoz *et al.* 2018).

Triglyceride assay

Hepatic triglyceride (TG) content was determined using a commercial kit according to the manufacturer's instructions (Laborlab). TG values were normalized to total liver weight.

Real-time PCR

Total RNA was isolated using the PureZOLTM reagent (BIO-RAD). A 2µg quantity of total RNA was used as a template for the synthesis of cDNA, according to the instructions of the kit (High Capacity cDNA Reverse Transcription, Applied Biosystems). Real-time PCR reactions were performed using 40 ng cDNA, 0.5 µL primers and 5 µL TaqMan Universal PCR Master Mix (Applied Biosystems). The primers used were *Fasn* (Mm00662319_m1),

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Scd1 (Mm00772290_m1), *Cpt1a* (Mm01231183), *Ppara* (Mm00440939_m1) and *Gapdh* (Mm99999915_g1). The relative content of mRNAs was determined after normalization with GAPDH using the ΔΔCT method.

Bioinformatics analysis

Correlation analyses were performed as previously described (Andreux *et al.* 2012) using hepatic mRNAs (EPFL/LISP BXD HFD Liver Affy Mouse Gene 1.0 ST (Aug18) RMA), proteome (bvEPFL/ETHZ BXD Liver, High Fat Diet (Jun16) Top100 SWATH) and phenotypes (BXD Published Phenotypes) of BXD inbred mice families fed with high-fat diet and are accessible on Genenetwork (http://www.genenetwork.org).

Statistical analysis

All results were presented as the mean ± standard error of the mean (S.E.M.). The Gaussian distribution of the data was assessed sing a Kolmogorov–Smirnov test. Data were analyzed using Student's *t*-test to compare two groups or ANOVA to compare 3+ groups, for data with Gaussian distributions in each of the groups. When the data were found to be not following a Gaussian distribution, the Mann–Whitney test was used (if homoscedasticity) or Welch's *t*-test technique was used (if heteroscedasticity) to compare 2 groups and Kruskal Wallis to compare 3+ groups. If found statistically significant, the one-way ANOVA test was followed by Bonferroni's *post hoc* test and the Kruskal–Wallis test followed by Dunn's multiple comparisons tests to compare between the different groups. Two-way ANOVA, with Bonferroni's correction for multiple comparisons, when appropriate, was used to analyze each point of ipPTT. The level of statistical significance used was $P<0.05$. The construction of the graphics and the statistical analysis were performed using GraphPad Prism 7.00.

Results

Short-term strength training reverses fasting hyperglycemia regardless of changes in body mass and adiposity

After 14 weeks of obesity induction, animals from the STO group started the short-term strength training. After 15 strength training sessions, the trained animals showed no statistical significant difference in body mass (Fig. 2A and B) and adiposity (Fig. 2E and F) when compared to the OB group. However, the hyperglycemia induced by obesity was reversed by strength training as observed in the STO animals, equating to that of the CTL group (Fig. 2C). The insulin concentration was higher for both obese groups. However, no statistical significant difference was observed between them (Fig. 2D). More details can be found in Supplementary Fig. 2.

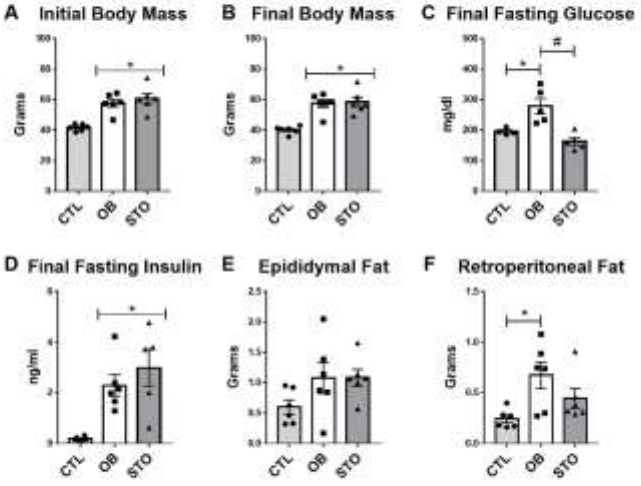

Figure 2
Physiological parameters of CTL, OB and STO groups. (A and B) Body mass of the animals at the beginning and at the end of the experiment. (C and D) Fasting glucose and insulinemia (after 8 h of fasting), respectively. (E and F) Adipose tissue weight of the epididymal and retroperitoneal regions, respectively. *$P<0.05$ vs CTL; #$P<0.05$ vs OB ($n = 4$–6 per group). We used Kruskal–Wallis test followed by Dunn's multiple comparisons tests in (B) and (D) and one-way ANOVA followed by Bonferroni's *post hoc* test in A, C, E and F.

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Short-term strength training decreases the fatty liver accumulation

The next step was to evaluate the lipid deposits and TG levels in the mice liver after short-term strength training. Initially, by the analyses of hematoxylin and eosin staining, the OB group presented higher lipid stocks when compared to the CTL group (Fig. 3A). However, the STO group presented an expressive reduction in lipid droplets size (Fig. 3A and C). These results were confirmed with oil red O staining for detection of neutral lipids, with a reduction in the stained area (Fig. 3A and D). Similar data were observed for the hepatic TG concentrations, in which the strength training reduced the TG levels in the liver of obese animals (Fig. 3B). More details can be found in Supplementary Fig. 3.

Short-term strength training reduces hepatic glucose production (HGP) and increases hepatic insulin sensitivity

The ipPTT was carried out 8 h after the last strength session for HGP control evaluation. Initially, we observed that the glycemic values of OB group were higher than CTL group at all times of the test (Fig. 4A). Moreover, obese trained animals presented lower glycemia at all points when compared to sedentary obese animals, with no statistically significant difference when compared to lean animals (Fig. 4A). Thus, the AUC of the OB group was higher during the test, while the STO group presented a reduction in these values compared to OB without significant differences compared to the CTL group (Fig. 4B). Interestingly, the increase in blood glucose during the test remained high until 90 min in the OB group but gradually increased and peaked at 60 min in the STO group and then gradually decreased (Fig. 4A), emphasizing the effectiveness of short-term strength training on glucose homeostasis.

Next, we evaluated the hepatic insulin sensitivity by Akt protein phosphorylation in response to insulin stimulus. The OB group reduced Akt phosphorylation at serine 473 residue, while the STO group reversed this result (Fig. 4C, D and E). More details can be found in Supplementary Fig. 4.

Once observed that HFD induced elevation in glycemia, reduced HGP control and decreased hepatic insulin sensitivity, bioinformatics analyses were performed to evaluate the relationship between fasting glycemia

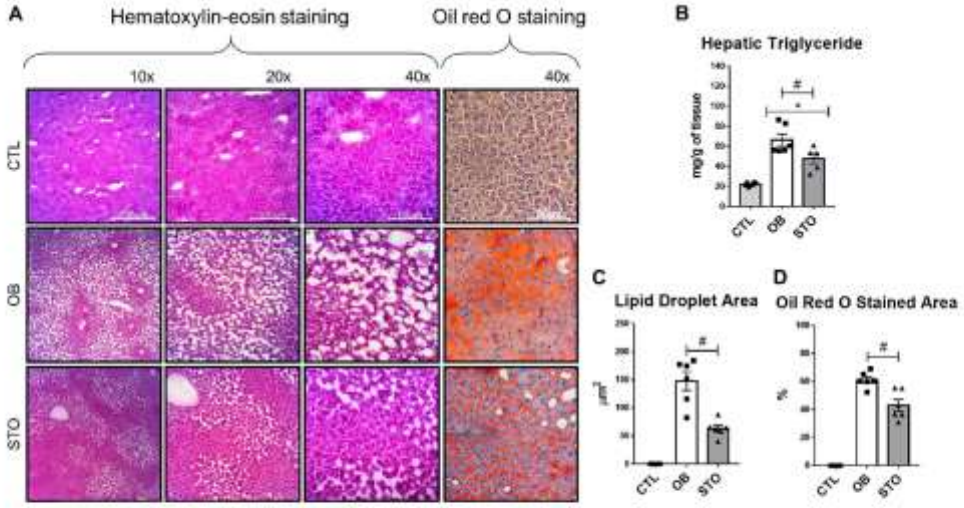

Figure 3
The hepatic fat content of CTL, OB and STO groups. (A) Hematoxylin and eosin staining and oil red O staining of the right lobe from three experimental groups. (B) TG content normalized for liver weight. (C) Liver lipid droplet area of the three groups, from oil red O staining. (D) Oil red O stained area of the three groups. *$P < 0.05$ vs CTL; #$P < 0.05$ vs OB ($n = 5$–6 per group). In (B), we used one-way ANOVA followed by Bonferroni's *post hoc* test. In (C) we used Mann–Whitney test between OB and STO groups. In (D) we used Student's *t*-test between OB and STO groups.

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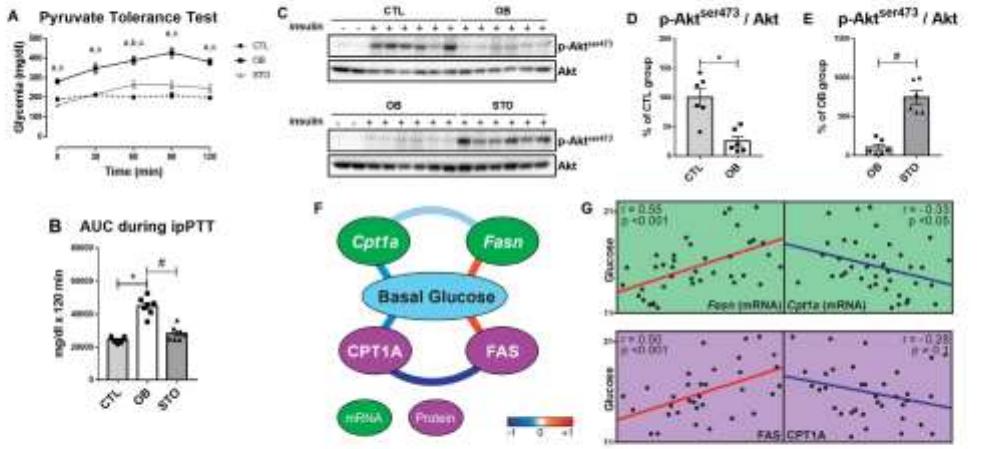

Figure 4

Hepatic glucose production during ipPTT and hepatic insulin sensitivity. (A) Glycemic curve during ipPTT. (B) The area under the curve during ipPTT. (C) Bands of p-Akt^{ser473} levels in the liver after insulin stimulus. (D) Quantification of hepatic p-Akt/Akt of CTL and OB groups. (E) Quantification of hepatic p-Akt of OB and STO groups. Only the bands of the animals stimulated with insulin were quantified. (F and G) Interaction network and correlation plots showing correlations between basal glucose levels (fasted state), hepatic mRNAs (shown in green) and proteins (shown in purple) of BXD mice fed with high-fat diet. Positive and negative Pearson's correlation coefficients are indicated by red and blue lines, respectively. Correlation plots of each analysis are also displayed, with Pearson's r and *P*-values indicated. In (A): $^{a}P < 0.05$ for CT vs OB; $^{b}P < 0.05$ for CT vs STO; $^{c}P < 0.05$ for OB vs STO. In (B, D and E): $^{*}P < 0.05$ vs CT; $^{\#}P < 0.05$ vs OB ($n = 7$ per group in A and B; $n = 6$ per group in D and E). In (A), we used two-way ANOVA test with Bonferroni's correction for multiple comparisons. In (B), we used Kruskal–Wallis test followed by Dunn's multiple comparisons tests. In (D and E), we used Student's *t*-test.

of obese animals with genes and proteins involved in hepatic lipid oxidation and synthesis. For this, families of isogenic mice were used as a reference to integrate transcriptome, proteome and phenotypes (Andreux *et al.* 2012). Fasting glycemia presented a negative correlation with lipid oxidation, which was assessed through analysis of the protein levels and mRNA of CPT1A. Coherently, fasting glycemia presented a positive correlation with hepatic lipogenesis, as assessed by the FAS (fatty acid synthase) content (Fig. 4F and G, Bioinformatics analysis – Supplementary Table 1).

Short-term strength training reduces lipogenesis, increases lipid oxidation and reduces inflammation in the liver

Finally, we investigated whether the short-term strength training led to molecular changes related to the synthesis and oxidation of lipids in the liver of obese mice. Initially, the obesity state increased the mRNA levels of lipogenic genes *Fasn* and *Scd1*, while the mRNA of oxidative genes *Cpt1a* and *Ppara* were reduced (Fig. 5A). Also, an obesity-induced reduction in ACC phosphorylation and an increase in total ACC and FAS content (Fig. 5B, C, D and E) were observed. However, the STO group reduced *Fasn* and *Scd1* and increased the *Cpt1a* and *Ppara* mRNA levels (Fig. 5I). Also, this group increased ACC phosphorylation and reduced ACC and FAS content (Fig. 5J, K, L and M). We also observed that obesity increased the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) levels (Fig. 5F, G and H) and the short-term strength training reversed this condition (Fig. 5N, O and P). More details can be found in Supplementary Fig. 5.

Discussion

Previous studies indicated hepatic insulin resistance was strongly associated with NAFLD (Petersen & Shulman 2017, Tilg *et al.* 2017). Approximately 70% of T2DM individuals (Leite *et al.* 2009) and 94% of obese diabetics (Silverman *et al.* 1990) are diagnosed with NAFLD. Here, we found that 14 weeks of exposure to HFD was efficient in generating our background of interest (obesity, T2DM and NAFLD). Also, we demonstrated strength training could be an effective alternative for reducing hepatic fat accumulation, reflecting a reduction in fasting glycemia,

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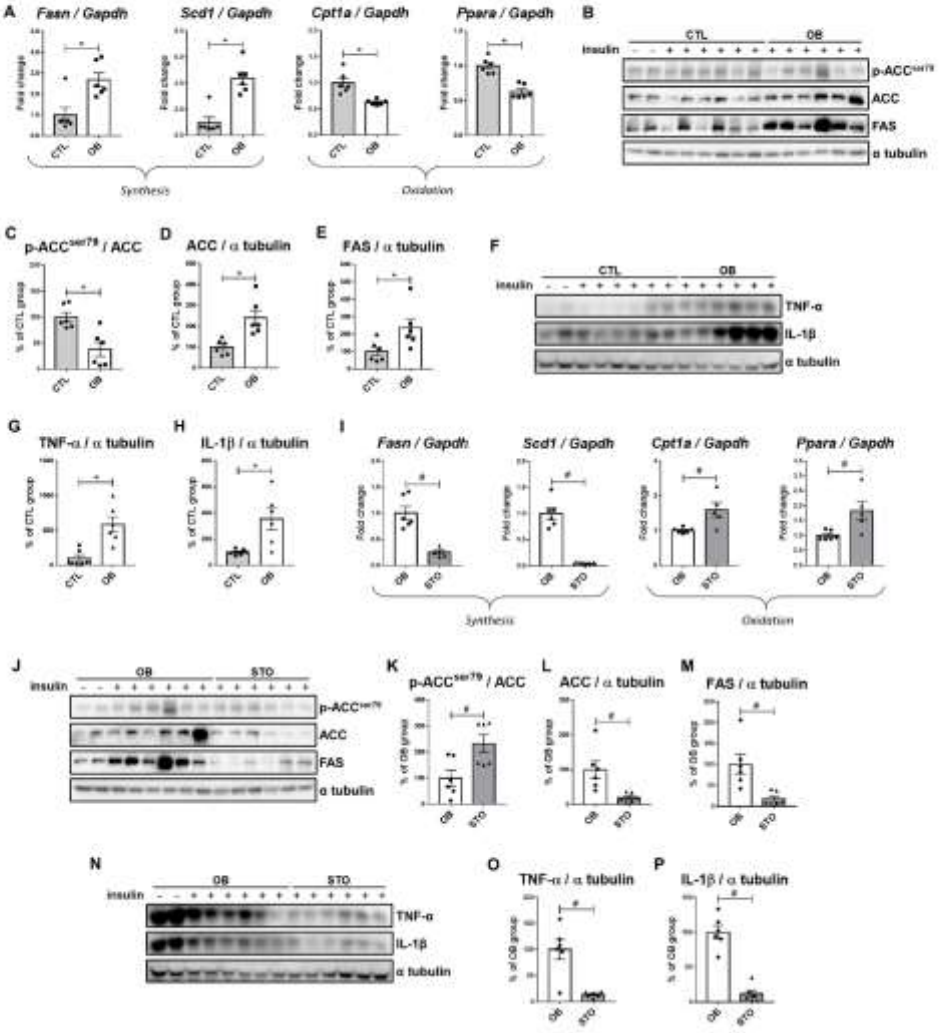

Figure 5

Parameters of hepatic lipogenesis, fat oxidation, and inflammation profile. (A) Levels of mRNA genes related to lipogenesis (*Fasn* and *Scd1*) and oxidation (*Cpt1α* and *Ppara*) of CTL and OB groups. (B) Bands of the lipogenic proteins in the liver of mice from CTL and OB groups after insulin stimulus. (C) Quantification of hepatic p-ACC^{ser79}/ACC of CTL and OB groups. (D and E) Quantification of hepatic ACC and FAS content, respectively, of CTL and OB groups. (F) Bands of the proinflammatory proteins in the liver of mice from CTL and OB groups after insulin stimulus. (G and H) Quantification of hepatic TNF-α and IL-1β content, respectively, of CTL and OB groups. (I) Levels of mRNA genes related to lipogenesis (*Fasn* and *Scd1*) and oxidation (*Cpt1α* and *Ppara*) of OB and STO groups. (J) Bands of the lipogenic proteins in the liver of mice from OB and STO groups after insulin stimulus. (K) Quantification of hepatic p-ACC^{ser79}/ACC of OB and STO groups. (L and M) Quantification of hepatic ACC and FAS content, respectively, of OB and STO groups. (N) Bands of the proinflammatory proteins in the liver of mice from OB and STO groups after insulin stimulus. (O and P) Quantification of hepatic TNF-α and IL-1β content, respectively, of OB and STO groups. Only the bands of the animals stimulated with insulin were quantified. *$P < 0.05$ vs CT; #$P < 0.05$ vs OB ($n = 5$–6 per group). In (A) (*Fasn*) and I (*Fasn*) we used Welch's t test. In (A) (*Cpt1α*), (C, D, E, H and I) (*Scd1* and *Cpt1α*), (K, L, O and P) we used Student's *t*-test. In the others, we used Mann–Whitney test.

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an increase in hepatic insulin sensitivity and, consequently, an improvement in the control of HGP. We observed the reduction of hepatic lipids occurred by the reduction in the protein content of ACC and FAS. All these results were observed independently of the body mass and adiposity reductions.

The literature provides evidence that aerobic training reduces NAFLD (Shen *et al.* 2015, Wu *et al.* 2015). Shen *et al.* (2015) observed reductions in liver fat depots in obese rodents which underwent aerobic exercise for 10 weeks. Also, aerobic training reduced TG and total cholesterol levels in hepatic and serum samples (Wu *et al.* 2015). However, the effects of strength training, with no change in adiposity on these parameters, had not yet been explored in the literature. Herein, the short-term strength training was effective in reducing both the size of fat deposits and TG levels in the liver. These results reinforce strength training may be another strategy for the treatment of NAFLD.

The reduction of hepatic lipogenesis in the STO group was linked to increased hepatic insulin sensitivity and reduced HGP. Previous studies have found lower glycemic values during the ipPTT for obese mice that swam for 8 weeks compared to the sedentary control group (Marinho *et al.* 2012, Souza Pauli *et al.* 2014). Nevertheless, both aerobic training and the acute aerobic session can improve hepatic insulin sensitivity (de Moura *et al.* 2013, Muñoz *et al.* 2018). However, little is known about strength training in this context. Recently, Botezelli *et al.* (2016) observed that long-term strength training (8 weeks) reduced the levels of hepatic steatosis and inflammation in the liver of rodents fed a high fructose diet. These animals showed better glycemic control in glucose tolerance and insulin tests. However, the control of hepatic glucose production during ipPTT and the activation of insulin pathway proteins in the liver of trained animals were not evaluated. In our study, we showed short-duration strength training was efficient in reversing inflammation (TNF-α and IL-1β) and hepatic insulin resistance provided by HFD-induced obesity, improving the HGP control during ipPTT.

The ACC protein catalyzes the acetyl-CoA carboxylation for the synthesis of malonyl-CoA and FFA, playing a key role in triacylglycerol synthesis. On the other hand, the ACC inhibits the oxidative activity of the cell by reducing carnitine palmitoyltransferase 1 A (CPT1A) (Kobayashi *et al.* 2010, Bechmann *et al.* 2012). Consistently, knockout mice for the isoform 2 of the *ACC* gene were protected from NAFLD induced by both diets high in saturated fat as high carbohydrate diets (Abu-Elheiga *et al.* 2012). Nevertheless, *ob/ob* mice with the deletion of ACC, specifically in the liver, did not present an increase in the accumulation of liver fat (Kim *et al.* 2017). Finally, subjects with hepatic steatosis treated with MK-4074 (liver-specific inhibitor of ACC1 and ACC2) had a 36% reduction in lipid content after 4 weeks of intervention (Kim *et al.* 2017). Thus, therapies that reduced the content levels activity of ACC in the liver were the subject of several studies for the prevention and treatment of NAFLD (Nuñez-Durán *et al.* 2018, Romier *et al.* 2018). In this context, aerobic training is known to provide an increase in the phosphorylation of ACC in the liver of obese animals, inhibiting its lipogenic action (Rector *et al.* 2008). Here, we showed for the first time the short-term strength training was able to reduce the activity and amount of ACC, increasing its phosphorylation and reducing its expression and total content.

In turn, the FAS protein is responsible for the synthesis of palmitic acid (Chakravarty *et al.* 2004), and it is identified as another key protein in the development of NAFLD (Dorn *et al.* 2010). Dorn *et al.* observed that *Fasn* expression was increased in mice with fatty liver (Dorn *et al.* 2010). The same study showed the hepatic expression of *Fasn* was positively correlated with NAFLD degree in humans. As ACC, FAS inhibition was also investigated to combat NAFLD. Chronic use of FAS inhibitor reduced lipogenesis in both mice and monkeys (Singh *et al.* 2016). Herein, the short-term strength training reduced the levels of mRNA and FAS contend, reinforcing the efficiency of this protocol in downregulating the synthesis of lipids in the liver of obese animals, regardless of body weight change.

Furthermore, besides the reduction of lipid synthesis, we verified an increase in the transcription of genes involved in lipid oxidation *Cpt1a* and *Ppara* in the liver of trained animals. Therefore, the oxidative mechanism may be considered as another positive effect by which strength training acts against obesity-induced NAFLD. Several studies investigated approaches to combat hepatic steatosis with these strategies. Recently, Hsiao *et al.* (2017) observed that the treatment of obese mice with pioglitazone, described as peroxisome proliferator-activated receptor gamma (PPARγ) agonist, increased hepatic levels of mRNA *Cpt1a* even without a significant increase in *Ppara*. These animals had hepatic steatosis attenuated. Similarly, a period of 8 weeks of aerobic training increased *Ppara* and *Cpt1a*, reducing the levels of hepatic lipids in obese mice (Muñoz *et al.* 2018).

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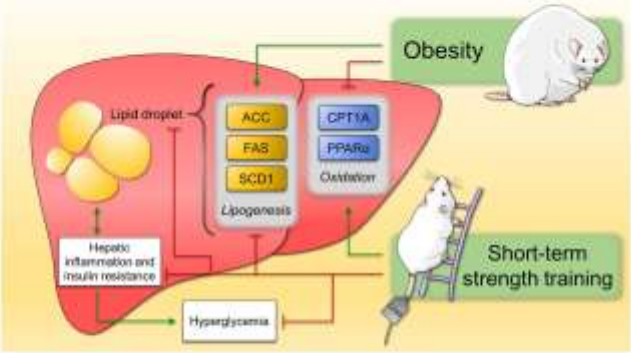

Figure 6
Role of short-term strength training on lipid metabolism in the liver, independent of body weight change. Obesity provides insulin resistance, fasting hyperglycemia and increase of lipid synthesis mechanisms in the liver, increasing inflammation and protein level of ACC and FAS, *Fasn* and *Scd1* transcription, and ACC activity. At the same time, this condition reduces the transcription of oxidative genes *Pparα* and *Cpt1α*. On the other hand, short-term strength training provided the opposite effects even without a reduction in body adiposity. Therefore, we demonstrated for the first time that the present short exercise protocol could be an important tool in the combat and/or treatment of NAFLD. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0567.

Here, we bring the first evidence that the short-term strength training provides an increase in lipid oxidation in the liver, countering obesity-induced NAFLD.

In summary, short-term strength training reduced hepatic fat accumulation and inflammation, increased hepatic insulin sensitivity and provided better control of the HGP, contributing to the reduction of obesity-induced hyperglycemia. These phenomena occurred through the reduction of both activity and content of the proteins involved with lipogenesis in the liver as well as the increase of transcription of genes involved with lipid oxidation, independently of changes in body mass and adiposity (Fig. 6). In summary, we provided new evidence supporting the practice of strength training as a strategy for the prevention and treatment of NAFLD.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-18-0567.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L P M designed the paper. R M P wrote the paper and had the overall responsibilities of the experiments in this study. K C C R, C P A, M R S, T D P C, R S C and D G M performed the experiments and data collection. C P A performed the histological experiments. M R S performed the PCR analysis. R S G performed the bioinformatics analysis. R A M and R S G performed the statistical analysis. A S R S, D E C, E R R, J R P and L P M contributed to discussion and supported the financial costs. All the authors have read and approved this manuscript.

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4.2 ARTIGO 2

Uma vez demonstrado que o protocolo de treinamento de força de curta duração era capaz de aumentar a sensibilidade à insulina e reduzir o acúmulo de gordura hepática em camundongos obesos, nosso próximo estudo teve como objetivo avaliar os efeitos do treinamento de força de curta duração sobre um dos principais mecanismos de controle da produção hepática de glicose afetados pela obesidade e resistência hepática à insulina, que culminam na hiperglicemia associada à obesidade: o eixo Akt / FOXO1.

Artigo em processo de finalização:

Short-term strength training reduces hepatic FOXO1 activity and hyperglycemia in obese mice

Short-term strength training reduces hepatic FOXO1 activity and hyperglycemia in obese mice

Running title: Strength training decreases hepatic nuclear FOXO1

Keywords: short-term strength training; diabetes; obesity; liver; insulin sensitivity, gluconeogenesis

Key points:

- Short-term strength training (STST) enhances the hepatic insulin sensitivity and the control of hepatic glucose production;
- Hepatic FOXO1 phosphorylation and nuclear extrusion is higher in strength-trained animals;
- STST reduces the gluconeogenic protein content of G6Pase and PEPCK;
- All these results occurred without corporal adiposity reduction.

ABSTRACT

Obesity is a worldwide health problem and is directly associated with insulin resistance and type 2 diabetes. The liver is an important organ for the control of healthy glycemic levels, since insulin resistance in this organ reduces phosphorylation of FOXO1 protein, leading to higher hepatic glucose production (HGP) and fasting hyperglycemia. Aerobic physical training is known as an important strategy in increasing the insulin action in the liver by increasing FOXO1 phosphorylation and reducing gluconeogenesis. However, little is known about the effects of strength training in this context. This study aimed to investigate the effects of short-term strength training on hepatic insulin sensitivity and GSK3β and FOXO1 phosphorylation in obese mice. To achieve this goal, obese Swiss mice performed the strength training protocol (1 daily session for 15 days). Short-term strength training increased the phosphorylation of Akt and GSK3β in the liver after insulin stimulus and improved the control of HGP during the pyruvate tolerance test. On the other hand, sedentary obese animals reduced FOXO1 phosphorylation and increased the levels of nuclear FOXO1 in the liver, increasing the PEPCK and G6Pase content. Bioinformatic analysis also showed positive correlations of hepatic FOXO1 levels and gluconeogenic genes, reinforcing our findings. However, strength trained animals

reverted this scenario, regardless of body adiposity changes. In conclusion, short-term strength training is an efficient strategy to enhance the insulin action in the liver of obese mice, contributing to glycemic control by reducing the activity of hepatic FOXO1 and lowering PEPCK and G6Pase contents.

INTRODUCTION

Obesity is considered a worldwide health problem and is directly associated with several metabolic disorders. Among these complications, insulin resistance stands out, because this is the initial phenomenon for the onset of type 2 diabetes mellitus (T2DM) (TEMPLEMAN et al., 2017). In this scenario, hepatic insulin resistance is considered one of the main key points for hyperglycemia associated to T2DM, since this hormone is responsible for the suppression of hepatic glucose production (HGP) (PETERSEN; SHULMAN, 2017). However, the mechanisms underlying how obesity is related to hepatic insulin resistance and collaborates with the perpetuation of fasting hyperglycemia are not fully understood.

The inhibition of HGP provided by insulin action requires the proper functioning of complex biomolecular machinery in this tissue. Once secreted under nutrient-abundant conditions, insulin binds to a tyrosine kinase receptor (IR) and initiates a signaling cascade that culminates in protein kinase B / Akt phosphorylation and activation (PERRY et al., 2014). Once activated, Akt inhibits HGP by two mechanisms: 1- stimulating glycogen synthesis by phosphorylation and inactivation of Glycogen Synthase Kinase-3β (GSK3β) protein, which once phosphorylated no longer inhibits the action of Glycogen Synthase (GS) (PERRY et al., 2014); 2- by phosphorylation and nuclear exclusion of the transcription factor Forkhead box protein 1 (FOXO1) in three phosphorilations sites (thr24, ser256 and ser319), thereby reducing transcription of the pro-gluconeogenic genes Phosphoenolpyruvate Carboxykinase (PEPCK) and Glucose- 6-Phosphatase (G6Pase). It is importante to highlight that among those phosphorilation sites in FOXO1 described above, ser256 is knowed as “gatekeeper”, necessary for the phosphorilation in the other sites, been the main target in several studies (BARTHEL; SCHMOLL; UNTERMAN, 2005). However, obesity can downregulate these two pathways. Initially, obese diabetes-prone mice have increased GSK3β activation (ELDAR-FINKELMAN et al., 1999), and its inhibition in liver provided an overall improvement for glycemic homeostasis

(MARINHO et al., 2012a). Therefore, GSK3β phosphorylation in response to insulin is considered an important marker of hepatic sensitivity to this hormone. Similarly, the hepatic nuclear FOXO1 phosphorylation is impaired in obese mice (WANG et al., 2018). Moreover, the selective pharmacological inhibition of FOXO1 reduced PEPCK and G6Pase expression in hepatocytes (LANGLET et al., 2017) and mice with liver-specific deletion of FOXO1 showed lower postprandial glycemic levels than wild type animals (LU et al., 2012). Therefore, the reduction of FOXO1 activity is also considered an important strategy to combat hyperglycemia in T2DM.

Several pieces of evidence have pointed out aerobic physical exercise as an important non-pharmacological strategy to increase hepatic insulin sensitivity (MARCINKO et al., 2015; SARGEANT et al., 2018a). Endurance physical training reversed the reduction in obesity-induced GSK3β phosphorylation by increasing glycogen content and improving HGP control (MARINHO et al., 2012a). Consistently, increased hepatic FOXO1 phosphorylation has also been observed following chronic aerobic exercise in obese rodents, culminating in a reduction in the protein content of PEPCK and G6Pase as well as in the glycemic levels of animals (MARINHO et al., 2012a).

On the other hand, strength training has gained attention because also provides benefits for liver metabolism, combating the deleterious effects associated with obesity and T2DM (DOS SANTOS et al., 2019; HONKA et al., 2016; PEREIRA et al., 2017a). Recently, regardless of the adiposity reduction, we observed that obese mice performing 15 strength exercise sessions increased hepatic Akt phosphorylation after insulin stimulation, culminating in better control of HGP (PEREIRA et al., 2019). Consistently, Honka and colleagues demonstrated that older women with hepatic insulin resistance reduced endogenous glucose production after four months in a strength training program (HONKA et al., 2016). However, the direct effects of strength training on the biomolecular mechanisms involved with HGP remain poorly explored. Therefore, our study aimed to investigate the direct effects of strength training on hepatic FOXO1 and GSK3β activity and, consequently, on the control of gluconeogenesis in obese mice, regardless of body composition alterations.

METHODS

Animals and diet

In the present study, we used male Swiss mice from the Unicamp Central Animal Facility (CEMIB) at eight weeks old. The animal experiments were carried out respecting the Brazilian legislation on the scientific use of animals (Law No. 11.794, of October 8, 2008). All experiments were accepted by the Ethics Committee on Animal Use (CEUA) of Biological Sciences (UNICAMP-Campinas-SP, number 4406-1). Animals were maintained individually in polyethylene cages with the enriched environment as previously described (PEREIRA et al., 2019). The light switched on at 06:00 and off at 18:00 h, the temperature was controlled at 22 ± 2° C, relative humidity maintained at 45-55%, and on-site noises below 85 decibels. One-hundred W lamps were used during the clear period of the day (Phillips® soft white light; 2700 K; 565-590 nm; 60 lux). Mice had free access to water and conventional diet.

At the beginning of the experiment, the animals were divided into two groups: the Control Lean group (CTL) fed a chow diet, and the Obesity group fed a high-fat diet (HFD). After 14 weeks of exposure to HFD, the animals of the obese group were equally redistributed according to body weight and fasting glycemia into two groups: a) Sedentary Obese (OB), which remained sedentary throughout the experiment; b) Strength Training Obese (STO), which performed a short-term strength training. The high-fat diet was prepared based on the American Institute of Nutrition (AIN-93G) guidelines (REEVES; NIELSEN; FAHEY, 1993) with an alteration to contain 35% of fat (4% soy oil and 31% of lard) (OLIVEIRA et al., 2015).

Experimental design and training protocol

As previously described (PEREIRA et al., 2019), mice performed the short-term strength training in a ladder with 1.5 cm distance between the steps and 70 cm of high, with a loading apparatus that was fixed with adhesive tape across the length of the tail of the animal, where the loads were coupled. The load apparatus was a conical plastic tube with approximately 7.5 cm of height and 2.5 cm of diameter.

Firstly, the animals were adapted to the apparatus for 5 consecutive days. On the first day, the animals were placed in a chamber at the top of the ladder for 60 seconds with the loading apparatus empty attached on its tail. For the first climbing attempt, the animal was placed on the ladder at 15 cm from the entrance of the chamber. For the second attempt, the animal has placed 25 cm away from the chamber. For the third attempt onwards, the animal was positioned at the base of the ladder, 70 cm away from the chamber. The attempts starting from the base of the

ladder and continued until the animal reaches the chamber three times without the need for any stimulus.

Forty-eight hours after the last day of adaptation, the animals underwent the maximal voluntary carrying capacity (MVCC) test. This is an incremental test to determine the maximum load in which each animal can climb the entire length of the ladder. The initial series was performed with an overload corresponding to 75% of the animal's body weight, and an incremental load of 5 g was added at each further attempt to climb until the animal could no longer complete the entire course. At each successful attempt, the animal was removed from the ladder and placed in an individual cage, where it rested for 5 minutes until the next attempt. The heaviest overload in which the animal performed a successful climb was considered the MVCC, and this value was used to prescribe the individual loads in the experiment.

Forty-eight hours after the MVCC determination, the strength training protocol was initiated. The exercise sessions consisted of 20 climbing series with an overload of 70% of the MVCC and with a rest interval of 60-90 seconds between sets. The animals were exercised for five consecutive days per week, followed by two days of rest, until they completed 13 sessions of physical exercise. Subsequently, mice were submitted to the pyruvate tolerance test. After 24 hours, the animals performed two more sessions of exercise, totaling 15 sessions, as showed in Figure 1.

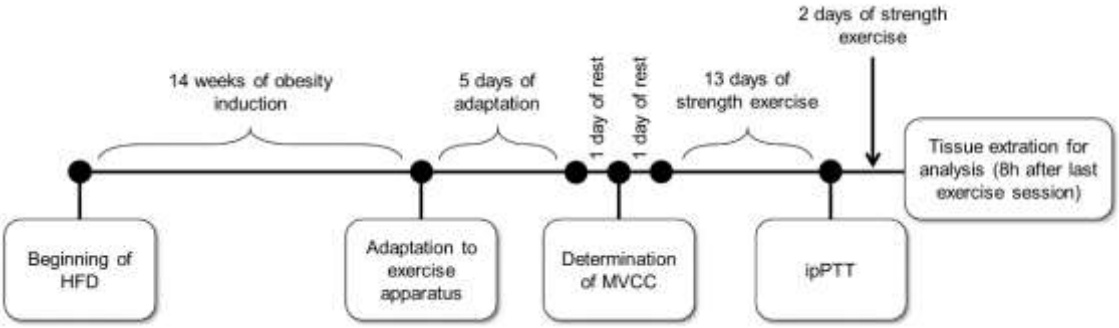

Figure 1: Experimental design. Schematic representation of the experiments. The ipPTT and tissue extraction were performed 8 hours after the exercise session and respecting a fast period of 8 hours.

Intraperitoneal Pyruvate Tolerance Test (ipPTT)

After the 13th exercise session, we performed the ipPTT to estimate HGP control. The animals were submitted to 8 hours of fasting, and the test was performed

8 hours after the end of the exercise session. The pyruvate was injected intraperitoneally (i.p.), 2.0 g of pyruvate/kg body weight, and the blood samples were collected at 30, 60, 90 and 120 min from the tail of the animal for blood glucose determination. Glucose levels were determined using a glucometer (Accu-Chek; Roche Diagnostics®). The results were evaluated determining the areas under the serum glucose curves (AUC) during the test by the trapezoidal method (MATTHEWS et al., 1990), using Microsoft Excel.

Tissue extraction and immunoblotting analysis

After the ipPTT, animals of STO group were submitted to two more exercise sessions and were anesthetized i.p. by the injection of *chloral hydrate* of ketamine (100 mg/kg, Parke-Davis, Ann Arbor, MI) and xylazine (10 mg/kg, Rompun, Bayer, Leverkusen), after 8 hours of fasting and 8 hours after the last exercise session. After the verification and assurance of the corneal reflexes, mice were injected i.p. with human insulin (8 U/kg body wt Humulin-R; Lilly, Indianapolis, IN) or saline. After 10 min, the liver was rapidly removed, snap-frozen in liquid nitrogen, and stored at -80° C until analysis. Also, the epididymal adipose tissue (right side) was removed and weighted. The liver was homogenized in extraction buffer [1% Triton-X 100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/mL] at 4° C with a TissueLyser II (QIAGEN®) operated at maximum speed for 120 s. The lysates were centrifuged (Eppendorf 5804R) at 12.851 × *g* at 4° C for 15 min to remove insoluble material, and the supernatant was used for the assay. The protein content was determined by the bicinchoninic acid method (WALKER, 1994). The samples containing 60 µg of total protein were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk at room temperature for 1 h and incubated with primary antibodies against the protein of interest. After that, a specific secondary antibody was used. The specific bands were labeled by chemiluminescence and visualization was performed by photo documentation system in G: box (Syngene). The bands were quantified using the software UN-SCAN-IT gel 6.1. The following primary antibodies were used: anti-Phospho-Akt ser473 (4060), anti-Akt (4685), anti-Phospho-GSK3β (5558), anti-GSK3β (5676), anti-Phospho-FOXO1 ser256 (9461), anti-FOXO1 (2880) and anti-GAPDH (2118) from Cell Signaling Technology®

(Beverly, MA), anti-PEPCK (LS-C178341) from LifeSpan BioSciences and anti-G6Pase (sc-398155) and anti-HISTONE3 (sc-517576) from Santa Cruz Biotechnology® (Santa Cruz, CA). The following secondary antibodies were used: Anti-rabbit IgG, HRP-linked Antibody (7074) and Anti-mouse IgG, HRP-linked Antibody (7076) from Cell Signaling Technology® (Beverly, MA).

Nuclear extraction

The liver samples were removed and homogenized in STE buffer [0.32 M of sucrose, 20 mM of Tris-HCL (pH 7.4), 2 mM of EDTA, 1 mM of DTT, 100 mM of sodium fluoride, 100 mM of sodium pyrophosphate, 10 mM of sodium orthovanadate, 1 mM of PMSF, and 0.1 mg aprotinin/mL] at 4˚C with a Polytron PTA 20S generator. The homogenates were centrifuged (1000 × g, 25 min, 4˚C), the obtained pellets were washed with STE buffer (1000 × g, 10 min, 4˚C), suspended in Triton buffer (1% TritonX-100, 20 mM of Tris HCl 9PH 7.4), 150 mL of NaCl, 2 mM of EDTA, 100 mM of NaF, 100 mM of sodium pyrophosphate, 10 mM of sodium orthovanadate, 1 mM of PMSF and 0.1 mg of aprotinin/mL), kept on ice for 30 minutes, and centrifuged (15000 × g, 30 min, 4˚C). The supernatant collected is referred to as a nuclear fraction.

Liver immunohistochemistry analysis

Liver samples were collected and fixed in isopentane for cryopreservation at −80° C. The tissue was sliced in a Leica™ Cryostat cryostat (CM1850, Heidelberg, Germany) to a thickness of 10 µm and placed on identified adhesion slides.

The slices of liver tissue derived from three animals in each experimental group were subject to immunohistochemistry. Antigenic recovery was performed in a pressure cooker (Electrolux Chef), and endogenous peroxidase activity was blocked with 3% of hydrogen peroxide diluted in methanol for 15 minutes. Subsequently, tissue was blocked with 3% bovine serum albumin (BSA), diluted in TBS-T (1% Triton X-100, 100 mM of Tris, pH 7.4) for one hour and incubated with polyclonal primary antibodies anti-Phospo-FOXO1 from Bioss (bs-3142r) diluted in 1% BSA overnight. The following day, the sections were incubated with secondary goat anti-rabbit HRP antibody (Santa Cruz sc-2030; 1:200) diluted in 1% BSA for 2 hours in room temperature. The liver sections of each experimental group were evaluated through the brownish precipitate of diaminobenzidine that was used as the chromogen

indicating immunoreactivity (DAB diluted 1:50 for 3 minutes) and after stained with *Harris hematoxylin* during five minutes. Positive and negative controls were performed.

Samples of the liver were acquired using photomicroscope Zeiss Axiophoto (Zeiss, Munich, Germany) at 40x magnification. The intensity of immunoreactivity of p-FOXO antigens was examined in 10 fields per animal using ImageJ software (version 1.50i), and the percentage of tissue marking was quantified for each image.

Triglyceride assay

Hepatic and serum triglyceride (TG) contents were determined using a commercial kit according to the manufacturer's instructions (Laborlab®). Hepatic TG values were normalized by total liver weight.

Bioinformatics analysis

Correlation analyses from BXD mice families (ANDREUX et al., 2012) and enrichment analyses (WANG et al., 2017) were performed as previously described. Databases from the liver (EPFL/LISP BXD HFD Liver Affy Mouse Gene 1.0 ST (Aug18) RMA) and phenotypes (BXD Published Phenotypes) are accessible on Genenetwork (http://www.genenetwork.org).

Statistical analysis

All results were presented as the mean ± standard error of the mean (SEM). The Gaussian distribution of the data was assessed using a Shapiro-Wilk test and analyzed by Student's *t*-test for parametric data to compare two groups when necessary. We used the one-way Analysis of Variance (ANOVA) test followed by Bonferroni's post-hoc test to compare more than two groups. Two-way ANOVA (with repeated measures when appropriate), with Bonferroni's correction for multiple comparisons, was used to analyze each point of ipPTT. The level of statistical significance was $P<0.05$. The construction of the graphics and the statistical analysis were performed using GraphPad Prism 7.00.

RESULTS

Short-term strength training reverses fasting hyperglycemia, reduces serum and hepatic TG levels and improves HGP control, regardless of body mass and adiposity alterations:

Initially, we observed that 14 weeks of obesity induction was efficient to increase body mass, adiposity, fasting glucose, as well as serum and hepatic TG levels (Fig. 2A-F). On the other hand, short-term strength training protocol was able to reverse obesity-induced hyperglycemia, without reducing the amount of adipose tissue (Fig. 2A-D). Besides, the strength training protocol was effective in reducing serum and hepatic TG levels in obese mice (Fig. 2E and F).

Our next step was to evaluate whether short-term strength training was able to improve the control of HGP in obese animals regardless of adiposity reduction. Eight hours after the 13th exercise training session, the animals underwent the ipPTT, and blood glucose was assessed every 30 min for 120 min. Initially, we observed that obesity worsened HGP control since the glycemia in the OB group was elevated at all points during the test when compared to the CTL group (Fig. 2A). However, animals from the STO group presented lower glycemic levels than the OB group, with a difference in all points (Fig. 2A). Consequently, the AUC during the test was elevated for the OB group and short-term strength training was able to reverse this condition (Fig.2B).

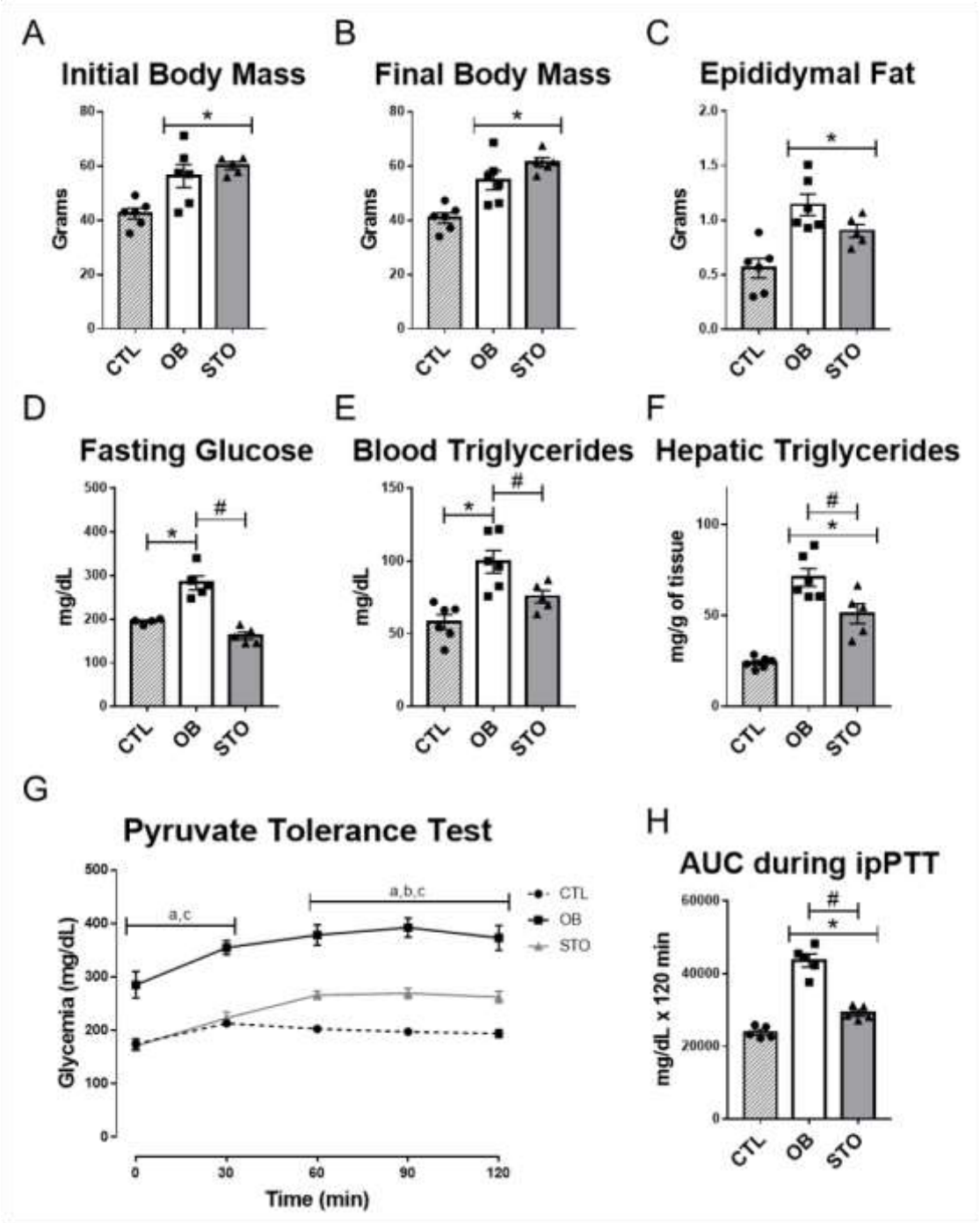

Figure 2: Physiological parameters of experimental groups. A and B) Initial and final body mass of three experimental groups. C) Adipose tissue weight of the epididymal region. D) Fasting glucose at the end of the experimental period. E and F) Blood and hepatic triglycerides levels, respectively. G) Glycemic curve during ipPTT. H) Area under the curve during ipPTT. In figure G: a=p<0.05 for CT vs OB; b=p<0.05 for CT vs STO; c=p<0.05 for OB vs STO. In others: *=p<0.05 vs CT; #=p<0.05 vs OB (n = 5-6 per group). In figure G we used two-way ANOVA test with Bonferroni's

correction for multiple comparisons. We used one-way ANOVA followed by Bonferroni's post-hoc test in others.

Short-term strength training increases hepatic insulin sensitivity and GSK3β phosphorylation:

To assess hepatic insulin sensitivity of these animals, the Akt activation levels were evaluated in the liver after i.p. insulin injection. As shown in Figure 3, obesity reduced p-Akt^{ser473} (Fig. 3A and B). On the other hand, short-term strength training reversed this situation, since the STO animals increased p-Akt^{ser473} levels when compared to sedentary obese animals (Fig. 3A and C). Consistent with these results, we also evaluated the activation of GSK3β protein, which is directly involved with glycogen synthesis control. Similar to the results observed for Akt activity, obesity decreased GSK3β^{ser9} phosphorylation in animals from the OB group, and short-term strength training reversed this parameter (Fig. 3).

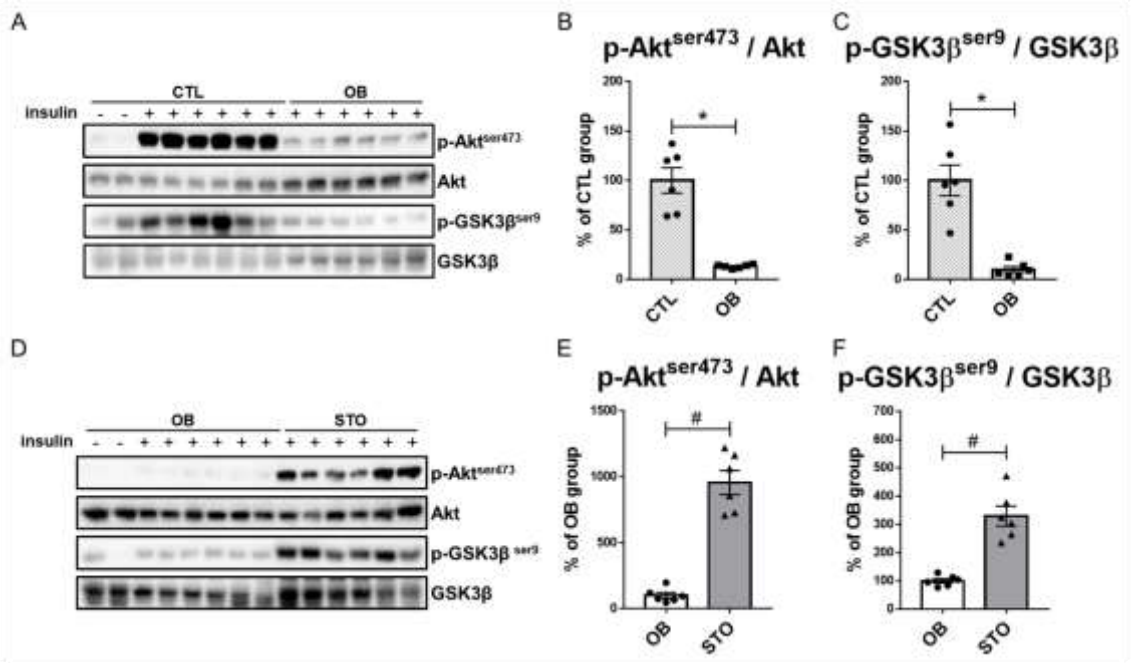

Figure 3: Parameters of hepatic insulin sensitivity. A) Bands of proteins related to hepatic insulin sensibility of mice from CTL and OB groups. B and C) Quantification of hepatic p-Akt^{ser473} and p-GSK3β^{ser9} contents of CTL and OB groups, respectively. D) Bands of proteins related to hepatic insulin sensibility of mice from OB and STO groups. E and F) Quantification of hepatic p-Akt^{ser473} and p-GSK3β^{ser9} contents of OB and STO groups, respectively. Only the bands of the animals stimulated with insulin

were quantified. *=p<0.05 vs CT; #=p<0.05 vs OB (n = 6 per group). We used Student's t-testz in these analyses.

Effects of short-term strength training in FOXO1 activity and bioinformatics analysis:

Once demonstrated that Akt protein activation was increased after short-term strength training, we investigated whether the increased Akt activation in response to insulin would reduce FOXO1 activity. Initially, we performed western blot analyses with an antibody anti - phosphorylated FOXO1 specific on serine residue 256 using whole-liver homogenates sample. As shown in Figure 4, obesity reduced the p- FOXO1^{ser256} (Fig. 4A and B); however, short-term strength training reversed this situation, since the STO animals increased FOXO1^{ser256} compared with the sedentary obese animals (Fig. 4A and C). Furthermore, once it is known that after being phosphorylated by Akt, FOXO1 translocate from the cell nucleus to the cytoplasm, we performed experiments to observe the amount of FOXO1 located in the nucleus and the cytoplasm. The OB group mice reduced the cytoplasmic FOXO1 levels and increased the nuclear FOXO1 content, indicating a greater translocation of FOXO1 from the cytoplasm to the nucleus (Fig. 4D and E). On the other hand, animals from the STO group increased cytoplasmic FOXO1 levels and reduced FOXO1 permanence in the cell nucleus when compared to sedentary obese animals (Fig. 4D and F), which indicates that animals submitted to the short-term strength training decreased FOXO1 translocation to the hepatocyte cell nucleus. Finally, immunohistochemistry analyses confirmed these results, with the STO group presenting higher amounts of p- FOXO1^{ser256} in the cytoplasm compared to the OB group (Fig. 4G).

Using a publicly accessible dataset from isogenic BXD mice, we correlated the FOXO1 levels in the liver of mice treated with a high-fat diet and the levels of genes related to gluconeogenesis (Fig. 4I). There were significant correlations between FOXO1 and *Gsk3b, G6pc* and *Pck1*, as well as with two phenotypes: running distance and glycemia. These correlations highlight our hypothesis that physical exercise (in our case, short-term strength training) could affect not only glycemia but also gluconeogenic targets, as well as FOXO1. Mammalian ontology analysis highlighted the evaluated genes as involved with abnormal glycogen homeostasis (Fig. 4J), also pointing towards strength training, as high-intensity exercise is known to deplete glycogen levels in a potent manner (NIELSEN et al., 2011). Finally,

enrichment analysis (Fig. 4K) added FOXO1 signaling pathway, as well as insulin signaling pathway and others, indicating that a treatment that affects the evaluated genes and ameliorates insulin sensitivity could also be affecting the FOXO1 pathway.

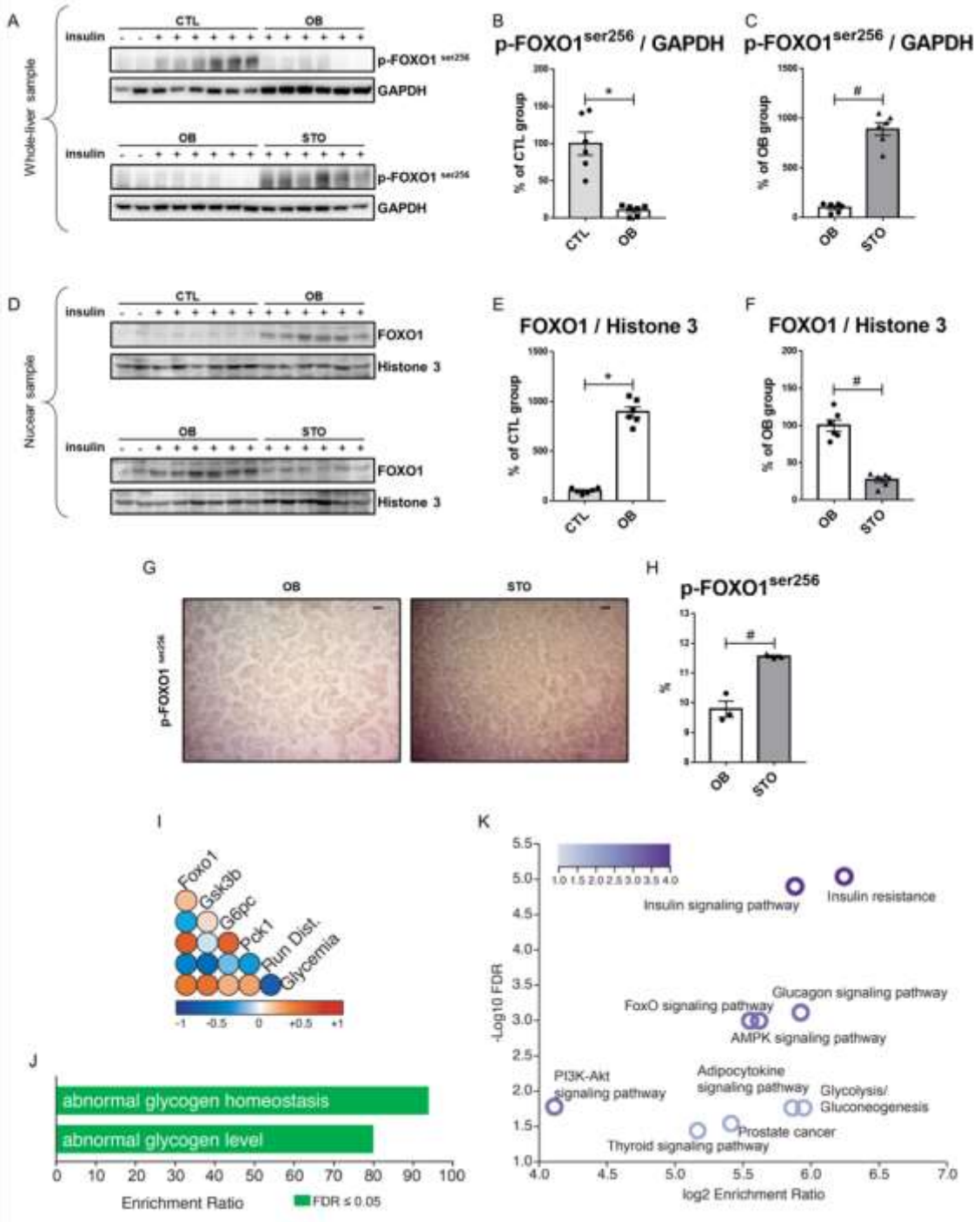

Figure 4: FOXO1 phosphorylation and nuclear extrusion of CT, OB and STO groups, and bioinformatics analysis. A) Bands of whole-liver p-FOXO1^{ser256} of mice from CTL and OB groups (up), and OB and STO groups (down). B) Quantification of p-FOXO1^{ser256} content of CTL and OB groups. C) Quantification of

p-FOXO1^{ser256} content of OB and STO groups. D) Bands of nuclear FOXO1 of mice from CTL and OB groups (up), and OB and STO groups (down). E) Quantification of nuclear FOXO1 content of CTL and OB groups. F) Quantification of nuclear FOXO1 content of OB and STO groups. G) Immunohistochemistry analyses of the liver of OB and STO group, magnification of 40X, bar=50µm. H) Marked area with p-FOXO1^{ser256}. Only the bands of the animals stimulated with insulin were quantified. I) Corrgram analysis from liver mRNA of BXD mice families fed with High Fat Diet and phenotypes. J) Mammalian Phenotype Analysis. K) Pathway Enrichment Analysis. *=p<0.05 vs CT; $^{\#}$=p<0.05 vs OB (n = 6 per group in A-F; n = 3 per group in G and H). We used Student's t-test in these analyses.

Short-term strength training reduces the content of gluconeogenesis proteins PEPCK and G6Pase in response to insulin:

Since short-term strength training provides improvements in HGP control, enhances hepatic insulin sensitivity, and increases the exclusion of FOXO1 from the nucleus, we evaluated the levels of gluconeogenesis pathway proteins that are transcribed by FOXO1. The results show that animals from the OB group increased the protein contents of PEPCK and G6Pase (Fig. 5A-C). In contrast, animals from the STO group presented reduced content of these two proteins, which are crucial for controlling hepatic gluconeogenesis (Fig. 5A-C).

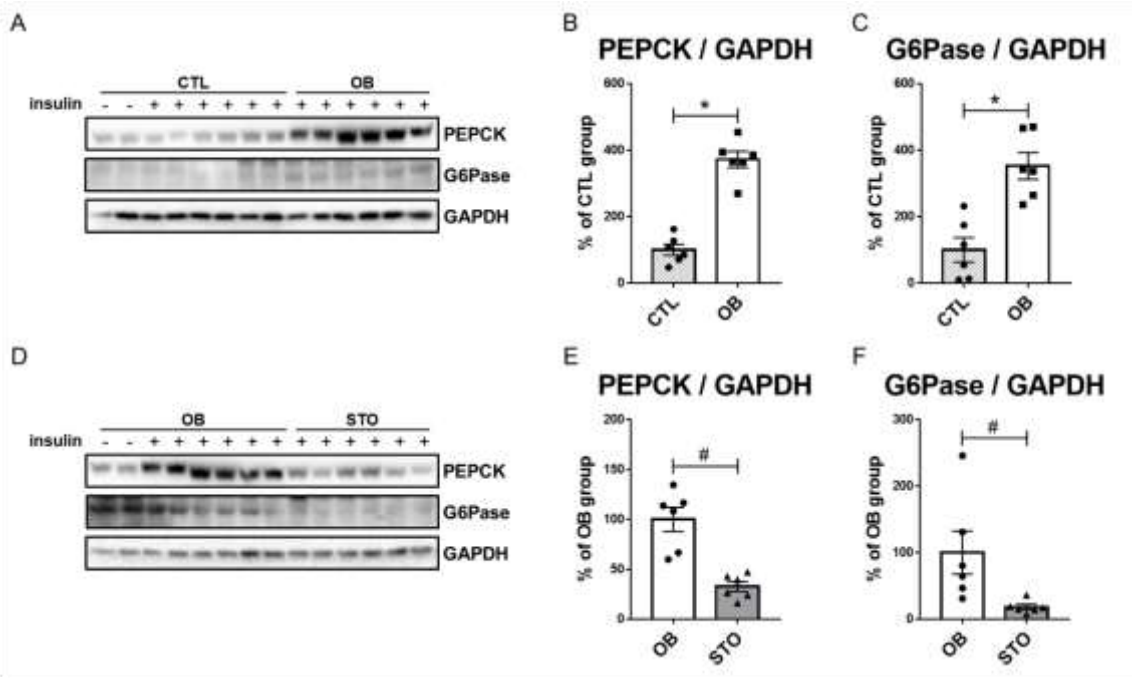

Figure 5: Gluconeogenic protein levels of CT, OB and STO groups. A) Bands of PEPCK and G6Pase of mice from CTL and OB groups. B) Quantification of PEPCK contents of CTL and OB groups. C) Quantification of G6Pase contents of CTL and OB groups. D) Bands of PEPCK and G6Pase of mice from OB and STO groups. E) Quantification of PEPCK contents of OB and STO groups. F) Quantification of G6Pase contents of OB and STO groups. Only the bands of the animals stimulated with insulin were quantified. *=$p<0.05$ vs CT; $^{\#}$=$p<0.05$ vs OB (n = 6 per group). We used Student's t-test in these analyses.

DISCUSSION

Obesity is closely associated with hepatic insulin resistance, which is one of the major phenomena responsible for fasting hyperglycemia and T2DM origin (PETERSEN; SHULMAN, 2017; TILG; MOSCHEN; RODEN, 2017). Thus, it is of great importance to identify new strategies to increase hepatic insulin sensitivity and to reduce hepatic gluconeogenesis. Here, we demonstrated that strength training could increase hepatic insulin sensitivity, resulting in greater GSK3β phosphorylation, increasing FOXO1 translocation from nucleus to the cytoplasm, which in turn reduces its activity and leads to a decrease of PEPCK and G6Pase. Thus, the HGP control has been improved in trained animals, culminating in the reduction of fasting hyperglycemia. Interestingly, these results were observed regardless of reduction in animals adiposity.

Aerobic training has been pointed as an efficient tool to combat obesity-associated liver metabolic complications (CHANG et al., 2006; MARCINKO et al., 2015; SARGEANT et al., 2018a; SHAH et al., 2009). Obese mice that performed aerobic swimming training for 8 weeks reduced the glycemic levels during ipPTT when compared to sedentary obese animals (MARINHO et al., 2012a). It has been recently shown that aerobic treadmill training prevents the reduction of Akt phosphorylation in the liver of HFD-induced obese mice (MUÑOZ et al., 2018a). Moreover, a recent meta-analysis concluded that aerobic training is an efficient strategy for increasing hepatic insulin sensitivity in overweight and obese subjects, being considered an important tool in T2DM treatment (SARGEANT et al., 2018a). On the other hand, despite the growing evidence that strength training has a protective effect against metabolic disorders (KLIMCAKOVA et al., 2006; PEREIRA

et al., 2017a), its effects on HGP mechanisms in obesity and T2DM remains poorly investigated. As commented above, in the present study we found that obese mice submited to short-term strength training increases liver Akt phosphorylation after insulin stimulus, reverting the obesity-induced hepatic insulin resistance. Thus, we can affirm that strength training can be a good tool to improve the insulin action in the liver in obese state.

As previously described, one of the main functions of insulin in the liver is to provide GSK3β phosphorylation and inactivation by mechanisms dependents of Akt activation, thereby increasing GS activity and initiating glycogen synthesis (PERRY et al., 2014). Thus, GSK3β activity has been described as a determinant in the genesis of complications associated with insulin resistance (NABBEN; NEUMANN, 2016; RAO et al., 2007). In their study, Rao and colleagues observed that the use of L803-mts (highly specific peptide inhibitor of GSK3) improved glycemic homeostasis and reduced hyperinsulinemia and hyperleptinemia in obese mice (RAO et al., 2007). Coherently, overexpression of constitutively activated GSK3β increased the degradation of key proteins for insulin signal transduction in hepatocytes, while inhibition of GSK3β kinase activity provided the opposite effect (LENG et al., 2010). Finally, obese and insulin-resistant animals decreased hepatic GSK3β phosphorylation in response to insulin (MARINHO et al., 2012a). Also, aerobic training was effective in reversing this situation, culminating in an increase in hepatic glycogen (MARINHO et al., 2012a). Herein, we demonstrated that 15 strength exercise sessions were able to augment GSK3β phosphorylation, which is a new biomolecular mechanism whereby strength training can provide benefits for hepatic glycemic metabolism, regardless of the reduction in body fat.

On the other hand, FOXO1 is known as a crucial protein for general metabolic control and hepatic gluconeogenesis, being activated under fasting conditions and inactivated by Akt-mediated phosphorylation (LU et al., 2012; PERRY et al., 2014). Furthermore, mice with liver-specific deletion of both Akt1 and Akt2 showed nuclear FOXO1 hyperactivation, followed by severe hyperglycemia, glucose intolerance, and hyperinsulinemia. In contrast, FOXO1 deletion in the same animal model reversed these metabolic complications (LU et al., 2012). Xiong and colleagues demonstrated that, after being exposed to HFD for 3 months, mice with deletion of hepatic FOXO1/3/4 were protected from T2DM, since they were normoglycemic and with greater insulin sensitivity than their wild type controls (XIONG et al., 2013). On

the other hand, mice overexpressing FOXO1 in the liver show increased gluconeogenesis, followed by glucose intolerance and fatty liver accumulation (QU et al., 2006). Thus, several studies have been conducted aiming to identify strategies to reduce FOXO1 activity in the liver (LANGLET et al., 2017; PAJVANI; ACCILI, 2015). Recently, Langlet and colleagues identified several FOXO1 inhibitors (LANGLET et al., 2017); however, the applicability of these inhibitors has some limitations: 1) the use of inhibitors has an undesirable side effect of increasing TG synthesis; 2) pharmacokinetic properties preclude their application in vivo. In contrast, we observed that strength training is an efficient strategy for reducing FOXO1 activity, ameliorating not only HGP and glycemia but also serum and hepatic TG levels.

Based on the fact that FOXO1 acts as a transcription factor for gluconeogenic genes (PUIGSERVER et al., 2003), the increase in its nuclear extrusion provided by strength training has resulted in a reduction of PEPCK and G6Pase protein contents, regardless of changes in the amount of adipose tissue. Several other studies that evaluated the effects of aerobic training in obese animals had already shown similar results. After 8 weeks of treadmill training, Chang and colleagues observed a significant reduction in PEPCK expression in obese Zucker rats, with a reduction in obesity-induced hyperglycemia (CHANG et al., 2006). Similarly, 8 weeks of swimming reversed the elevation of both PEPCK and G6Pase in the liver of HFD-fed swiss mice (Marinho *et al.*, 2012). However, despite an increase in hepatic Akt phosphorylation in response to insulin after short-term strength training in obese mice (PEREIRA et al., 2019), the mechanisms involved in HGP control had not been investigated. Thus, for the first time, we highlighted that strength training could increase FOXO1 translocation from the nucleus to the cytoplasm, reducing the content of PEPCK and G6Pase and consequently reducing the hyperglycemia of obese and diabetic animals. Again, these positive results occurred regardless of adiposity reduction. Furthermore, even though mice lacking hepatic G6Pase have Glycogen storage disease type 1a and hepatocarcinoma (MUTEL et al., 2011), it has recently been shown that G6Pase partial reduction protected the mice against gains in adiposity and aging-related insulin resistance (KIM et al., 2015).

In summary, short-term strength training improved hepatic insulin sensitivity, culminating in increased Akt activation and, consequently, in greater GSK3β inactivation and FOXO1 nuclear extrusion. Thus, PEPCK and G6Pase levels were reduced, improving HGP control (Fig. 6). These phenomena were observed

regardless of body adiposity reduction, which is a new straight mechanism by which strength training becomes an important strategy in combating hyperglycemia and hypertriglyceridemia associated with obesity and T2DM.

***Insert Figure 6 here.**

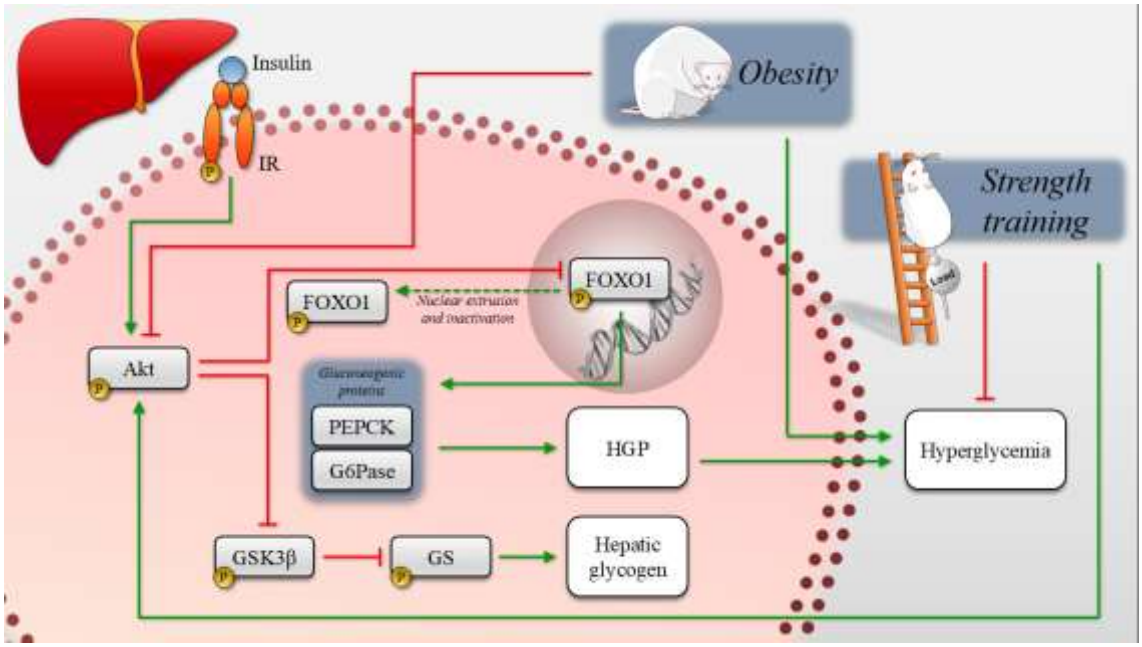

Figure 6: Role of short-term strength training on glucose metabolism in the liver, independent of body weight change. The diet-induced obesity led to hepatic liver resistance in mice, providing hyperglycemia and reduction in the control of HGP, reducing AKT^{ser473}, GSK3β^{ser9} and FOXO1^{ser256} phosphorylation. Thus, FOXO1 was maintained in the cellular nucleus, inducing an increase in PEPCK and G6Pase levels. However, short-term strength training restored liver insulin sensitivity, increasing the FOXO1 nuclear extrusion and the control of HGP. FOXO1: Forkhead box protein 1; G6Pase: Glucose- 6-Phosphatase; GS: Glycogen Synthase; GSK3β: Glycogen Synthase Kinase-3β; IR: Insulin receptor; HGP: Hepatic glucose production; PEPCK: Phosphoenolpyruvate Carboxykinase.

4.3 ARTIGO 3

Embora já bem-descrita a participação da proteína FOXO1 no controle da produção hepática de glicose, mais uma proteína vem ganhando destaque nesse contexto: a proteína Piruvato Carboxilase (PC), que atua no início do processo de gliconeogênese e tem sua atividade aumentada no fígado de diabéticos. Portanto, nosso próximo estudo teve como objetivo investigar os efeitos do treinamento de força de curta duração sobre os níveis de PC no fígado de camundongos obesos.

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Journal of Endocrinology | R M Pereira, K C C Rodrigues *et al.* | Strength training reduces hepatic PC levels | 247:2 | 127–138

RESEARCH

Strength exercise reduces hepatic pyruvate carboxylase and gluconeogenesis in DIO mice

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*(R M Pereira and K C C Rodrigues contributed equally to this work)

Abstract

Obesity is linked to a reduction in the control of hepatic glucose production, which is the primary mechanism related to fasting hyperglycemia and the development of type 2 diabetes mellitus (T2DM). The main system involved in hepatic gluconeogenesis synthesis is controlled by pyruvate carboxylase (PC), which increases in obesity conditions. Recently, we showed that short-term strength training is an important tool against obesity-induced hyperglycemia. As aerobic exercise can reduce the hepatic PC content of obese animals, we hypothesized that strength exercise can also decrease this gluconeogenic enzyme. Therefore, this study investigated whether the metabolic benefits promoted by short-term strength training are related to changes in hepatic PC content. Swiss mice were divided into three groups: lean control (Ctl), obese sedentary (ObS), and obese short-term strength training (STST). The STST protocol was performed through one session/day for 15 days. The obese exercised animals had reduced hyperglycemia and insulin resistance. These results were related to better control of hepatic glucose production and hepatic insulin sensitivity. Our bioinformatics analysis showed that hepatic PC mRNA levels have positive correlations with glucose levels and adiposity, and negative correlations with locomotor activity and muscle mass. We also found that hepatic mRNA levels are related to lipogenic markers in the liver. Finally, we observed that the obese animals had an increased hepatic PC level; however, STST was efficient in reducing its amount. In conclusion, we provide insights into new biomolecular mechanisms by showing how STST is an efficient tool against obesity-related hyperglycemia and T2DM, even without body weight changes.

Key Words

- strength training
- pyruvate carboxylase
- obesity
- liver
- T2DM

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Introduction

De novo synthesis of glucose from glycan precursors (non-sugars or non-carbohydrates), such as lactate, amino acids, and glycerol, is defined as gluconeogenesis. This synthesis is controlled by the liver and the cortex of the kidneys and is important for the maintenance of glycemic homeostasis, mostly under fasting conditions (Zhang *et al.* 2018). Due to insulin resistance, obese individuals present uncontrolled hepatic gluconeogenesis; thus, any derangement in this endogenous glucose production may contribute to the maintenance of fasting hyperglycemia and, consequently, to the development of type 2 diabetes mellitus (T2DM) (Magnusson *et al.* 1992, Gastaldelli *et al.* 2000, Basu *et al.* 2005). Therefore, it is of paramount importance to understand the mechanisms that underlie hepatic gluconeogenesis for the treatment and prevention of T2DM, providing new perspectives for glycemic control, since most of the current strategies are based on modulating insulin secretion and reducing insulin resistance.

Initially, there are mainly two mechanisms for hepatic gluconeogenesis to occur. First, inside the mitochondria, the main mechanism is controlled by the enzyme pyruvate carboxylase (PC), which forms oxaloacetate (OXA) from the carboxylation of pyruvate and provides substrate for the second step to start. In the cytosol, the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) continue the synthesis of a new glucose molecule from the OXA generated by the PC (Weber & Cantero 1954, McGilvery & Mokrasch 1956, Utter & Keech 1960). In 2001, the enzymes PEPCK and G6Pase were listed as the main ones responsible for increasing hepatic gluconeogenesis (Yoon *et al.* 2001). However, in 2009, it was shown that fasting hyperglycemia was not associated with these enzymes in humans (Samuel *et al.* 2009), highlighting that more attention should be directed to the role of PC in the liver gluconeogenesis process (Jitrapakdee *et al.* 2008). This is because PC, by providing the substrate, is an indispensable enzyme for gluconeogenesis.

Gluconeogenesis, hepatic glucose production (HGP), and hepatic PC might be regulated by an indirect effect of insulin on suppressing white adipose tissue (WAT) lipolysis, which in turn decreases hepatic acetyl-CoA (an allosteric activator of PC), leading to reduced hepatic PC (Perry *et al.* 2015). On the other hand, high fat-fed rats showed insulin resistance in WAT, and increased PC activity; however, when these animals were treated with atglistatin, an inhibitor of adipose triglyceride lipase, they showed normalized hepatic acetyl-CoA content, PC activity, and HPG (Perry *et al.* 2015). An elegant study showed that diabetic animals have around 2.5 times more PC than control lean animals (Weinberg & Utter 1980). In 2013, Kumashiro and collaborators showed that obese animals had increased hepatic PC levels and, after reducing its synthesis (using a specific antisense oligonucleotide – ASO), it was possible to observe an improvement in insulin sensitivity, a reduction in endogenous glucose production, and, consequently, a reduction in hyperglycemia in obese rodents (Kumashiro *et al.* 2013). Furthermore, the authors showed that the relative hepatic PC levels were directly correlated with serum glucose and glycated hemoglobin levels in humans (Kumashiro *et al.* 2013), unlike the association of PEPCK and G6Pase with serum glucose (Samuel *et al.* 2009). Therefore, as PC has been highlighted as the main molecule involved in hepatic gluconeogenesis, studies aiming to reduce its activity are crucial in trying to control fasting hyperglycemia in diabetic subjects.

In this context, it is known that the regular practice of aerobic exercise is an important non-pharmacological tool that assists in the control of fasting hyperglycemia. Muñoz and colleagues showed that an 8-week aerobic training protocol decreased body weight, fasting hyperglycemia, and hyperinsulinemia in obese mice, as well as improving insulin signaling. Interestingly, these data were accompanied by a reduction in hepatic PC levels (Muñoz *et al.* 2018). On the other hand, the effects of strength physical exercise and body weight changes on PC levels were not addressed. Recently, we verified that a short-term strength exercise protocol reduced HGP, ameliorating glycemic homeostasis without body weight changes (Pereira *et al.* 2019). However, it was not described whether this improvement in glucose metabolism is linked to PC levels. Therefore, the present study evaluated the effects of short-term strength exercise on the hepatic PC levels and glucose homeostasis of diet-induced obese (DIO) mice, with no differences in body adiposity.

Material and methods

Animals and diet

All the animal procedures were previously approved by the Ethics Committee on Animal Use (CEUA) in Biological Sciences (UNICAMP – Campinas – SP, case number 4406-1) and carried out according to the Brazilian legislation on the scientific use of animals (Law No. 11,794, of October 8, 2008). In the present study, 8-week-old male

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Swiss mice were used, provided by the Multidisciplinary Center for Biological Research/UNICAMP. The animals arrived at 4 weeks old and were maintained in individual polyethylene cages with an enriched environment as previously described (Pereira *et al.* 2019). Briefly, animals were kept with a ratio of 12 h light:12 h darkness, the temperature was controlled at 22 ± 2°C, and water and food (chow or high-fat diet) were offered ad libitum. More details about animal conditions were published before (Pereira *et al.* 2019).

The first step of this study was to induce obesity through diet, and at this point, the animals were distributed into two groups: control lean group (Ctl), fed a chow diet, and DIO group, fed a high-fat diet (HFD). The diet-induced obesity protocol lasted 14 weeks, and for the second step of this study, the obese group was equally redistributed according to the mice's body weight and fasting glycemia into two groups: (1) obese sedentary (ObS), consisting of obese mice that remained sedentary throughout the experiment, and (2) short-term strength training (STST), consisting of mice that performed the strength training protocol. The HFD was prepared according to the American Institute of Nutrition (AIN-93G) guidelines (Reeves *et al.* 1993), and it was modified to contain 35% fat (4% soy oil and 31% lard) (Oliveira *et al.* 2015).

Short-term strength training protocol

The short-term strength training protocol was already published in detail by our research group (Pereira *et al.* 2019). Briefly, the protocol was performed on a ladder where the mice carried the load apparatus fixed with adhesive tape to their tails. Before the strength training protocol, the animals were adapted to the ladder and load apparatus for 5 consecutive days.

After the adaptation protocol, the mice rested for 48 h. Then, the rodents were subjected to the maximal voluntary carrying capacity (MVCC) test to determine the maximum load with which each animal could climb the entire length of the ladder. The load for the first attempt at the MVCC test was equivalent to 75% of the animals' body weight. In the subsequent attempts, an incremental overload (5 g) was added in each further attempt to climb until the animal could no longer complete the entire course. The mice rested for 5 min in an individual cage between each attempt, and the heaviest overload carried in the last successful attempt was considered the animal's MVCC, and this value was used to prescribe the individual loads in the experiment.

The short-term strength training protocol started 48 h after the MVCC determination. The STST consisted of 20 climbing series with an overload of 70% of the MVCC and with a rest interval of 60–90 s between sets. The mice were exercised for 5 consecutive days a week, followed by 2 days of rest, until they completed 13 sessions of physical exercise. After that, the mice underwent the pyruvate tolerance test or insulin tolerance test, and after 24 h, the animals performed two more training sessions, totaling 15 sessions, and then they were euthanized (Fig. 1).

Insulin tolerance test (ITT) and intraperitoneal pyruvate tolerance test (ipPTT)

Eight hours after the end of the 13th exercise session, some of the animals were subjected to the ITT and the rest were subjected to the ipPTT, after 8 h of fasting. For the ITT, the mice received an intraperitoneal injection of recombinant human insulin (Humulin R) from Eli Lilly at a concentration of 1.5 U/kg of body weight. Blood samples were collected from the tail at 0, 5, 10, 15, 20, 25, and 30 min to determine the glycemic levels using a glucometer (Accu-Chek; Roche Diagnostics). Time 0 was measured before the insulin injection. The rate constant for plasma glucose disappearance (kITT) was calculated using the formula 0.693/biological half-life ($t_{1/2}$). Plasma glucose $t_{1/2}$ was calculated from the slope of the least squares analysis of the serum glucose concentration during the linear decay phase (Bonora *et al.* 1989). For the ipPTT, the animals were subjected to an intraperitoneal injection (i.p.) of pyruvate (2 g/kg of sodium pyruvate) (Azis Cientifica®, Cotia, SP, Brazil). The blood samples from the animals were drawn from the tail before the pyruvate injection, which was considered as time 0, and 30, 60, 90, and 120 min after the injection to determine the blood glucose concentration. The ITT and ipPTT results were evaluated based on the areas under the serum glucose curves (AUC) during the test using the trapezoidal method (Matthews *et al.* 1990) in Microsoft Excel (2013) (Microsoft Corporation).

Tissue extraction and immunoblotting analysis

The euthanasia was performed 8 h after the 15th exercise session, and during those 8 h the animals were also fasted. Ten minutes before the euthanasia, the animals received either human insulin (8 U/kg body weight Humulin-R; Lilly) or saline via i.p. The animals were anesthetized via i.p. by the injection of chloral hydrate of ketamine (100 mg/kg, Parke-Davis, Ann Arbor, MI, USA) and xylazine (10 mg/kg, Rompun, Bayer),

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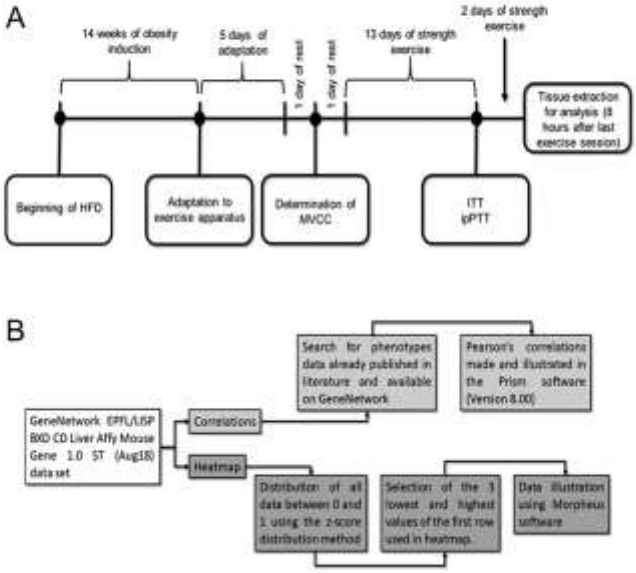

Figure 1
Experimental design and pipeline schematic regarding bioinformatics analysis. (A) Schematic representation of the experiments. The insulin tolerance test (ITT), intraperitoneal pyruvate tolerance test (ipPTT), and tissue extraction were performed 8 h after the exercise session and 8 h of fasting. (B) Pipeline schematic describing the steps performed.

and after verification and assurance of the lack of corneal reflexes, the liver was collected and rapidly snap-frozen in liquid nitrogen and stored at -80°C until analysis. The epididymal and retroperitoneal adipose tissue (right side) were removed and weighed to measure the fat depots. The liver was homogenized in extraction buffer (1% Triton-X 100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.1 mg aprotinin/mL) at 4°C with a TissueLyser II (QUIAGEN®) operated at maximum speed for 120 s. The lysates were centrifuged (Eppendorf 5804R) at 12,851 ***g*** at 4°C for 15 min to remove insoluble material, and the supernatant was used for the assay. The protein content was determined by the bicinchoninic acid method (Walker, 1994). The samples containing 60 µg of total protein were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk at room temperature for 1 h and incubated with primary antibodies against the protein of interest. After that, a specific secondary antibody was used. The specific bands were labeled by chemiluminescence, and visualization was performed by the G:BOX photodocumentation system (Syngene). The bands were quantified using the ImageJ 1.51s software. The primary antibodies used were: anti-Phospho-Akt ser473 (4060), anti-Akt (4685), and anti-β-actin (3700) from Cell Signaling Technology®, and anti-PC (sc271493) from Santa Cruz Biotechnology®. The secondary antibodies used were anti-rabbit and anti-mouse, from Cell Signaling Technology® . More details about the antibodies used can be found in Supplementary Table 1 (see section on supplementary materials given at the end of this article).

Reverse transcription and quantitative polymerase chain reaction (RTqPCR)

Total RNA was extracted from the tissues using the TRIzol methodology (Invitrogen). The cDNA was synthesized using the High Capacity cDNA RT Kit (Thermo Fisher Scientific). The qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The following primers were evaluated: *PC* – forward: GACGGCGAGGAGATAGTGTC; reverse: CATGGACTGTTCGGAACTTCA and *Actb* – forward: TGTCGAGTCGCGTCCA; reverse: TCATCCATGGCGAACTGGTG (used as the normalizing gene). Samples for this reaction, in triplicate, were made in a 96-well plate (MicroAmp, Applied Biosystems) for amplification and reading in the Step One Plus Real-Time

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PCR System (Applied Biosystems). The primers used were designed using the Primer-BLAST software (NCBI).

Bioinformatics analysis

All the data used in this bioinformatics analysis were provided by GeneNetwork (http://www.genenetwork.org/) using the EPFL/LISP BXD CD Liver Affy Mouse Gene 1.0 ST (Aug 18) data set. The Pearson's correlation was calculated using Prism (8.0.1) GraphPad Software with phenotypes provided by previous experiments available in GeneNetwork (Philip *et al.* 2010, Andreux *et al.* 2012, Williams *et al.* 2016) and heat maps were made using the Morpheus software (https://software.broadinstitute.org/morpheus/). To better describe the bioinformatics analysis, a pipeline illustration was drawn and can be found in Fig. 1B. Briefly, the same data set was used to devise the correlations and the heat map. The *PC* levels were correlated with phenotypes related to body composition, glucose metabolism, and locomotor activity. Moreover, to make the heat map, all data used were distributed in values between 0 and 1 using the z-score distribution method, and then the data were organized in ascending order and the three lowest and highest values of the first row (locomotor activity or fat mass) were used to create the heat map.

Statistical analysis

The results were shown as the mean ± S.E.M.. The Shapiro–Wilk test was used to evaluate the Gaussian distribution of the data, and Student's *t*-test was used to compare the two groups with parametric data when necessary. Furthermore, to compare more than two groups, the one-way ANOVA test was performed, followed by Bonferroni's post hoc test. To analyze the points of the ITT and ipPTT, the two-way ANOVA (with repeated measures when appropriate) was used, with Bonferroni's correction for multiple comparisons. The statistical significance level considered was $P < 0.05$. The graphics were created and the statistical analysis performed using Prism (7.00) GraphPad Software.

Results

Short-term strength training reduces blood glucose levels and increases insulin sensitivity in obese mice, even without changes in body adiposity

After obesity induction with a HFD, the animals in the ObS group presented increased body mass, fasting glycemic levels, and body adiposity compared to the Ctl animals (Fig. 2A, B, C and D). However, after 15 strength training

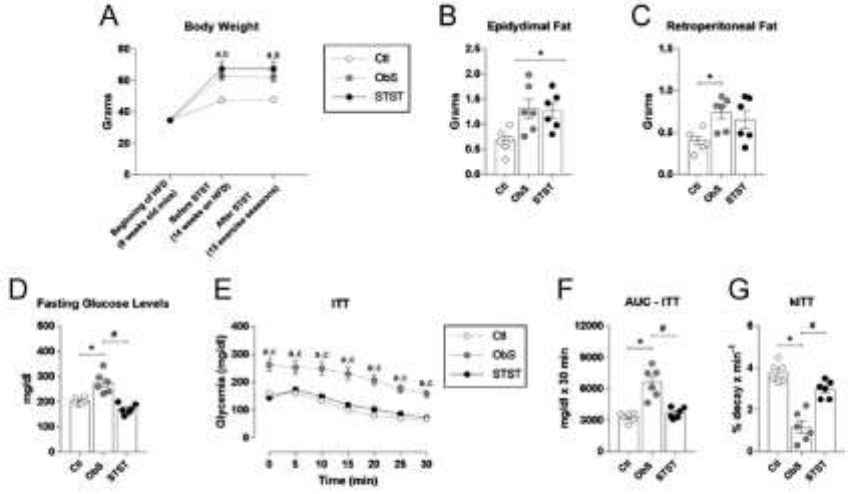

Figure 2
Physiological parameters of experimental groups. (A) Body weight at beginning of HFD, before and after STST protocol. (B and C) Weight of epididymal and retroperitoneal fat, respectively. (D) Fasting glucose levels after 8 h of fasting. (E) Glucose levels during ITT. (F) Area under the curve during ITT. (G) Rate constant for plasma glucose disappearance. In A and E: $^{a}P < 0.05$ for Ctl vs ObS; $^{b}P < 0.05$ for Ctl vs STST; $^{c}P < 0.05$ for ObS vs STST. In B, C, D, F, and G: $^{*}P < 0.05$ vs Ctl; $^{\#}P < 0.05$ vs ObS ($n = 6$ per group).

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sessions, the animals in the STST group showed identical fasting glycemic levels to the Ctl group, even without changes in body composition (Fig. 2A, B, C and D). After insulin injection, the animals in the ObS group showed higher glycemic levels and impaired kITT compared to the Ctl group, reinforcing the insulin resistance status in these animals (Fig. 2E, F and G). But the STST group showed the same pattern as the lean animals, with no difference in the AUC and kITT (Fig. 2E, F and G). More details about Fig. 2 can be found in Supplementary Figure 2.

Obese trained animals showed increased hepatic insulin sensitivity and better control of HGP compared to sedentary obese animals

In the next step, the effects of obesity and short-term strength training were checked using parameters related to hepatic insulin sensitivity and control of HGP. Eight hours after the 13th strength training session, the animals from the three experimental groups were subjected to the ipPTT, receiving an intraperitoneal injection of pyruvate solution and having their glycemic values checked every 30 min, from 0 to 120 min into the experiment. As shown in Fig. 3A, the animals in the ObS group showed increased glycemic values compared to the animals in the Ctl group throughout the 120 min of the test. However, the animals in the STST group showed a reduction in these values at all points. Consequently, the AUC during the test was higher for the ObS group compared to the other two groups, while the STST group showed no difference from the Ctl group (Fig. 3B).

To assess the hepatic insulin sensitivity, we measured hepatic pAkt^{ser473} levels after an intraperitoneal insulin injection, since Akt activation is a critical step of the insulin-signaling pathway in the liver (Kubota *et al.* 2017). We observed that the ObS group animals had significant hepatic insulin resistance, with reduced levels of pAkt^{ser473} even without changes in total Akt levels (Fig. 3C, D and E). On the other hand, the trained animals presented increased Akt levels and phosphorylation compared to the animals in the ObS group (Fig. 3F, G and H), showing that STST is a new strategy to increase hepatic insulin sensitivity in obese animals. More details about Fig. 3 can be found in Supplementary Figure 3.

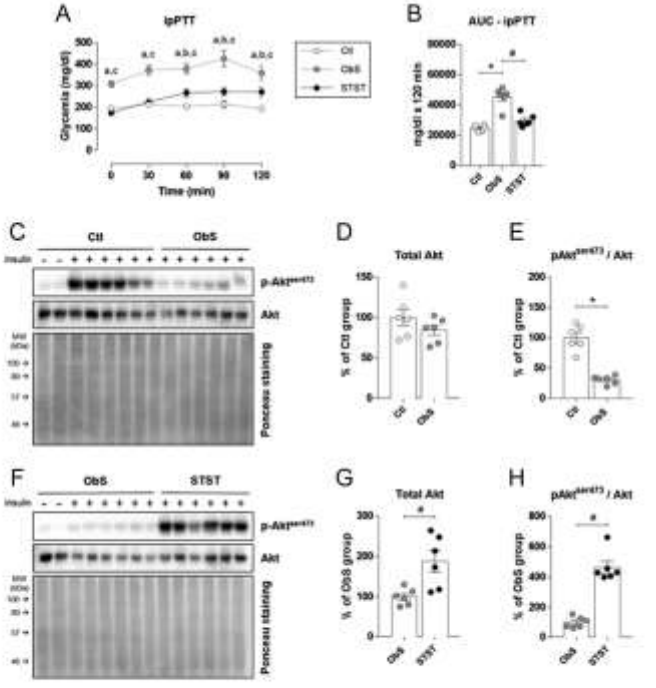

Figure 3
ipPTT and hepatic insulin sensitivity. (A) Glucose levels during ipPTT. (B) Area under the curve during ipPTT. (C) Bands of hepatic pAkt^{ser473} and total Akt of Ctl and ObS groups after insulin injection. (D) Quantification of total Akt levels of Ctl and ObS groups. (E) Quantification of hepatic pAkt^{ser473}/Akt of Ctl and ObS groups. (F) Bands of hepatic pAkt^{ser473} and total Akt of ObS and STST groups after insulin injection. (G) Quantification of total Akt levels of ObS and STST groups. (H) Quantification of hepatic pAkt^{ser473}/Akt of ObS and STST groups. Only the bands of the animals stimulated with insulin were quantified. The quantification of total Akt levels were normalized by Ponceau staining, and pAkt^{ser473} was normalized by total Akt quantification. In A: $^{a}P < 0.05$ for Ctl vs ObS; $^{b}P < 0.05$ for Ctl vs STST; $^{c}P < 0.05$ for Ob5 vs STST. In B, D, E, G, and H: $^{A}P < 0.05$ vs Ctl; $^{B}P < 0.05$ vs ObS ($n = 6$ per group).

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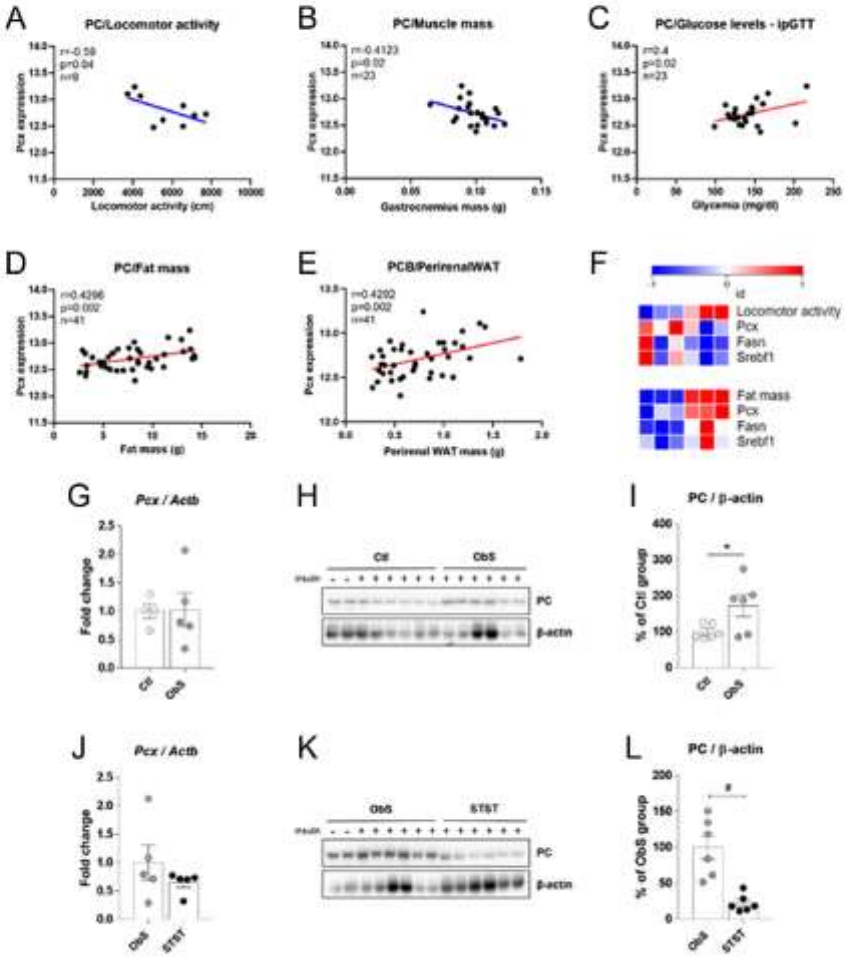

Figure 4

Bioinformatics analyses and hepatic PC levels. (A) locomotor activity; (B) muscle mass; (C) blood glucose levels in GTT 60 min after glucose intraperitoneal injection; (D) fat mass; (E) perirenal WAT mass. Blue lines represent negative correlations and red lines represent positive correlations. (F) Heat map showing how the lipogenic genes decrease with more activity stimulus and increase with fat accumulation. (G) Hepatic *PC/Actb* mRNA levels of Ctl and ObS groups ($n = 4$–5 per group). (H) Western blot analysis of hepatic PC and β-actin of Ctl and ObS groups ($n = 6$ per group). (I) Quantification of bands of hepatic PC/β-actin of Ctl and ObS groups. (J) Hepatic *PC/Actb* mRNA levels of ObS and STST groups ($n = 4$–5 per group). Only the bands of the animals stimulated with insulin were quantified. (K) Western blot analysis of hepatic PC and β-actin of ObS and STST groups ($n = 6$ per group). (L) Quantification of bands of hepatic PC/β-actin of ObS and STST groups. *P < 0.05 vs Ctl; #P < 0.05 vs ObS. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0193.

Bioinformatics analysis and effects of obesity and short-term strength training on hepatic PC levels

To check the relevance of PC in overall metabolic control, we first evaluated the correlation between hepatic *PC* levels and several phenotypes using a public database with a large panel of isogenic BXD mice strains. We found that the expression of this *PC* gene was positively correlated with glucose levels, fat mass, and peritoneal WAT, revealing the harmful effects of obesity on PC levels

(Fig. 4A, B, C, D and E). On the other hand, the *Pc* expression was negatively correlated with locomotor activity and muscle mass, indicating that physical activity might be an alternative to reduce PC levels. Moreover, the first heat map (Fig. 4F) shows that with an increase in locomotor activity, both *Pc* expression and lipogenic genes (*Fasn* and *Srebpf1*) decrease. In contrast, the opposite effect is shown in the second heat map, where the expression of those genes increases with the highest fat mass levels.

Finally, we investigated whether obesity and STST can change PC levels in mice hepatocytes. Initially, we observed that obesity increased the hepatic PC protein levels of obese mice (Fig. 4H and I), even without changing the mRNA levels of *Pc* (Fig. 4G). In contrast, after 15 sessions of strength training, the trained animals presented reduced PC protein levels (Fig. 4K and L), also without changes in *Pc* mRNA levels (Fig. 4J). More details about Fig. 4 can be found in Supplementary Figure 4.

Discussion

Insulin resistance is indicated as the main factor responsible for the onset of T2DM and cardiovascular diseases related to hyperglycemia (Patel & Goyal 2019). By 2050, it is estimated that 33% of the US population will be diagnosed with insulin resistance, and it will be one of the main causes of death (Boyle *et al.* 2010). In this context, hepatic insulin resistance plays a fundamental role in hepatic glucose production (HGP), since it is known that the failure of insulin to inhibit HGP is a crucial factor in the perpetuation of hyperglycemia in diabetic patients (Magnusson *et al.* 1992, Petersen & Shulman 2017). Several studies demonstrate that obesity is strongly associated with hepatic insulin resistance, and despite the significant advances of medicine (Tilg *et al.* 2017, Foretz *et al.* 2019), lifestyle changes such as physical training, leading to increased energy expenditure and reduced body adiposity, are still the most promising interventions with the best results (Bacchi *et al.* 2013, Sargeant *et al.* 2018, Pereira *et al.* 2019). However, the biomolecular mechanisms involved in the increased hepatic insulin sensitivity in obese patients provided by physical exercise remains poorly understood. In the present study, we demonstrated that a short period of strength training was able to increase insulin sensitivity and hepatic Akt phosphorylation in response to insulin in obese mice, culminating in the improved control of HGP and normalization of fasting blood glucose levels. Furthermore, we also showed that the training reversed the increment in hepatic PC content induced by obesity, indicating a new mechanism by which strength training can be classified as an essential strategy against hepatic insulin resistance. Another important point of our study is that we did not observe any reduction in fat deposits after the exercise protocol, showing that the aforementioned effects were directly caused by the training, and were not side effects related to the decrease in body adiposity after the long-term exercise protocol.

Previously to short-term strength training, the animals were subjected to DIO. We observed that the sedentary obese animals showed an increase in both body mass and body adiposity, providing an increase in fasting glucose. However, after 15 sessions of strength training, the glycemic values of the trained animals returned to normal levels, showing no difference from the lean animals, thus corroborating data previously found both in rodents subjected to the same conditions (Pereira *et al.* 2019) and in women with liver insulin resistance who performed strength training for 4 months (Honka *et al.* 2016). We also observed that the animals in the ObS group showed reduced insulin sensitivity, with higher glycemic levels and less glucose uptake after intraperitoneal insulin injection, as is well described in the literature (Oliveira *et al.* 2015, Roden & Shulman 2019). Previous studies have already shown that strength training can be an important tool against obesity-associated insulin resistance (Klimcakova *et al.* 2006, Tang *et al.* 2014). After 8 weeks of strength training, obese rats showed both an improvement in insulin action and higher glucose tolerance, assessed by the ipITT and oral glucose tolerance test, respectively (Tang *et al.* 2014). Similar, data were found in a study involving obese male subjects that showed increased insulin sensitivity in the euglycemic-hyperinsulinemic clamp after 3 months of dynamic strength training (Klimcakova *et al.* 2006). In the present study, we showed that a short period of strength training is also able to increase insulin action in obese mice, providing higher glucose uptake and reversing hyperglycemia.

Similarly, the trained animals also showed more activation of Akt and better control of HGP compared to the animals in the ObS group, reversing obesity-induced liver insulin resistance. There are already a large number of studies showing that aerobic training provides several benefits to the liver in obesity conditions (Pereira *et al.* 2017, Sargeant *et al.* 2018). After 8 weeks of swimming, obese mice showed higher phosphorylation of hepatic Akt after insulin injection, also with reduced fasting glycemia and independently of any adiposity reduction

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(Marinho *et al.* 2012). The authors also observed improved control of HGP in the trained animals, with lower glycemic values at all points in the ipPTT (Marinho *et al.* 2012). A recent meta-analysis involving 94 participants from six different studies showed that aerobic training can increase insulin action in the liver of overweight subjects (Sargeant *et al.* 2018). Another important finding was that strength training also provided an increase in total Akt levels. It is widely demonstrated that despite the lower activation of Akt in the liver of obese animals with non-alcoholic fatty liver disease (NAFLD), their total protein levels remain unchanged (Xiao *et al.* 2018, Xu *et al.* 2020). Similarly, several studies using obese rodents subjected to aerobic exercise have not observed changes in Akt levels, not even after an acute session (Ropelle *et al.* 2009) such as long-term aerobic training (Oliveira *et al.* 2011, Muñoz *et al.* 2018). However, in the present study, the animals subjected to the short-term strength training protocol showed an increase in total hepatic Akt content, revealing another mechanism by which strength training contributes to an increase in insulin action in NAFLD. However, despite the initial evidence showing that strength training provides significant metabolic improvements in obesity conditions (Klimcakova *et al.* 2006, Pereira *et al.* 2017), the mechanisms related to these benefits remain poorly explored.

Recently, our research group demonstrated that after 15 sessions of strength training, obese mice showed reduced hepatic lipogenesis, fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC) contents, and increased ACC phosphorylation, suppressing their lipogenic activity (Pereira *et al.* 2019). Thus, the hepatic lipid content was reduced, increasing insulin action and the control of HGP. However, the mechanisms that control hepatic gluconeogenesis in these animals have not been explored. We suggest that short-term strength training can provide better control of HGP, reducing hepatic PC content. As previously discussed, PC is known to be an essential protein in HGP control, mediating the conversion of pyruvate to OXA and initiating gluconeogenesis (Utter & Keech 1960). Kumashiro and colleagues observed that HFD-induced obesity increased hepatic PC content, although their transcripts did not change (Kumashiro *et al.* 2013). However, the use of ASO to reduce PC expression concomitant with a HFD provided better control of HGP in rodents, since they showed reduced glycemic values at all points during the ipPPTT, increased Akt phosphorylation, and lower basal endogenous glucose production, confirming the relevant participation of PC in maintaining healthy blood glucose levels (Kumashiro *et al.* 2013). Similarly, our bioinformatics analysis, which includes a large panel of isogenic BXD mice strains, revealed that mice with higher hepatic *PC* transcription had elevated glycemic values during a glucose tolerance test, thus corroborating our data showing increased PC levels and impaired insulin sensitivity in sedentary obese mice. Furthermore, our bioinformatics analysis also demonstrated a strong negative correlation between *PC* levels and both locomotor activity and muscle mass, two phenotypes commonly associated with physical exercise (Ross *et al.* 2019), thus reinforcing our hypothesis that the metabolic benefits provided by strength training could be mediated by mechanisms involving PC.

Finally, we assessed whether strength training was able to alter the protein content of hepatic PC. Muñoz and colleagues demonstrated that aerobic exercise can lead to an improvement in the hepatic metabolism of obese rodents by reducing PC levels (Muñoz *et al.* 2018). However, the effects of strength training in this context and without changes in body adipose remain unexplored. In the mitochondria, when there is an excess of energy, high levels of Acetyl-CoA activate PC and stimulate the conversion of pyruvate to OXA to initiate the formation of a new glucose molecules. When there are high levels of mitochondrial NADH, the mitochondrial malate dehydrogenase converts OXA to malate so it can be carried to the cytoplasm. Then, in the reduction process (NAD^+ to NADH), cytosolic malate dehydrogenase converts malate into OXA again (Jitrapakdee *et al.* 2008). Furthermore, this cytoplasmic OXA is converted to phosphoenolpyruvate by PEPCK and, after passing through a reverse glycolysis process, the G6Pase enzyme is able to remove a phosphate group from the glucose-6-phosphate molecule, facilitating its access to the circulatory system (Weber & Cantero 1954). In the present study, it was possible to observe that only 15 strength exercise sessions were sufficient to reduce the HGP of obese animals. According to the data obtained in this study, we suggest that this improvement in the animals' fasting hyperglycemia was mediated by the reduction in PC levels. Because the exercised obese animals showed a reduction in PC content, consequently, it is estimated that there was a reduction in the intracellular OXA levels and, then, lower substrate availability for gluconeogenesis. Nevertheless, these animals showed an improvement in hepatic insulin sensitivity, which may have favored the attenuation of PEPCK and G6Pase synthesis, thus contributing to a reduction in gluconeogenesis.

Therefore, we provide fresh evidence about a new mechanism by which strength training acts against

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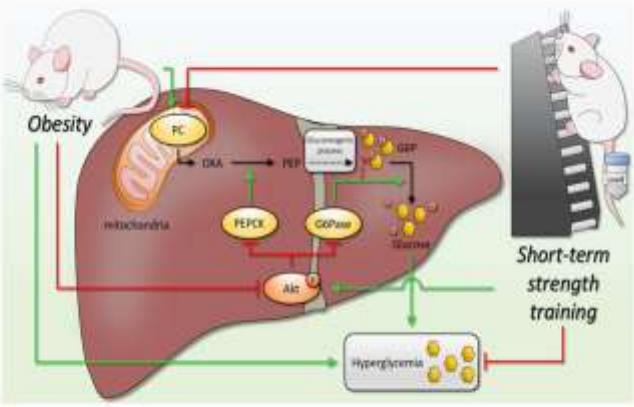

Figure 5
Schematic mechanism of the effect of short-term strength training on hepatic PC levels, independent of body weight changes. Obesity increases hepatic PC levels and reduces hepatic insulin action and HGP control, causing hyperglycemia. Short-term strength training reduces hepatic PC levels and counteracts these adverse metabolic effects in obese mice, even without changes in adiposity. This is a new biomolecular mechanism by which short-term strength training is an efficient tool against obesity-related hyperglycemia and T2DM. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0193.

metabolic complications. As already aforementioned, previous studies have shown that strength training can reduce the protein content and the activity of lipogenic proteins in obese mice (Pereira *et al.* 2019), corroborating previous data observed in humans (Bacchi *et al.* 2013), obese rats (dos Santos *et al.* 2019), and ovariectomized rats (Domingos *et al.* 2012) after long-term strength training. With the reduction in liver fat, the insulin action and the control of HPG in these animals were improved (Pereira *et al.* 2019); however, the relationship between NAFLD and liver insulin resistance remains poorly understood. As shown in the heat map in Fig. 4F, mice with reduced levels of *PC* mRNA also showed a reduction in the expression of lipogenic genes (*Fasn* and *Srebf1*). Therefore, these data allow us to conclude that changes in the hepatic content of PC mediated by obesity may be related to the control of both HGP and fat liver accumulation. On the other hand, even though our bioinformatics analysis showed a positive correlation between hepatic levels of *PC* mRNA and both total fat mass and perineal white adipose tissue, the STST animals showed reduced PC protein content without reducing adiposity, indicating that strength exercise might change PC metabolism through a non-adiposity related mechanism. Interestingly, we found no changes in *PC* mRNA levels in the ObS and STST groups, confirming previous data found in human livers (Kumashiro *et al.* 2013) and suggesting that changes in liver PC protein levels caused by obesity and strength training are related to post-transcriptional changes. However, more studies are needed to understand these mechanisms better.

In conclusion, short-term strength training reduced hepatic PC levels in obese mice, increasing insulin action in the liver, and counteracting HFD-induced insulin resistance and hyperglycemia, as summarized in Fig. 5. Therefore, we suggest a new mechanism by which short-term strength training can be an efficient tool against hepatic metabolic disorders associated with obesity. Importantly, all these results were observed independently of a reduction in body adiposity.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JOE-20-0193.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
L P M designed the paper. R M P and K C C R wrote the paper and had the overall responsibilities for the experiments and the truth of the results in this study. R M P, K C C R, M R S, G F P, and A P M performed the experiments and data collection. R M P performed the statistical analysis. A P M performed the RTqPCR analysis. G F P performed the bioinformatics analysis. L P M contributed to the financial support. F M S, A S R S, D E C E R R, J R P, and L P M contributed to the discussion and provided laboratory support. All the authors have read and approved this manuscript.

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4.4 ARTIGO 4

Uma conhecida proteína capaz de regular negativamente a ação da insulina no tecido hepático é a Proteina Tirosina Fosfatase 1B (PTP1B), induzida por inflamação e largamente relacionada à hiperglicemia. Já é sabido que camundongos idosos resistentes à insulina apresentam redução do conteúdo hepático de PTP1B, porém os efeitos do treinamento de força de curta duração sobre a PTP1B hepática ainda é desconhecido. Portanto, o objetivo do nosso próximo estudo foi avalizar se os efeitos benéficos do treinamento de força no metabolismo hepático de camundongos obesos está relacionado a alterações no conteúdo de PTP1B hepática.

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Short-Term Strength Exercise Reduces Hepatic Insulin Resistance in Obese Mice by Reducing PTP1B Content, Regardless of Changes in Body Weight

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Abstract: Obesity is closely related to insulin resistance and type 2 diabetes genesis. The liver is a key organ to glucose homeostasis since insulin resistance in this organ increases hepatic glucose production (HGP) and fasting hyperglycemia. The protein-tyrosine phosphatase 1B (PTP1B) may dephosphorylate the IR and IRS, contributing to insulin resistance in this organ. Aerobic exercise is a great strategy to increase insulin action in the liver by reducing the PTP1B content. In contrast, no study has shown the direct effects of strength training on the hepatic metabolism of PTP1B. Therefore, this study aims to investigate the effects of short-term strength exercise (STSE) on hepatic insulin sensitivity and PTP1B content in obese mice, regardless of body weight change. To achieve this goal, obese Swiss mice were submitted to a strength exercise protocol lasting 15 days. The results showed that STSE increased Akt phosphorylation in the liver and enhanced the control of HGP during the pyruvate tolerance test. Furthermore, sedentary obese animals increased PTP1B content and decreased IRS-1/2 tyrosine phosphorylation; however, STSE was able to reverse this scenario. Therefore, we conclude that STSE is an important strategy to improve the hepatic insulin sensitivity and HGP by reducing the PTP1B content in the liver of obese mice, regardless of changes in body weight.

Keywords: strength exercise; obesity; liver; insulin signaling; diabetes; PTP1B; gluconeogenesis

1. Introduction

In general, proinflammatory proteins reduce insulin signaling, contributing to the establishment of insulin resistance in different tissues and collaborating with the development of type 2 diabetes *mellitus* (T2DM) in obese subjects [1]. In the hepatocytes, the insulin, after binding to its receptor, initiates a signaling cascade, which will culminate in the phosphorylation of nuclear transcription factor Forkhead Box Protein O1 (FOXO1) promoting

its translocation from the cell nucleus to the cytoplasm [2,3]. One of the main functions of FOXO1 in the hepatocyte cell nucleus is to ally itself with Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha (PGC1-α), which is a nuclear transcription cofactor, and initiate the transcription of molecules that will collaborate with the gluconeogenesis process in the organ [4]. However, when there is insufficient insulin responsiveness, the FOXO1 phosphorylation is reduced causing transcription of gluconeogenic genes and perpetuating exacerbated gluconeogenesis even in postprandial periods [5]. Thus, identifying proteins that negatively interfere in the hepatic insulin sensitivity and strategies to reduce their content become extremely important to improve the quality of life of obese and diabetic people.

Tyrosine-protein phosphatase non-receptor type 1, also known as protein-tyrosine phosphatase 1B (PTP1B), is a member of the protein tyrosine phosphatase (PTP) family [6]. PTPs are proteins with high potential to negatively regulate insulin transduction since PTP1B binds to and dephosphorylates and inactivates important proteins of the insulin pathway such as Insulin Receptor (IR), as well as Insulin Receptor Substrate (IRS) 1 and 2 [7,8]. The elevation of PTP1B in the liver of obese mice, caused by the administration of Tumor Necrosis Factor-alpha (TNF-α), culminated in severe insulin resistance [9]. On the other hand, when PTP1B was deleted in hepatic tissue, the glycemic homeostasis was improved [10,11]. The authors showed that PTP1B reduces the spread of insulin signaling when PTP1B content is high; it allows greater transcription of gluconeogenic genes. Differently, when it was deleted, this condition was reverted. In this sense, it is evident that PTP1B is an important molecule responsible for glycemic homeostasis, thus strategies that are effective in reducing its content might be relevant to improving insulin sensitivity in obese and diabetic individuals [8]. Since the use of medications can bring unwanted side effects, non-pharmacological strategies that may reduce PTP1B content are of paramount importance.

Regular physical exercise is well known to reduce inflammation resulting from obesity and, consequently insulin resistance in both peripheral and central tissues [12–14]. In 2013, Moura and colleagues showed that aged animals presented an elevation of hepatic PTP1B levels and after only one bout of aerobic physical exercise, it was possible to observe PTP1B reduction in this tissue and consequent reduction in the hepatic glucose production (HGP) [15]. In 2015, Passos and co-authors observed that obese animals present increased PTP1B and, after being submitted to a chronic aerobic exercise protocol (8 weeks), presented a reduction in PTP1B and an improvement in glycemic homeostasis [16]. On the other hand, researches involving strength physical exercise, and insulin sensitivity are still little explored in the literature. Recently, we showed that a short period of strength physical exercise was effective in reducing insulin resistance and fat accumulation in the liver of obese mice [17]. However, it is not known whether these findings are linked to PTP1B metabolism. We hypothesize that diet-induced obesity would increase the PTP1B content in the liver of obese animals and impair hepatic insulin sensitivity due to the reduction of IRS-1/2 phosphorylation. On the other hand, we also hypothesize that the short-term strength exercise would reverse this scenario, reducing levels of PTP1B, allowing increased phosphorylation of IRS-1/2 in Y612 residue, even without promoting body weight change. In this sense, since there are no studies showing the direct effects of strength training on the metabolism of PTP1B, this study aimed to investigate the effects of STSE on hepatic insulin sensitivity and whether a short period of strength physical exercise can decrease PTP1B content and increased tyrosine phosphorylation of IRS-1, allowing increased activity of Akt, which culminate in lower fasting glycemia and better HGP control, regardless of changes in body weight.

2. Results

2.1. Short-Term Strength Exercise Ameliorates Glucose Sensitivity without Reducing Body Weight and Fat Depots

To evaluate the effects of STSE in obese mice the first step of this study was to induce obesity using HFD for 14 weeks. After that, the obese animals were divided into sedentary

and exercised mice. The exercised animals were submitted to 15 sessions of strength exercise which consisted of 20 climbing series with an overload of 70% of the maximum voluntary carrying capacity (MVCC) and with a rest interval of 60–90 s between sets. After the experimental period, the results demonstrate that the diet-induced obesity protocol was effective in increasing body weight gain and impairing glucose sensitivity (Figure 1A,B,E). Besides, 15 sessions of strength training were able to revert the hyperglycemia caused by obesity regardless of body weight chance or epididymal fat and retroperitoneal fat reduction (Figure 1A–E).

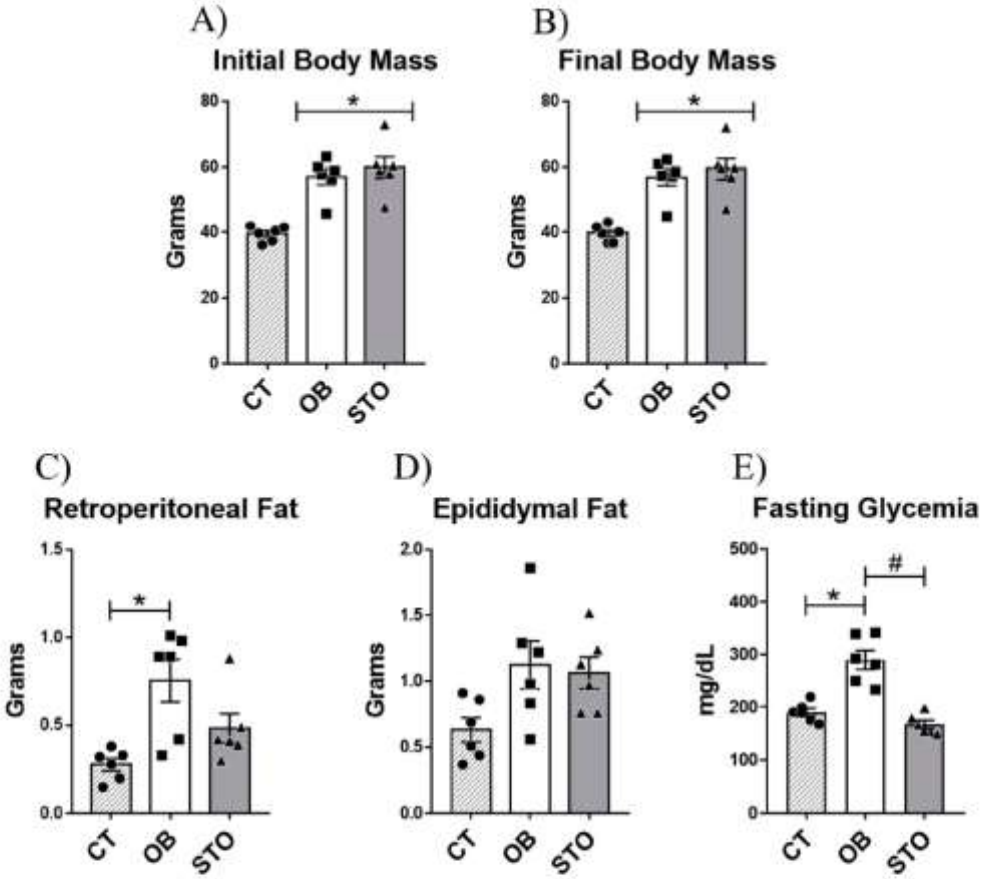

Figure 1. Physiological parameters. Initial body mass: body weight of the animals after diet-induced obesity protocol and before starting the exercise protocol (**A**), Final body mass: body weight of the animals after short-term strength exercise protocol (15 exercise sessions) and before euthanasia (**B**), Retroperitoneal fat (**C**), Epididymal fat (**D**), and Fasting glycemia (**E**). $n = 6$ per group. CT = Control group; OB = Obese Sedentary Group and STO = Strength Training Obese. * $p < 0.05$ vs. CT; # $p < 0.05$ vs. OB ($n = 6$ per group).

2.2. Short-Term Strength Exercise Improves Insulin Sensitivity and Reduces Hepatic Glucose Production in Obese Mice

Once it was observed that obesity impaired fasting glycemia, it was sought to investigate whether hepatic glucose production was affected by obesity. The results of the

intraperitoneal pyruvate tolerance test (ipPTT) demonstrated that obese sedentary animals presented increased hepatic glucose production, as the blood glucose was higher at all time points of the test when compared to the control (Figure 2A), culminating in a greater area under the curve (AUC) during the ipPTT (Figure 2B). In contrast, short-term strength exercise ameliorates HGP control, reducing blood glucose during ipPTT as well as the AUC of the test (Figure 2A,B). Also, obesity impaired insulin signaling in the liver of mice (Figure 2C,D); however, exercised mice restored Akt phosphorylation, demonstrating an improvement in hepatic insulin signaling (Figure 2C,E).

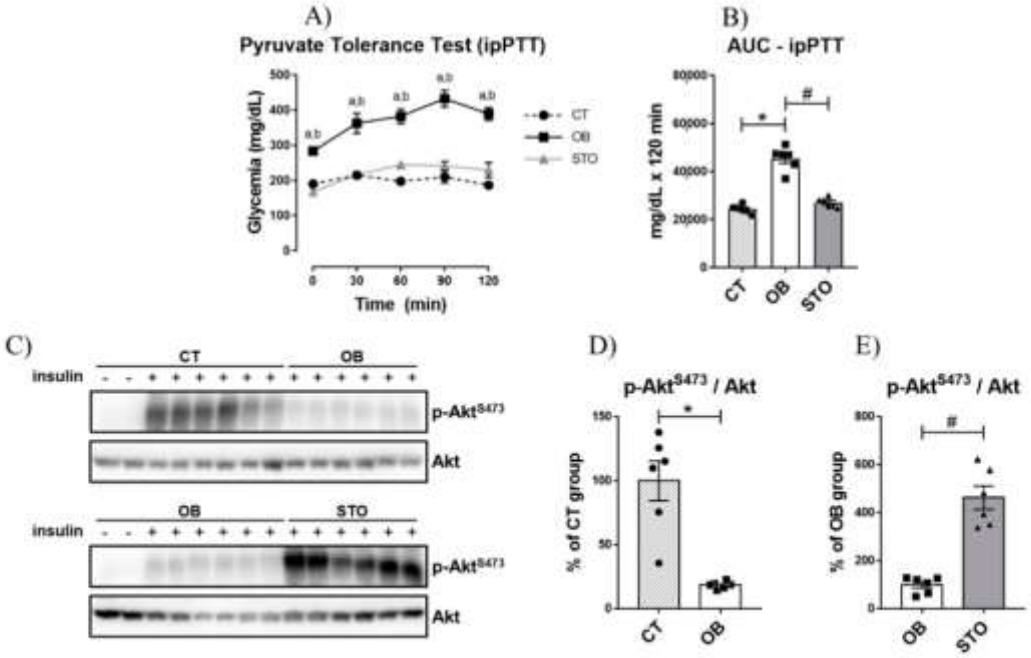

Figure 2. Hepatic Glucose Production and Hepatic Insulin Signaling. Glycemic curve during Intraperitoneal Pyruvate Tolerance Test (ipPTT) (**A**); Area Under the Curve (AUC) during ipPTT (**B**), Bands of phospho-Akt^{S473} and total Akt (**C**), Quantification of p-Akt^{S473}/Akt of CT and OB groups (**D**); Quantification of p-Akt^{S473}/Akt of OB and STO groups (**E**). $n = 6$ per group. CT = Control group; OB = Obese Sedentary Group and STO = Strength Training Obese. In (**A**): a = $p < 0.05$ for CT vs. OB; b = $p < 0.05$ for OB vs. STO. In (**B**,**D**,**E**): * $p < 0.05$ vs. CT; # $p < 0.05$ vs. OB. CT: $n = 8$ (2 saline injection + 6 insulin injection)—OB: $n = 8$ (2 saline injection + 6 insulin injection)—STO $n = 6$ (all insulin injection). The statistical analysis was performed with insulin injected mice.

2.3. Strength Exercise Reduces PTP1B Content in the Liver of Obese Mice

The next step of this study was to evaluate the effects of two interventions—obesity and exercise—in the basal level of PTP1B. To achieve this aim, we measured the protein content of PTP1B in control, sedentary obese, and strength training obese animals. The results in Figure 3A,B show that diet-induced obesity (DIO) robustly increased the basal PTP1B content in sedentary animals, however, STSE reduced the basal PTP1B levels. Subsequently, our objective was to investigate the effects of obesity and physical exercise on obese animals stimulated with insulin. When comparing the PTP1B levels in CT and OB mice stimulated with insulin, it is possible to observe that PTP1B is increased in OB mice (Figure 3C,D), similarly as observed in the PTP1B basal levels (without insulin stimulation).

Consequently, it was possible to observe that obese animals showed a reduction in p-IRS-$1/2^{Y612}$ levels after insulin stimulus (Figure 3C,F). Furthermore, by evaluating the effects of physical exercise in insulin-stimulated animals, the STSE was able to reduce the PTP1B content when compared to sedentary obese animals (Figure 3G,H). As a consequence of the reduction in PTP1B caused by exercise, the p-IRS-$1/2^{Y612}$ levels were elevated (Figure 3G,J). Moreover, to assess if there is a correlation between PTP1B and insulin signaling in this study, the protein levels of PTP1B were correlated with p-Akt content. The protein content of PTP1B was negatively correlated with p-Akt in both comparisons CT × OB (Figure 3E) and OB × STO (Figure 3I).

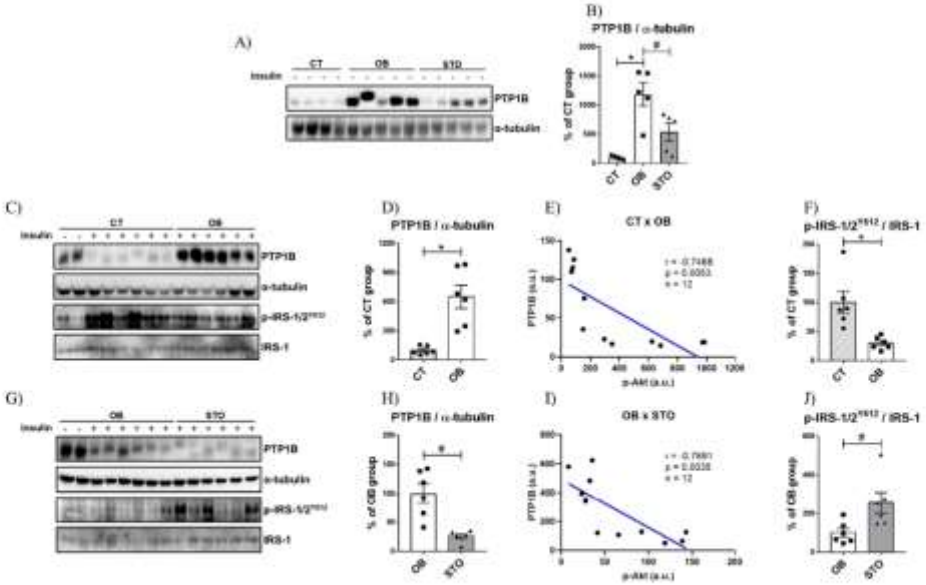

Figure 3. PTP1B content is increased in obese mice and STO downregulates PTP1B content. Bands of basal PTP1B, β-actin p-IRS-1/2 (Y612) and total IRS-1 of CT, OB, and STO groups (**A**), Quantification of basal PTP1B normalized with α-tubulin of CT, OB, and STO groups (**B**), Bands of PTP1B and α-tubulin of CT and OB groups (**C**), Quantification of PTP1B normalized with α-tubulin of CT and OB groups (**D**), Correlation between the protein content of PTP1B and p-Akt of CT and OB groups (**E**), Quantification of p-IRS-1/2 (Y612) normalized with total IRS-1 of CT and OB groups (**F**), Bands of PTP1B and α-tubulin of OB and STO groups (**G**), Quantification of PTP1B normalized with α-tubulin of OB and STO groups (**H**), Correlation between the protein content of PTP1B and p-Akt of CT and OB groups (**I**), Quantification of p-IRS-1/2 (Y612) normalized with total IRS-1 of OB and STO groups (**J**). CT = Control group; OB = Obese Sedentary Group and STO = Strength Training Obese. * $p < 0.05$ vs. CT; # $p < 0.05$ vs. OB. Panels A–B: $n = 4$–6 animals per group—all saline-injected animals. Panels C–H: CT: $n = 8$ (2 saline injection + 6 insulin injection)—OB: $n = 8$ (2 saline injection + 6 insulin injection)—STO $n = 6$ (all insulin injection). The statistical analysis in panels C–H was performed with insulin-injected mice.

Furthermore, a publicly accessible dataset from human liver samples was used to evaluate the expression of PTP1B, lipogenic, and inflammatory genes. It was observed that when there is a high amount of *Ptp1b* mRNA, the expression of *Acaca*, *Fasn*, *Tnf*, and *Il1b* is also increased (Figure 4A). Moreover, using obese mice liver dataset, it was evaluated if there could be a correlation between *Ptp1b* levels in the liver of those mice with the following phenotypes: Blood glucose, locomotor activity, liver mass, and body weight (Figure 4B–E). Almost all phenotypes evaluated showed a positive correlation with PTP1B, except for the locomotor activity, highlighting our hypothesis that physical exercise might

be an interesting strategy to reduce PTP1B content and, consequently, ameliorating hepatic glycemic control.

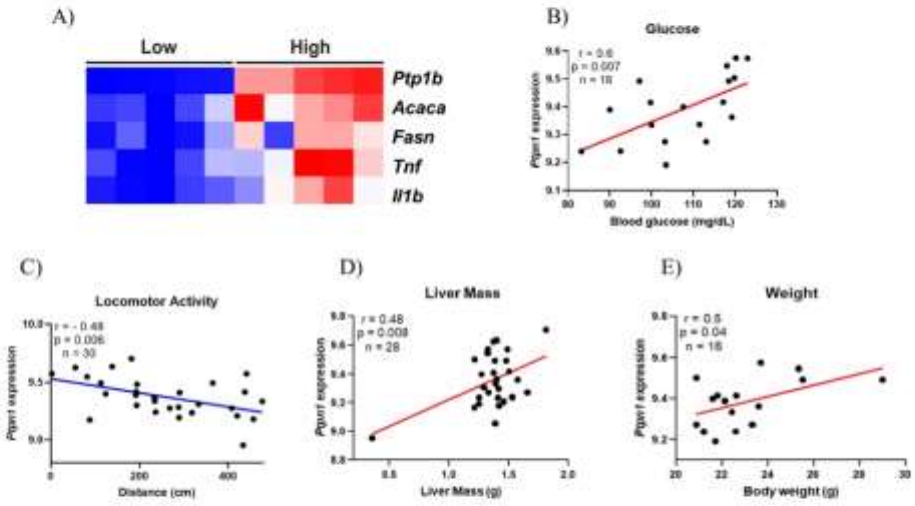

Figure 4. Bioinformatics analysis. Heatmap illustrating the lower and higher expression of Protein-Tyrosine Phosphatase 1B (PTP1B) and the influence in lipogenic genes [Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthase (FAS)] and inflammatory genes [Tumor Necrosis Factor (TNF) and interleukin 1β (IL1β)] on human liver tissue. The figure illustrates clusters between the groups Low and High showing that they are directly related (**A**); Correlation between PTP1B gene expression and Blood Glucose levels (n = 18) (**B**); Correlation between PTP1B gene expression and locomotor activity (n = 30) (**C**); Correlation between PTP1B gene expression and liver mass (n = 28) (**D**); Correlation between PTP1B gene expression and body weight (n = 16) (**E**).

3. Discussion

Obesity is a major risk factor to the development of hepatic insulin resistance culminating in fasting hyperglycemia and T2DM genesis [18,19]. The liver is a key organ for controlling glucose homeostasis since it regulates gluconeogenesis and glycogenolysis, controlling the hepatic glucose release [20]. Also, the hepatic insulin resistance contributes to uncontrolled HGP, which in turn, is majorly responsible for hyperglycemia in T2DM patients [21]. Therefore, it is important to find new strategies to increase hepatic insulin sensitivity and to reduce hepatic gluconeogenesis. Also, studies with animals [22,23] and humans [24] demonstrated that physical exercise is a great strategy to improve hepatic insulin sensitivity. Zhang and colleagues evaluated the effects of acute and chronic aerobic exercise on hepatic insulin sensitivity of diet-induced obese rats and observed that both protocols were able to increase Akt phosphorylation and insulin release [23].

Moreover, the Otsuka Long-Evans Tokushima Fatty (OLETF) rats were another model of obesity and type 2 diabetes used to evaluate the role of aerobic exercise on hepatic insulin sensitivity [22]. The authors submitted OLEFT animals to a voluntary running wheel for 20 weeks and observed that exercised animals increased the phosphorylation of Akt in both Threonine 308 and Serine 473 residues [22]. Furthermore, Malin and colleagues evaluated the effects of 12 weeks of aerobic treadmill walking in 20 older adults, and they observed that exercise was able to reduce hepatic insulin resistance, which was assessed using the euglycemic–hyperinsulinemic clamp [24]. On the other hand, there is a lack of studies on the role of strength exercise in hepatic insulin sensitivity in the literature. In this study, we showed that even without reducing body weight, a short-term strength

exercise protocol improved hepatic insulin signaling since STSE animals decreased PTP1B content and increased phosphorylation of IRS-1, allowing increased activity of Akt, which culminated in lower fasting glycemia and better HGP control.

An interesting target to improve hepatic insulin signaling is PTP1B since this phosphatase can dephosphorylate the insulin receptor and its substrates and block insulin signaling [25]. Panzhinskiy and colleagues observed that PTP1B whole-body knockout mice attenuated the harmful effects of 20 weeks of HFD, such as body weight gain, adiposity, glucose intolerance, and hepatic steatosis when compared with wild-type C57BL/6J mice that were also fed an HFD, suggesting that PTP1B is an important protein in obesity development [26]. Studies with liver-specific knockout mice have shown an overall glucose homeostasis improvement, including enhanced hepatic insulin signaling and increased suppression of hepatic glucose production in insulin-resistant and high fat diet-induced obesity models [11,27]. Moreover, there is evidence in the literature showing that Akt might impair PTP1B function as a positive feedback mechanism of insulin action since PTP1B could impair the upstream sites to inhibit insulin signaling [28]. Using the cell culture model, Ravichandran and colleagues showed that Akt can phosphorylate PTP1B at Serine 50 after insulin stimulation, and they also observe that mutations at Serine 50 affect the ability of PTP1B to dephosphorylate insulin receptor (IR) and IRS [28]. As observed in our data, STSE increased Akt phosphorylation which can have reduced PTP1B activity in the cellular cytosol, culminating in increased IRS-1/2 tyrosine phosphorylation and better hepatic insulin signaling. Interestingly, our data showed a negative correlation between the PTP1B protein content and Akt phosphorylation, when comparing CT vs. OB and OB vs. STO groups, which indicates that those proteins are related to each other.

Physical exercise seems to be a great strategy to reduce PTP1B content and improve insulin signaling. Moura and colleagues evaluated the effects of acute aerobic exercise on the liver content of PTP1B of aged mice and observed that two bouts of swimming exercise were able to reverse the increased content of PTP1B due to the aging process. Moreover, aged-exercised mice enhanced insulin signaling and reduced the gluconeogenesis pathway, suggesting that PTP1B content reduction mediated by physical exercise might be related to better glucose homeostasis [15]. In contrast, little is known about the effects of strength exercise on hepatic insulin sensitivity and HGP. Botezelli and colleagues compared the effects of chronic (8 weeks) aerobic, strength, and combined exercise in fructose-fed rats, and observed that the strength exercise protocol (which consists of series of jumps in tanks of water) showed improvements in glucose homeostasis, insulin sensitivity and the content of lipids in hepatic tissue, which highlights the significance of investigating this type of exercise [29]. Recently our research group showed that short-term strength exercise reduced the HGP and improved insulin signaling in the liver [17]; however, there is a gap in the literature about the role of this type of exercise on PTP1B association with proteins of the insulin pathway. This is the first study showing the efficiency of strength exercise to reduce PTP1B content in obese mice without body weight interference, and it seems to be an important non-pharmacological option to treat the complications of obesity and T2DM. Moreover, it is important to highlight the isolated effect of STSE impacting the PTP1B levels, since the basal levels of PTP1B in non-insulin stimulated animals were also reduced in exercised mice when compared with sedentary obese animals.

In conclusion, short-term strength exercise can improve hepatic insulin signaling, increasing IRS-1/2 tyrosine phosphorylation, and Akt activation, as well as lowering hepatic glucose production and fasting glycemia. Also, we showed that hepatic insulin sensitivity was increased due to a reduction in PTP1B content, as observed in exercised animals, reducing IRS-1/2 tyrosine dephosphorylation (Figure 5). It is important to highlight that these benefits were found regardless of body weight reduction in obese mice. Therefore, strength exercise may be considered an important strategy to prevent and treat the side effects of obesity and associated diseases such as T2DM, improving metabolic health even in the earlier stages of the treatment.

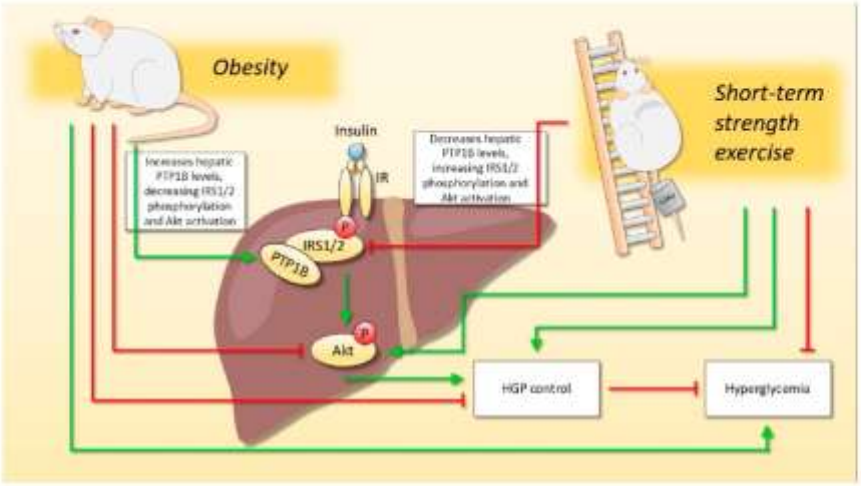

Figure 5. The effect of short-term strength exercise on hepatic glucose metabolism, regardless of body weight change. Diet-induced obesity impaired hepatic insulin signaling due to increased PTP1B content, which results in hyperglycemia and impaired control of hepatic glucose production (HGP). On the other hand, short-term strength exercise was able to reverse the harmful effects of obesity since exercised animals reduced the PTP1B content. Moreover, even though the exercised animals did not reduce body weight, the hepatic insulin signaling was improved, and they demonstrated better HGP control and reduced fasting glycemia.

4. Materials and Methods

4.1. Animals and Diet

Male Swiss mice at eight weeks old were used in the present study. The animals were from the Unicamp Central Animal Facility (CEMIB), and all animals experiments were previously approved by the Ethics Committee on Animal Use (CEUA) of Biological Sciences (UNICAMP-Campinas-SP, number 4406-1) and carried out according to the Brazilian legislation on the scientific use of animals (Law No. 11.794, of 8 October 2008). The animals arrived at four-week-old and were maintained in individual polyethylene cages with an enriched environment, and with inside light, noise, humidity, and temperature control, as we previously described [17]. Water and conventional food were offered ad libitum.

Initially, the animals were divided into two groups: the Control Lean (CT) group (n = 8) was fed a chow diet, and the Obesity group was fed a high-fat diet (HFD). The diet-induced obesity protocol lasted 14 weeks, and after that, the animals of the obese group were equally redistributed, considering their body weight and fasting glycemia, into two groups: (a) Sedentary Obese (OB) (n = 8), which remained sedentary throughout the experiment and (b) Strength Training Obese (STO) (n = 6), which was submitted to a short-term strength training. The high-fat diet was prepared according to the American Institute of Nutrition (AIN-93G) guidelines [30], modified to contain 35% of fat (4% soy oil and 31% of lard) [31]. Moreover, it is important to highlight that all experiments were repeated to evaluate the basal levels of PTP1B, therefore, the diet-induced obesity protocol and the short-term strength training were performed twice. The number of animals used in the second experiment was: CT—n = 4, OB—n = 5, and STO—n = 5.

4.2. Experimental Design and Exercise Protocol

The short-term strength training was performed on a ladder with a 1.5 cm distance between the steps and 70 cm high (AVS projects, São Carlos, Brazil), and the mice carried the

load apparatus fixed with adhesive tape across the length of their tail. The load apparatus was a conical plastic tube with around 7.5 cm of height and 2.5 cm of diameter.

The first step was the adaptation to the apparatus that lasted five consecutive days. On the first day, the animals were placed in a chamber at the top of the ladder for 60 s, with the loading apparatus empty attached on its tail. The animals were progressively placed away from the chamber. For the first climbing attempt, the animal was placed on the ladder at 15 cm from the entrance of the chamber. For the second attempt, the animal was placed 25 cm away from the chamber. For the third attempt onwards, the animal was positioned at the base of the ladder, 70 cm away from the chamber. The attempts started from the base of the ladder and continued until the animal reached the chamber three times without the need for any stimulus.

Then, 48 h after the last day of adaptation, the animals were submitted to the maximal voluntary carrying capacity (MVCC) test to determine the maximum load with which each animal could climb the entire length of the ladder. The MVCC started with an overload corresponding to 75% of the animal's body weight, and an incremental load of 5 g was added at each further attempt to climb until the animal could no longer complete the entire course. At the end of each successful attempt, the animal rested in an individual cage for 5 min until the next attempt. The heaviest overload in which the animal performed a successful climb was considered the MVCC and this value was used to prescribe the individual loads in the experiment.

The strength exercise protocol began 48 h after the MVCC determination. The exercise sessions consisted of 20 climbing series with an overload of 70% of the MVCC and with a rest interval of 60–90 s between sets. The animals were exercised for five consecutive days per week, followed by two days of rest, until they completed 13 sessions of physical exercise. Subsequently, mice were submitted to the pyruvate tolerance test. After 24 h, the animals performed two more sessions of exercise, totaling 15 sessions, as demonstrated in Figure 6.

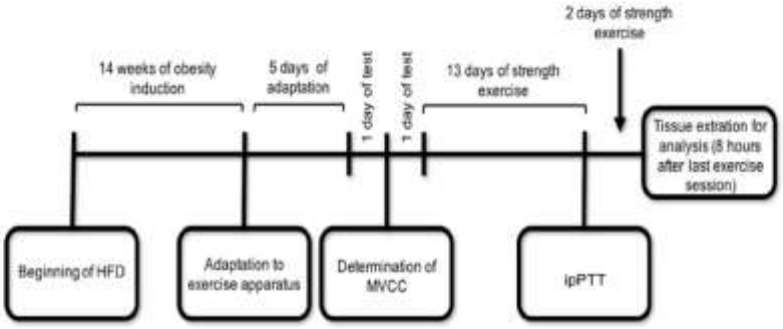

Figure 6. Schematic representation of the experimental design. The ipPTT and tissue extraction were performed 8 h after the exercise session and 8-h fasting. HFD = High Fat Diet; MVCC = Maximum Voluntary Carrying Capacity; ipPTT = intraperitoneal Pyruvate Tolerance Test.

4.3. Intraperitoneal Pyruvate Tolerance Test (ipPTT)

To estimate the hepatic glucose production (HGP) control, an ipPTT was performed after 13 exercise sessions. The ipPTT protocol consists of intraperitoneal injection of 2.0 g of pyruvate/kg body weight after 8 h fasting and 8 h after the last exercise session. The blood samples were collected at 0, 30, 60, 90, and 120 min from the tail of the animal for blood glucose determination. Point 0 was collected before pyruvate (Êxodo Científica, Sumaré, SP, Brazil) injection. The results were evaluated by determining the areas under the blood glucose curves (AUC) during the test by the trapezoidal method [32], using Microsoft Excel (Version 2013).

4.4. Fasting Glycemia Assay

Eight hours after the 15th session of STSE, and 8 h fasting, the blood was collected from the tail of the animals to determine the fasting glycemia. The blood glucose was determined using a glucometer (Accu-Chek; Roche Diagnostics, Indianapolis, IN, USA).

4.5. Tissue Extraction and Immunoblotting Analysis

After 8 h fasting and 8 h after the last exercise session, before receiving saline or insulin, all animals were anesthetized via i.p. by the injection of chloral hydrate of ketamine (50 mg/kg, Parke-Davis, Ann Arbor, MI, USA) and xylazine (20 mg/kg, Rompun, Bayer, Leverkusen). After the verification and assurance of the corneal reflexes, mice were injected via i.p. with human insulin (8 U/kg body wt Humulin-R; Lilly, Indianapolis, IN, USA) or saline. The first time that the experiment was performed 2 animals from the CT group and 2 animals from the OB group received saline and 6 animals from each group (CT, OB, and STO) received insulin. On the other hand, in the second time that this experiment was conducted all animals received only the saline injection (CT—$n = 4$, OB—$n = 5$, and STO—$n = 5$) because this study aimed to analyze the basal levels of PTP1B. After 10 min, the liver was rapidly removed and snap-frozen in liquid nitrogen and stored at −80 °C until analysis and epididymal and retroperitoneal adipose tissue (right side) were removed and weighed. The liver was homogenized in an extraction buffer [1% Triton-X 100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/mL] at 4 °C with a TissueLyser II (QIAGEN®) operated at maximum speed for 120 s. The lysates were centrifuged (Eppendorf 5804R) at 12.851 g at 4 °C for 15 min to remove insoluble material, and the supernatant was used for the assay. The protein content was determined by the bicinchoninic acid method [33]. The samples containing 60 µg of total protein were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk at room temperature for 1 h and incubated with primary antibodies against the protein of interest. After that, a specific secondary antibody was used. The specific bands were labeled by chemiluminescence and visualization was performed by a photo documentation system in G: box (Syngene). The bands were quantified using the software UN-SCAN-IT gel 6.1. The primary antibodies used were: anti-Phospho-Akt S473 (4060), anti-Akt (4685) and anti- α-Tubulin (2144) from Cell Signaling Technology® (Beverly, MA, USA) and anti-PTP1B (sc14021), anti-IRS-1 (sc559), and Phospho-IRS-1/2 Y612 (sc17195) from Santa Cruz Biotechnology® (Santa Cruz, CA, USA). The secondary antibody used was the Anti-rabbit IgG, from Cell Signaling Technology® (Beverly, MA, USA).

4.6. Bioinformatics Analysis

A heatmap was made using the data collected in Gene Network (http://www.genenetwork.org/, accessed on 2 October 2019) using the GTExv5 Human Liver RefSeq (Sep15) RPKM log2 data set of mRNA expression [34]. Correlations were made using obese mice liver dataset "EPFL/LISP BXD HFD Liver Affy Mouse Gene 1.0 (Aug18) RMA", also available on GeneNetwork. Phenotypes were only included when data were collected at the same time, excluding time-courses and also, excluding data when mice had interventions such as stress, drug injections, or exposure to different types of environments. Data were normalized and distributed according to the z-score distribution, using R Project studio (Version 1.2.5033). More details of the steps of the bioinformatic analysis can be found in the Supplementary Material.

4.7. Statistical Analysis

All results were presented as the mean ± standard error of the mean (SEM). The Gaussian distribution of the data was assessed using a Shapiro–Wilk test and analyzed by Student's *t*-test for parametric data to compare two groups when it was necessary. We used the one-way Analysis of Variance (ANOVA) test followed by Bonferroni's post-hoc

test to compare more than two groups. Two-way ANOVA (with repeated measures when appropriate), with Bonferroni's correction for multiple comparisons, was used to analyze each point of ipPTT. The level of statistical significance used was $p < 0.05$. The construction of the graphics and the statistical analysis was performed using GraphPad Prism 7.00.

Supplementary Materials: Supplementary materials can be found at https://www.mdpi.com/article/10.3390/ijms22126402/s1.

Author Contributions: L.P.d.M. designed the paper. R.M.P. and K.C.d.C.R. wrote the paper and had the overall responsibilities of the experiments in this study. R.M.P., K.C.d.C.R., M.R.S., L.W.T.B., V.R.M. and A.P.M. performed the experiments and data collection. R.M.P. performed the statistical analysis. G.F.P. performed the bioinformatics analysis. F.M.S., A.S.R.d.S., D.E.C., E.R.R., J.R.P. and L.P.d.M. contributed to the discussion and supported the financial costs. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee on Animal Use (CEUA) of Biological Sciences (UNICAMP-Campinas-SP, number 4406-1).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors of this study have no competing interests to declare.

Abbreviations

T2DM	Type 2 Diabetes *Mellitus*
FOXO1	Forkhead Box Protein O1
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha
PTP1B	Protein-Tyrosine Phosphatase 1B
PTP	Protein Tyrosine Phosphatase
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
TNF-α	Tumor Necrosis Factor-alpha
HGP	Hepatic Glucose Production
STSE	Short-term strength exercise
ipPTT	intraperitoneal Pyruvate Tolerance Test
AUC	Area Under the Curve
ACC	Acetyl-CoA Carboxylase
FAS	Fatty Acid Synthase
IL1β	Interleukin 1β
OLETF	Otsuka Long-Evans Tokushima Fatty
CEMIB	Unicamp Central Animal Facility
CEUA	Ethics Committee on Animal Use
CT	Control Lean
HFD	High-fat diet
OB	Sedentary Obese
STO	Strength Training Obese
AIN-93	American Institute of Nutrition
MVCC	Maximal Voluntary Carrying Capacity
SEM	Standard error of the mean
ANOVA	Analysis of Variance

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4.5 ARTIGO 5

Ao longo dos anos, alguns estudos trouxeram evidências de que a combinação de diferentes modalidades de exercício (aeróbio e força) pode prejudicar as adaptações promovidas pela prática de somente uma dessas modalidades de modo isolado. Porém, estudos em animais investigando vias biomoleculares utilizando o protocolo de treinamento combinado são excassos. Recentemente, nosso laboratório demonstrou que o treinamento combinado de curta duração é eficiente em reduzir a hiperglicemia e aumentar a ação da insulina no fígado de camundongos obesos, porém os efeitos dessa nova modalidade de treinamento sobre o acúmulo de gordura hepática ainda não haviam sido explorados. Assim, o objetivo do nosso próximo estudo foi investigar os efeitos do treinamento combinado de curta duração sobre o acúmulo de gordura hepática e sobre as vias de síntese e oxidação de gordura em camundongos obesos.

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Short-term combined training reduces hepatic steatosis and improves hepatic insulin signaling

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Diabetes

ABSTRACT

Hepatic steatosis is directly associated with hepatic inflammation and insulin resistance, which is correlated with hyperglycemia and type 2 diabetes mellitus (T2DM). Aerobic and strength training have been pointed out as efficient strategies against hepatic steatosis. However, little is known about the effects of the combination of those two protocols on hepatic steatosis. Therefore, this study aimed to evaluate the impact of short-term combined training (STCT) on glucose homeostasis and in the synthesis and oxidation of fat in the liver of obesity-induced mice with hepatic steatosis. Swiss mice were distributed into three groups: control lean (CTL), sedentary obese (OB), and combined training obese (CTO). The CTO group performed the STCT protocol, which consisted of strength and aerobic exercises in the same session. The protocol lasted seven days. The CTO group reduced the glucose levels and fatty liver when compared to the OB group. Interestingly, these results were observed even without reductions in body adiposity. CTO group also showed increased hepatic insulin sensitivity, with lower hepatic glucose production (HGP). STCT reduced the expression of the lipogenic genes *Fasn* and *Scd1* and hepatic inflammation, as well as increased the ACC phosphorylation and the oxidative genes *Cpt1a* and *Ppara*, reverting the complications caused by obesity. Since this protocol increased lipid oxidation and reduced hepatic lipogenesis, regardless of body fat mass decrease, it can be considered an effective non-pharmacological strategy for the treatment of hepatic steatosis.

1. Introduction

Hepatic steatosis is the most common liver disease in the Western world [1], is characterized by excessive fat accumulation and is directly associated with hepatic inflammation and insulin resistance [2]. Liver insulin resistance is one of the main factors triggering hyperglycemia in type 2 diabetes *mellitus* (T2DM), since the liver is responsible for 90% of the endogenous glucose production, and insulin is one of the primary hormones responsible for controlling this phenomenon [3,4]. A recent study has shown that obese patients with hepatic steatosis have more severe insulin resistance and increased insulin secretion than obese with normal hepatic lipid levels and glucose tolerance, resulting in impaired glucose homeostasis [5].

A large number of studies in the area have shown that lifestyle interventions are considered one of the main treatments for hepatic steatosis [6,7]. Thoma and colleagues observed that strategies

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increasing physical activity levels and reducing energy consumption are effective in diminishing hepatic lipid levels in patients with hepatic steatosis and improving hepatic insulin sensitivity [8]. Knowing physical exercise is a great strategy to reduce body fat [9], energy consumption [10,11], and hepatic steatosis [9,12], it is essential to understand the mechanisms underlying these benefits promoted by physical exercise.

Several types of exercise have been pointed out as efficient strategies against hepatic steatosis [13–15]. Studies using rodent models have shown that eight weeks of treadmill aerobic training reduced the expression of several lipogenic genes [16–18]. Furthermore, human studies have shown that aerobic training reduces hepatic steatosis in both overweighted children [19] and obese elderly [20], improving glycemic homeostasis. Botezelli and colleagues observed that 8 weeks of strength exercise is able to improve insulin sensitivity, glucose homeostasis and reduce the hepatic lipid levels of obese rats [14]. Recently, regardless of adiposity reduction, obese mice reduced hepatic lipid level and several lipogenic proteins after a short-term strength training protocol [12]. In addition, Bacchi and colleagues revealed that both aerobic and strength training (separately) was effective in reducing fatty liver in diabetic subjects after four months of intervention [13].

The combined training (i.e., the combination of aerobic with strength exercise) has been gaining the attention of the scientific community for providing several metabolic benefits [15,21]. After a combined training protocol, obese volunteers reduced body weight, body mass index (BMI), fasting blood glucose levels, and insulin resistance [21]. Moreover, after 12 weeks of combined training, obese adolescent girls reduced central adiposity, hyperinsulinemia, and hyperleptinemia, as well as increased adiponectin levels [22]. However, the direct effects of combined training on hepatic lipid accumulation are scarce. High-fructose diet-induced hepatic steatosis rats performing both swimming and strength training during eight weeks reduced fatty liver and inflammation similarly to rats submitted only to swimming training [14]. In a recent study, Franco and colleagues found that six months of combined training was effective in reducing hepatic steatosis in subjects with disease classified as moderate to severe [6]. However, the effects of combined training on biomolecular mechanisms underlying the hepatic lipid synthesis and oxidation remain unexplored. Recently, a study evaluated the effect 7 days of treadmill walking in obese adult human and, even without reducing body weight nor body fat, those subjects increased hepatic insulin extraction and insulin sensitivity, showing that short-term exercise in able to reverse several side effects of hepatic steatosis [23]. The hypothesis of the present study is that short-term combined training can reduce hepatic lipid levels in obese mice, independently of changes in body adiposity. Moreover, the aim of this study is to evaluate the direct impact of short-term combined training (STCT) on glucose homeostasis and in the synthesis and oxidation of fat in the liver of obesity-induced mice with hepatic steatosis.

2. Material and methods

2.1. Animals

Eight weeks old male Swiss mice from the Multidisciplinary Center for Biological Research (CEMIB - UNICAMP) were used in the present study. All procedures were conducted according to the Brazilian legislation on the scientific use of animals (Law No. 11.794, of October 8, 2008). Moreover, all experiments were accepted by the Ethics Committee on Animal Use (CEUA) of Biological Sciences (UNICAMP-Campinas-SP, number 4773-1/2018).

Initially, four-week-old animals were maintained in individual polyethylene cages with the enriched environment (PVC pipes were sawed in the middle generating a shelter of 10 × 10 cm of the base and 5 cm of height) and under controlled conditions of the light–darkness cycle (12/12 h), temperature (22 ± 2 °C), relative humidity maintained at 45–55%, and on-site noises below 85 dB. Furthermore, mice had water and conventional food ad libitum.

Eight-week-old animals were first distributed into two groups: the lean control group (CTL) that fed a chow diet and the obesity group that fed a high-fat diet (HFD). Moreover, the high-fat diet was prepared according to the American Institute of Nutrition (AIN-93G) guidelines [24], modified to contain 35% of fat (4% soy oil and 31% of lard) [25]. Ten weeks after the beginning of HFD diet exposure, the obese animals were equally distributed according to body weight and fasting glycemia into two groups: 1 – sedentary obese (OB), which remained sedentary throughout the experiment; 2 – combined training obese (CTO), which underwent a short-term combined training.

2.2. Combined training

To avoid body weight loss, a short-term protocol lasting seven days was chosen. In the first step of this protocol, the animals performed the strength exercise, which consisted of 10 climbs carrying a weight corresponding to 70% of Maximum Voluntary Carrying Capacity (MVCC) with 90 s of rest between the series [12]. The adaptation to the apparatus and the MVCC test was performed as proposed by Pereira and colleagues [12]. Briefly, the animals were adapted to the ladder (AVS projetos®, São Carlos, SP, Brazil) for five consecutive days, where they gradually increase the distance to reach the chamber until they get 70 cm away from the entrance [12,26,27]. Two days after the adaptation protocol, mice were submitted to an incremental test (MVCC) to determine the maximal individual load to climb 70 cm of the ladder. Lastly, a particular load corresponding to 70% of MVCC was weighed and placed in a conical tube and attached to mice's tail to perform the climbs during the training protocol.

Following strength training, animals commenced aerobic exercise which consisted of a treadmill running for 30 min at 75% of exhaustion velocity. To determine the exhaustion velocity, firstly, the animals were adapted for five consecutive days in a treadmill (AVS projetos®, São Carlos, SP, Brazil) for 10 min/day at 6 m/min [27]. Two days after the adaptation protocol, the animals performed an incremental test to define exhaustion velocity. Briefly, the animals started the test running at 6 m/min, and the speed increased 3 m/min every 3 min until the animal could no longer run. The highest speed reached by each animal was considered its exhaustion velocity, and 75% of this velocity was used to determine the running velocity. More details can be found in Fig. 1.

2.3. Intraperitoneal pyruvate tolerance test (ipPTT)

The animals were submitted to intraperitoneal Pyruvate Tolerance Test (ipPTT) 16 h after the 5th exercise training session and with eight hours of fasting to evaluate the hepatic glucose production (HGP). The dose used was 2.0 g of pyruvate per kilogram of body weight (sodium pyruvate Axis Científica®, Cotia, SP, Brazil). Time 0 was collected before the pyruvate injection and represented the basal glucose levels. After the pyruvate injection, the blood samples were collected at 30, 60, and 120 min from the tail of the animals to measure the blood glucose. To determine the glucose levels, a glucometer (Accu-Chek®; Roche Diagnostics, Indianapolis, IN, USA) was used, and the results were evaluated identifying the areas under the blood glucose curves (AUC) during the test by the trapezoidal method [28].

2.4. Liver hematoxylin-eosin histology and oil red O staining

The liver fragments were fixed in isopentane (Synth®, Diadema, SP, Brazil) to cryopreserve the samples at −80 °C. The liver samples were sliced in a Leica Cryostat model CM1850 (Leica Biosystems®, Buffalo Grove, IL, USA) to a thickness of 10 μm and placed on identified adhesion slides. The slices (n = 6 per group) were stained to the hematoxylin-eosin (H&E) and oil red O methods. Firstly, the slices were stained with hematoxylin for 10 min or with oil red O solution (Sigma-Aldrich®, Saint Louis, MO, USA) for 25 min. Then, the slices were washed and

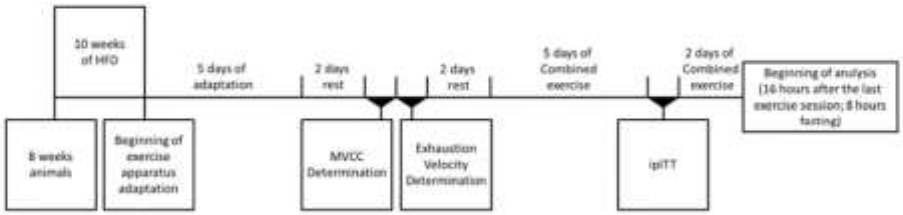

Fig. 1. Experimental design. Schematic representation of the experiments during the short-term combined training. The physiological test ipPTT and tissue collection were performed 16 h after the previous exercise session, 8-h fasting.

stained with eosin for 5 min. Furthermore, images with 40× zoom [14,29] of the slices stained with Oil red O were used to analyze the red-stained area and the lipid droplets area using the program ImageJ [30] (Raw Data Material Fig. 3A).

2.5. Physiological parameters

Blood glucose levels were determined using a glucometer (Accu-Chek®; Roche Diagnostics, Indianapolis, IN, USA) before the tissue collection, and serum insulin was measured by ELISA kit (Crystal Chem®, Elk Grove Village, IL, USA), according to the manufacturer's instructions.

2.6. Western blotting

Animals were anesthetized via intraperitoneal injection using chloral hydrate of ketamine (300 mg/kg, Parke-Davis, Ann Arbor, MI, USA) and xylazine (30 mg/kg, Rompun, Bayer, Leverkusen). Furthermore, the euthanasia was performed 16 h after the last combined exercise session and with 8 h of fasting. The corneal reflexes were checked to ensure the efficiency of the anesthesia, and the human insulin (8 U/kg body wt Humulin-R®; Lilly, Indianapolis, IN, USA) or saline was injected intraperitoneally into the mice ten minutes prior the liver harvest, which was snap-frozen in liquid nitrogen and stored at −80 °C until the molecular analysis. Moreover, the right side of the epididymal and retroperitoneal fat were harvested and weighted to compare the fat depots between the groups.

The Western blotting technique was performed as previously described by our research group [12]. The primary antibodies used were anti-phospho-Akt ser473 (#4060), anti-phospho-Acetyl-CoA Carboxylase ser79 (#3661), anti-acetyl-coA carboxylase (#3662), anti-GAPDH (#5147) from Cell Signaling Technology® (Danvers, MA, USA), anti-TNF-α (Cat # 506101) and IL-1β (Cat # 503501) from BioLegend® (San Diego, CA, USA) and anti-Akt (sc-8312) from Santa Cruz Biotecnology® (Dallas, TX, USA). After a specific secondary antibody was incubated, the specific bands were labeled by chemiluminescence, and the photo documentation system in G:box (Syngene®, Frederick, MD, USA) performed the visualization of the bands. The software UN-SCAN-IT gel 6.1 was used to quantify the bands. The secondary antibodies used were anti-rabbit IgG, HRP-linked antibody (#7074), and anti-mouse IgG, HRP-linked antibody (#7076) from Cell Signaling Technology ® (Danvers, MA, USA).

2.7. Reverse transcription – quantitative polymerase chain reaction (RT-qPCR)

The total RNA was isolated using the PureZOL™ reagent (BIO-RAD ®, Hercules, CA, USA) and converted to cDNA following the instructions of the High Capacity cDNA Reverse Transcription (Applied Biosystems ®, Foster City, CA, USA) kit, using 2 μg of total RNA as a template for the synthesis of cDNA. Furthermore, real-time PCR reactions were performed using 40 ng cDNA, 0.5 μL primers, and 5 μL TaqMan Universal PCR Master Mix (Applied Biosystems®, Foster City, CA, USA). The primers used were *Fasn* (Mm00662319_m1), *Scd1* (Mm00772290_m1), *Cpt1a* (Mm01231183), *Ppara* (Mm00440939_m1) and *Gapdh* (Mm99999915_g1). The relative content of mRNAs was determined after normalization with GAPDH using the ΔΔCT method [31].

2.8. Statistical analysis

All results were presented as the mean ± standard error of the mean (SEM). The Gaussian distribution of the data was assessed using the Kolmogorov–Smirnov test. Data were analyzed using Student's *t*-test to compare two groups or ANOVA to compare 3+ groups for data with Gaussian distributions in each of the groups. When the data were found to be not following a Gaussian distribution, the Mann–Whitney test was used (if homoscedasticity), or Welch's *t*-test technique was used (if heteroscedasticity) to compare 2 groups and Kruskal Wallis to compare 3+ groups. When appropriate, the one-way ANOVA test was followed by Bonferroni's post hoc test and the Kruskal–Wallis test followed by Dunn's multiple comparisons tests to compare between the different groups. Two-way ANOVA, with Bonferroni's correction for multiple comparisons, when appropriate, was used to analyze each point of ipPTT. The level of statistical significance used was $p < 0.05$. The construction of the graphics and the statistical analysis was performed using Prism (7.00) GraphPad Software, San Diego, CA, USA.

3. Results

3.1. Short-term combined training reduces obesity-related hyperglycemia even without changes in body adiposity

Initially, we evaluated the effects of HFD and the combined training protocol on parameters related to body composition and glycemic homeostasis. After ten weeks of HFD-feeding, the animals showed higher body mass, fasting hyperglycemia, and hyperinsulinemia, as well as a higher volume of adipose tissue in the epididymal and retroperitoneal deposits (Fig. 2A–F). After the combined training protocol, fasting blood glucose returned to typical values, since the CTO group did not show any difference between the CTL group (Fig. 2G). No differences in body mass and body adiposity between CTO and OB groups were observed. Although there was no statistical difference, we noted a tendency towards a reduction in fasting insulin levels in CTO animals when compared to the OB group ($p = 0.0799$).

3.2. Short-term combined training reduces hepatic steatosis

The lipid content analysis revealed higher fat accumulation in the liver of OB group animals when compared to the CTL group (Fig. 3A). However, combined training was effective in reversing this scenario, showing a meaningful improvement in liver fat accumulation observed through H&E and Oil red O stained areas, which makes CTO group similar to CTL mice for this parameter (Fig. 3A and B). Next, Oil red O staining was used to analyze the lipid droplet area in OB and CTO

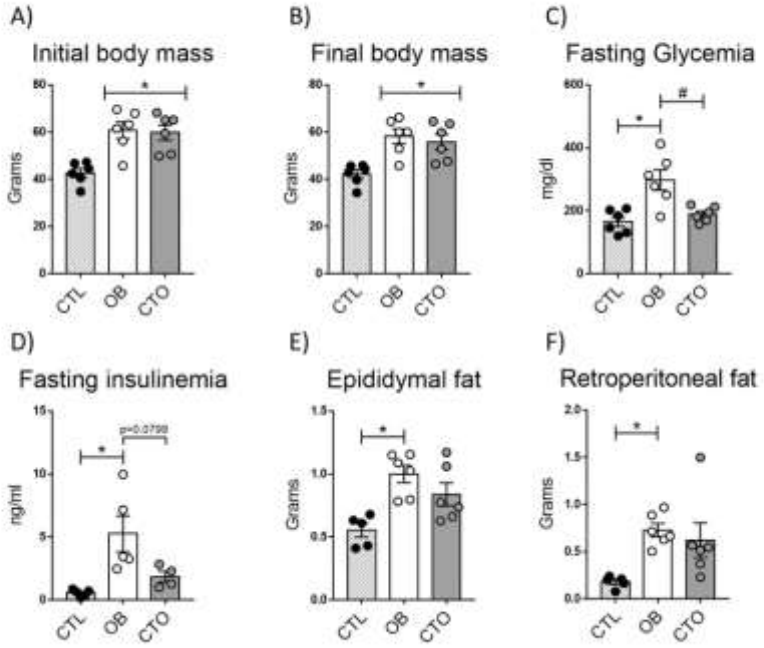

Fig. 2. Metabolic parameters and fat weight of all experimental groups. A and B) Body mass at the beginning and the end of the experimental period. C and D) Fasting glycemia and insulinemia, respectively. E and F) Fat weight of epididymal and retroperitoneal regions, respectively. *p < 0.05 vs CTL; #p < 0.05 vs OB (n = 4–6 per group). In Fig. F Kruskal-Wallis test was used followed by Dunn's multiple comparisons tests, and one-way ANOVA followed by Bonferroni's post-hoc test in others.

groups. The CTO group showed an important reduction in lipid droplet area mean values, with most of the lipid droplets with less than 20μm² (Fig. 3C and D).

3.3. Short-term combined training increases hepatic insulin signaling and improves the control of HGP

Next, we assessed whether combined training could increase hepatic insulin signaling and contribute to better control of HGP in obese mice. Initially, we performed the ipPTT 16 h after the last exercise session. We observed that obese animals presented glycemic values higher than the lean controls during all-time points (Fig. 4A). On the other hand, animals in the CTO group showed lower glycemic values than the OB group at all-time points (Fig. 4A). Thus, the AUC during the test was higher for the OB group compared to the CTL group, and the combined training protocol was efficient in reducing this value (Fig. 4B), demonstrating that trained animals have better control of HGP.

To assess hepatic insulin sensitivity, animals received an intraperitoneal injection of insulin 10 min before liver collection. Initially, insulin was efficient in providing Akt phosphorylation at serine 473 in the animals of the CTL group (Fig. 4C and D). However, the animals of the OB group showed a reduction in this phosphorylation, indicating an impaired hepatic insulin signaling associated with obesity (Fig. 4C and D). Importantly, the combined strength training increased Akt phosphorylation in response to insulin (Fig. 4E and F).

3.4. Short-term combined training reduces hepatic inflammation and lipogenesis

Finally, we evaluated the impact of combined training on the biomolecular pathways involved with hepatic lipogenesis. Obesity increased the levels of mRNA-lipogenic genes *Fasn* and *Scd1*, while the oxidative genes *Cpt1a* and *Ppara* were decreased (Fig. 5A-D). The animals in the OB group also reduced the phosphorylation of ACC, even with no change in their total content (Fig. 5E-F). Obese animals also had more significant liver inflammation, with a higher pro-inflammatory protein content of IL-1β (Fig. 5E and I). On the other hand, the combined training reversed this condition. Genetically, STCT decreased *Fasn* and *Scd1* levels (Fig. 5Fig. J and K) and increased *Cpt1a* and *Ppara* (Fig. 5L and M). Considering the total protein content, STCT increased ACC phosphorylation and decreased its total content (Fig. 5N-P), as well as decreased IL-1β and TNF-α levels (Fig. 5N and R).

4. Discussion

Hepatic steatosis is currently known as the leading cause of liver disease, and the practice of aerobic and strength training is an effective strategy in reducing liver fat in obese state [9,15,32]. However, little is known about the effects of combined training on hepatic steatosis and the pathways of lipogenesis and lipid oxidation in hepatic tissue. In the present study, we demonstrated that combined training is effective in reducing hepatic steatosis. Here, trained animals reduced the hepatic lipid content, which resulted in increased hepatic insulin signaling and better HGP control, decreasing obesity-associated hyperglycemia. Furthermore, liver inflammation (TNF-α and IL-1β) and the lipogenic stimulus provided by insulin were reduced with training. Moreover, obesity is one of the risk factors to increase the lipid droplets (LD), and the formation of very large lipid droplets in liver is a hallmark of hepatic steatosis [33]. In this study we showed that short-term combined training reduced the size of hepatic LD, demonstrating its efficiency to

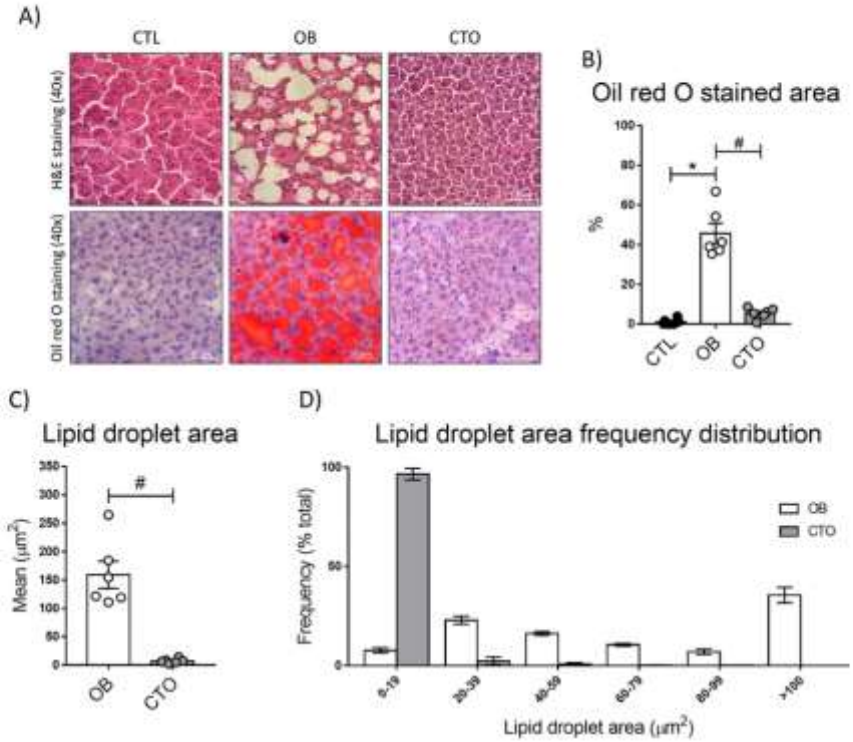

Fig. 3. Effects of short-term combined training in hepatic steatosis. A) H&E staining and Oil red O staining of the right lobe. B) Oil red O stained area of the three groups. C) Lipid droplet area of OB and CTO groups, from Oil red O staining. D) Liver droplet area frequency distribution of OB and CTO groups, from Oil red O staining. *$p < 0.05$ vs CTL; #$p < 0.05$ vs OB ($n = 6$ per group). In Fig. B one-way ANOVA was used followed by Bonferroni's post-hoc test and in Fig. C Student's *t*-test was used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

treat comorbidities associated with obesity, such as hepatic steatosis.

There is a high number of evidence supporting the benefits of aerobic training in hepatic steatosis. Recently, a meta-analyses showed that aerobic training is efficient in reducing several hepatic steatosis markers and liver damage caused by obesity [9,34]. Similar to aerobic, strength training might also reduce liver lipids [13], corroborating data from another recent meta-analysis [35]. Finally, some evidence about combined training in hepatic steatosis also exists. In one of the pioneering studies, Antunes and collegues observed that 20 weeks of combined training reduced body adiposity, markers of hepatic steatosis, total cholesterol, and low-density lipoprotein cholesterol (LDL-c) levels in obese adolescents [36]. Subsequently, it was demonstrated that ten weeks of combined training was able to reduce fatty liver in diabetic women, culminating in reductions in fasting glucose and insulinemia [37]. Finally, we showed that seven sessions of combined training led to similar results in an animal model of obesity-induced hepatic steatosis. It is essential to highlight that our effects were observed regardless of changes in body weight and body fat.

Previous studies have already shown that aerobic training is efficient in increasing hepatic insulin sensitivity and mitigating liver dysfunction both in animal models [38,39] and humans [20,40]. Obese mice submitted to six weeks of high-intensity aerobic training reduced HGP when compared to sedentary animals, with more significant suppression provided by insulin [38]. Similarly, after six months in an aerobic training program associated with therapy diet, obese older adults showed consistent reductions in lipids profile and liver damage markers, with increased insulin sensitivity [20]. Despite the smaller volume of studies, there is evidence that strength training is also able to improve insulin action in the liver. Obese mice submitted to 15 sections of strength exercise showed better control of HGP during ipPTT, with increased Akt phosphorylation after insulin injection [12]. Coherently, older women showed a reduction in endogenous glucose production after insulin stimulus in response to 4 months of strength training [41].

In a study with rodents, Botezelli and collegues demonstrated that eight weeks of combined training are effective in reversing metabolic complications induced by a high-fructose diet, reducing hyperglycemia, hyperinsulinemia, insulin resistance and glucose intolerance [14]. Although some human studies have also shown improvements in glycemic homeostasis after combined training [20–22], the effects of this modality on HGP control and hepatic insulin sensitivity have not been explored yet. In the present study, short-term combined training provided better control of HGP in obese mice submitted to seven sessions of exercise. Coherently, after insulin injection, trained animals showed higher hepatic Akt phosphorylation when compared to obese animals that remained sedentary throughout the experimental period. Thus, regardless of changes in body composition, we can conclude short-term combined training can increase liver insulin action in obese mice, reversing the loss of HGP control provided by obesity.

However, one of the main results in the present study was that short-term combined training could reduce lipogenic stimulus in the liver

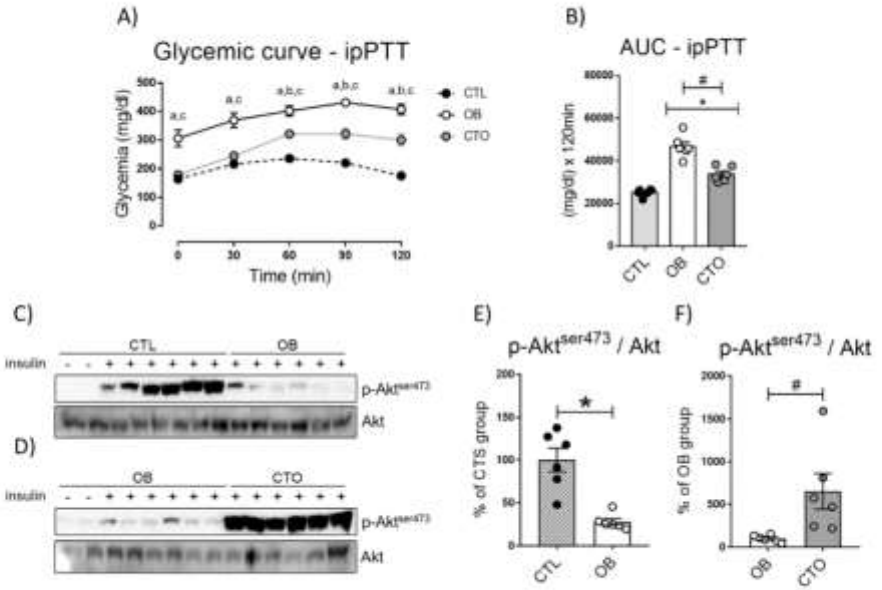

Fig. 4. HGP control and hepatic insulin sensitivity. A and B) Glycemic curve and AUC during ipPTT, respectively. C) Bands of hepatic p-Akt^{ser473} and total Akt of CTL and OB groups after insulin stimulus. D) Bands of hepatic p-Akt^{ser473} and Akt of OB and CTO groups after insulin stimulus. E) Quantification of hepatic p-Akt^{ser473}/Akt of CTL and OB groups. F) Quantification of hepatic p-Akt^{ser473}/Akt of OB and CTO groups. Only the bands of the animals stimulated with insulin were quantified. In Fig. A: $^{a}p < 0.05$ for CT vs OB; $^{b}p < 0.05$ for CT vs CTO; $^{c}p < 0.05$ for OB vs CTO (n = 6). In B, E and F: $^{a}p < 0.05$ vs CT; $^{*}p < 0.05$ vs OB (n = 6). In fig. A, it was used a two-way ANOVA test with Bonferroni's correction for multiple comparisons. In fig. B, it was used one-way ANOVA followed by Bonferroni's post-hoc test. In fig. E and F, it was used the Student's t test.

provided by insulin since trained animals reduced the lipogenic genes *Fasn* and *Scd1*, liver inflammation, as well as activity and protein content of ACC. Aerobic training is an efficient strategy to minimize lipogenic machinery in liver tissue [42–44]. In their study, Yasari and colleagues observed both lean and obese rats reduced SCD1 protein content after eight weeks of aerobic treadmill training [18]. Subsequently, obese mice that underwent swimming training for ten weeks showed the same result, with reduced expression of both *Scd1* and *Fasn* [45]. Similarly, Kalaki-Jouybari and colleagues observed consistent reductions in *Fasn* and *Acc* expressions in the liver of obese rats trained for eight weeks at a high-intensity protocol [17]. In addition, eight weeks of treadmill aerobic training provided increased ACC inhibition in the liver of obese mice [43].

Recently, our research group demonstrated that 15 sessions of strength training could reduce the hepatic lipogenesis of obese mice, also without changes in the animals' body composition [12]. However, the effects of the combination of these two training models on liver lipogenesis had never been investigated. Thus, in the present study, obese mice reduced hepatic lipogenesis, both at the mRNA and protein levels, after seven sessions of combined training.

In addition to lowering the synthesis pathways, short-term combined training also increased the expression of the oxidative genes *Cpt1a* and *Ppara*. These are genes with a well-described involvement with β-oxidation, being the subject of several studies to control the hepatic steatosis [46,47]. Both aerobic [39] or strength training [12] can increase the expression of *Cpt1a* and *Ppara* in the liver of obese rodents. Here, for the first time, we demonstrated short-term combined training could reduce the hepatic lipid levels by providing increased expression of the oxidative genes Cpt1a and Ppara, which is another efficient strategy to hepatic steatosis control by exercise.

5. Conclusion

In conclusion, short-term combined training reduced hepatic steatosis and hepatic inflammation, increasing hepatic insulin sensitivity, and promoting better HGP control. We found a reduction in lipogenic genes (i.e., *Fasn* and *Scd1*) and an increase in ACC phosphorylation, decreasing lipogenic pathways. We also verified a rise in oxidative genes (i.e., *Cpt1a* and *Ppara*). These phenomena occurred independently of changes in body mass and adiposity. Thus, combined training can be pointed out as a good strategy against hepatic steatosis.

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CRediT authorship contribution statement

LPM designed the paper. RMP and KCCR wrote the paper and had the overall responsibilities of the experiments in this study. RMP, KCCR, MRS, GFP, CPA, TDPC, RSC, and DGM performed the experiments and data collection. RMP and MRS performed the histological experiments. MRS performed the PCR analysis. RMP performed the statistical analysis. FMS, ASRS, DEC, ERR, JRP, and LPM contributed to discussion and supported the financial costs. All the authors have read and approved this manuscript.

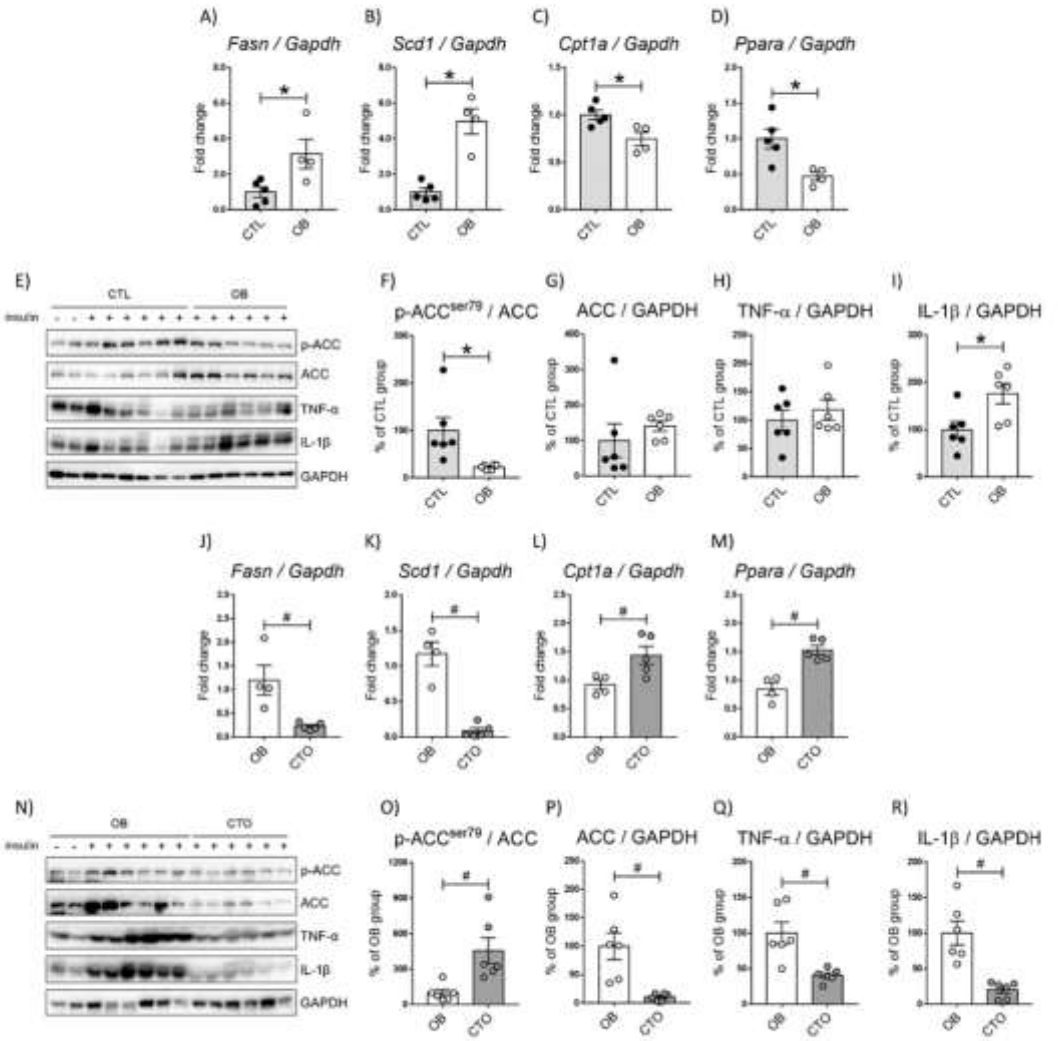

Fig. 5. Effects of short-term combined training in hepatic lipogenesis, oxidation, and inflammation. A-D) mRNA levels of genes *Fasn*, *Scd1*, *Cpt1a*, and *Ppara*, respectively of CTL and OB groups. E) Protein bands of the lipogenic and inflammatory proteins in the liver of mice from CTL and OB groups after insulin stimulus. F–I) Quantification of hepatic p-ACC^{ser79}, total ACC, TNF-α, and IL-1β, respectively, of CTL and OB groups. J–M) mRNA levels of genes *Fasn*, *Scd1*, *Cpt1a*, and *Ppara*, respectively of OB and CTO groups. N) Protein bands of the lipogenic and inflammatory proteins in the liver of mice from OB and CTO groups after insulin stimulus. O–R) Quantification of hepatic p-ACC^{ser79}, total ACC, TNF-α, and IL-1β, respectively, of OB and CTO groups. Only the bands of the animals stimulated with insulin were quantified. $^{*}p < 0.05$ vs CT; $^{\#}p < 0.05$ vs OB (n = 4–6 per group). In fig. O, it was used the Mann-Whitney test. Student's *t*-test was used in the others.

Declaration of competing interest

The authors of this study have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2021.120124.

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4.6 ARTIGO 6

Já é bem descrita a participação da clusterina hepática em proporcionar redução da lipogênese hepática e proteção contra o acúmulo excessivo de gordura nesse órgão. Similarmente, o treinamento de força também é uma importante estratégia tanto para a prevenção quanto tratamento da NAFLD. Entretanto, ainda não existem estudos investigando os efeitos do treinamento de força sobre os níveis de clusterina no fígado de indivíduos obesos. Portanto, nosso próximo estudo teve como objetivo investigar os efeitos do treinamento de força de curta duração sobre os níveis de clusterina no fígado de camundongos obesos.

Artigo em processo de finalização:

Short-term strength training increases hepatic clusterin and reduces NAFLD in obese mice.

Short-term strength training increases hepatic clusterin and reduces NAFLD in obese mice.

INTRODUCTION

Clusterin is a disulfide-linked glycoprotein of 75 - 80 kDa and its predominant isoform is a glycoprotein secreted as a heterodimer composed of two chains of monomers joined by five disulfide bridges (RIZZI; COLETTA; BETTUZZI, 2009). Clusterin has a well-described participation in several physiological processes such as anti-apoptotic and anti-inflammatory actions, oxidative stress reduction, tissue differentiation and remodeling and HDL efflux (PARK; MATHIS; LEE, 2014; PEREIRA et al., 2018; TROUGAKOS, 2013). However, by still unknown mechanisms, clusterin can escape its secretion pathway and be found in the cytosol, originating its cytoplasmic isoform (TROUGAKOS, 2013).

Recently, an elegant study demonstrated that the liver is the main tissue responsible for clusterin synthesis, defining clusterin as a new hepatokine and highlighting the importance of this protein for the control of the liver metabolic functions (SEO et al., 2020). And previous studies shown that hepatic clusterin has an important role in lipogenesis control in this tissue. In 2013, it was demonstrated that hepatic clusterin has a suppressive role in Sterol regulatory element-binding protein-1c (SREBP-1c) (SEO et al., 2013). When the authors overexpressed clusterin in AML-12 hepatocytes, they observed reduction both in SREBP-1c and their lipogenics transcripts Fatty Acid Synthase (FAS), Acetyl-CoA carboxylase (ACC) e Stearoyl-CoA desaturase (SCD). Coherently, rodents overexpressing clusterin were protected of non-alcoholic fatty liverdisease (NAFLD), with lower leves of hepatic triglycerides e SREBP-1c expression (SEO et al., 2013). Furthermore, mice overexpressing clusterin specifically in hepatocytes were protected from nonalcoholic steatohepatitis (NASH), with less inflammation, macrophage infiltration and hepatic triglycerides (PARK et al., 2018). Thus, knowing the relationship of NAFLD with the failure of hepatic insulin action and induction of hyperglycemia and type 2 diabetes *mellitus* (T2DM) in obese state (TILG; MOSCHEN; RODEN, 2017), hepatic clusterin can be an important therapeutic target for the management of hepatic fat accumulation and associated metabolic complications.

In this context, recent publications show that strength training is an important strategy against NAFLD and hepatic insulin resistance. One of the pioneering studies in the field demonstrated that 4 months of strength training reduced hepatic fat accumulation in diabetic subjects (BACCHI et al., 2013). Similarly, 2 months of strength training besides reducing hepatic fat in men with NAFLD, also reduced markers of liver damage alanine transaminase (ALT) and aspartate transaminase (AST) (SHAMSODDINI et al., 2015). However, the biomolecular mechanisms by which strength training fights NAFLD remain unkown. Obese rats who underwent strength training for 12 weeks showed reduction in SREBP-1c and hepatic fibrosis (DOS SANTOS et al., 2019). Similarly, short-term strength training promoted reductions in lipogenic gene expression, protein content and activity in the liver of obese mice, increasing hepatic insulin sensitivity and better control of hepatic glucose production (HGP) (PEREIRA et al., 2019). However, the effects of strength training on proteins that regulate liver lipogenesis such as clusterin remain unexplored. Therefore, the aim of this study was to investigate the effects of strength training on hepatic clusterin levels in obese mice.

MATERIAL AND METHODS

Animals and diet

All the animals' procedures were previously approved by the Ethics Committee on Animal Use (CEUA) of Biological Sciences (UNICAMP-Campinas-SP, number 4406-1) and carried out according to the Brazilian legislation on the scientific use of animals (Law No. 11.794, of October 8, 2008). In the present study, eight weeks old male Swiss mice were used, and the animals were provided by the Multidisciplinary Center for Biological Research / UNICAMP. The animals arrived at four-week-old and were maintained in individual polyethylene cages with an enriched environment as previously described (PEREIRA et al., 2019). Briefly, the light was switched on at 06:00 and off at 18:00 h, the temperature was controlled at 22 ± 2 °C and water and food (chow or high-fat diet) were offered ad libitum. More details about animal conditions were published before (PEREIRA et al., 2019).

The first step of this study was to induce obesity by diet, and at this point, the animals were distributed into two groups: Control Lean group (CTL), fed a chow diet, and DIO group, fed a high-fat diet (HFD). The diet-induced obesity protocol

lasted 14 weeks, and for the second step of this study, the obese group was equally redistributed considering the mice's body weight and fasting glycemia into two groups: a) Obese Sedentary (OBSed), obese mice that remained sedentary throughout the experiment and b) Obese Strength Training (OBStr), mice that performed the strength training protocol. The HFD was prepared according to the American Institute of Nutrition (AIN-93G) guidelines (REEVES; NIELSEN; FAHEY, 1993), and it was modified to contain 35% of fat (4% soy oil and 31% of lard) (OLIVEIRA et al., 2015).

Short-term Strength Training protocol

The short-term strength training protocol was already published in detail by our research group (PEREIRA et al., 2019). Briefly, the protocol was performed in a ladder and the mice carried the load apparatus fixed with adhesive tape in their tail. Before the strength training protocol, the animals were adapted to the ladder and load apparatus for 5 consecutive days.

After the adaptation protocol, the mice rested for 48 hours. Then, the rodents were submitted to the Maximal Voluntary Carrying Capacity (MVCC) to determine the maximum load in which each animal can climb the entire course of the ladder. The load of the first attempt of the MVCC is equivalent to 75% of the animals' body weight. In the subsequent attempts, an incremental overload (5 grams) was added at each further attempt to climb until the animal could no longer complete the entire course. The mice rested for 5 minutes in an individual cage between each attempt, and the heaviest overload carried in the last successful attempt is considered the MVCC of the animal, and this value is used to prescribe the individual loads in the experiment.

The short-term strength training protocol started 48 hours after the MVCC determination. The STST consists of 20 climbing series with an overload of 70% of the MVCC and with a rest interval of 60-90 seconds between sets. The mice were exercised for five consecutive days a week, followed by two days of rest, until they completed 13 sessions of physical exercise. After that, mice underwent the pyruvate tolerance test, and after 24 hours, the animals performed two more training sessions, totaling 15 sessions, and were euthanized.

Intraperitoneal Pyruvate Tolerance Test (ipPTT)

Eight hours after the end of 13th exercise session, the animals were submitted to ipPTT, 8 hours fasting. The animals were submitted to an intraperitoneal injection of pyruvate (2 g/kg of sodium pyruvate Azis Científica®, Cotia, SP) and the blood samples of the animals were drawn from the tail before the pyruvate injection, which was considered the time 0, and after 30, 60, 90, and 120 min of the injection to determine the blood glucose concentration. The results of ITT and ipPTT were evaluated using the areas under the serum glucose curves (AUC) during the test by the trapezoidal method (MATTHEWS et al., 1990) by Microsoft Excel (2013), Microsoft Corporation, Redmond, WA,USA.

Tissue extraction and immunoblotting analysis

The euthanasia was performed 8 hours after the 15th exercise session, and the animals also were fasted for 8 hours. Ten minutes before the euthanasia, the animals received either human insulin (8 U/kg body wt Humulin-R; Lilly, Indianapolis, IN) or saline via i.p. The animals were anesthetized via i.p. by the injection of chloral hydrate of ketamine (100 mg/kg, Parke-Davis, Ann Arbor, MI) and xylazine (10 mg/kg, Rompun, Bayer, Leverkusen), and after the verification and assurance of the lack of corneal reflexes, the liver was collected and rapidly snap-frozen in liquid nitrogen and stored at -80 °C until analysis. The epididymal and retroperitoneal adipose tissue (right side) were removed and weighted to measure the fat depots. The liver was homogenized in extraction buffer [1% Triton-X 100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/mL] at 4° C with a TissueLyser II (QUIAGEN®) operated at maximum speed for 120 s. The lysates were centrifuged (Eppendorf 5804R) at 12.851 × *g* at 4 °C for 15 min to remove insoluble material, and the supernatant was used for the assay. The protein content was determined by the bicinchoninic acid method (WALKER, 1994). The samples containing 60 µg of total protein were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk at room temperature for 1 h and incubated with primary antibodies against the protein of interest. After that, a specific secondary antibody was used. The specific bands were labeled by chemiluminescence, and visualization was performed by photo documentation system in G: box (Syngene). The bands were quantified using the software UN-SCAN-IT gel 6.1

Statistical analysis

The results were shown as the mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to evaluate the Gaussian distribution of the data, and Student's *t*-test was used to compare two groups with parametric data when it was necessary. Furthermore, to compare more than two groups, the one-way Analysis of Variance (ANOVA) test followed by Bonferroni's post-hoc test was performed. To analyze the points of ipPTT, the Two-way ANOVA (with repeated measures when appropriate), with Bonferroni's correction for multiple comparisons was used. The statistical significance level considered was $P<0.05$. The construction of the graphics and the statistical analysis were performed using Prism (7.00) GraphPad Software San Diego, CA, USA.

RESULTS

Short-term strength training reverses hiperglycemia, without changes in adiposity:

At the end of the experimental period, the animals in the OBSed group showed an increase in body mass and adiposity, with an increase in fasting glycemic levels (Fig 1 A-D). However, the animals submitted to short-term strength training showed similar glycemic values to lean animals of CTL group, even without changes in body composition. (Fig 1 A-D).

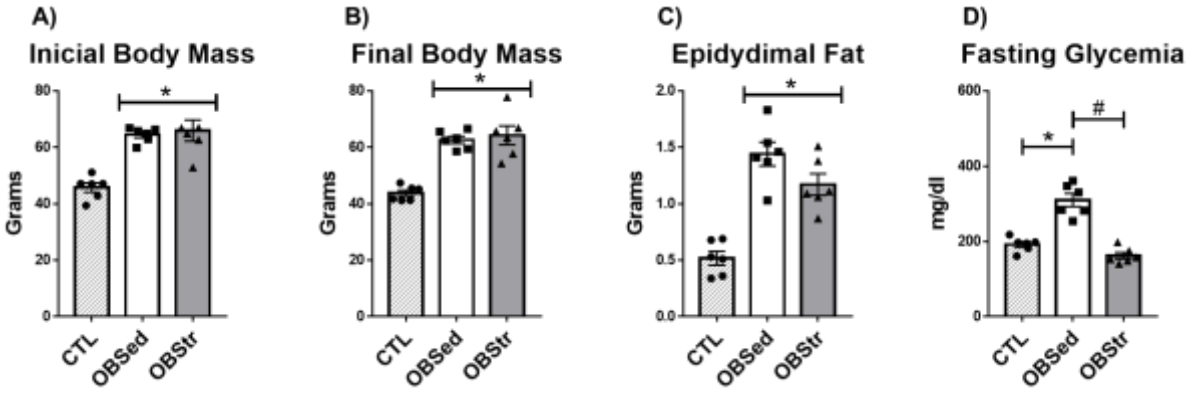

Figure 1: Physiological parameters of CTL, OBS and OBStr groups. A and B) Body mass of the animals at the beginning and at the end of the experiment. C) Adipose tissue weight of the epididymal region. D) Fasting glycemia (after 8 h of fasting). *P < 0.05 vs CTL; #P < 0.05 vs OBSed (n = 6 per group).

Short term strength training increases HGP control and reduces NAFLD in obese mice:

Our next step was to analyze HGP control and fat liver in all experimental groups. Initially, the animals were submitted to ipPTT to evaluate HGP control. After pyruvate injection, we observed that the animals in OBSed group showed a robust increase in glycemic values, which remained high during all the subsequent 120 minutes in the testing (Fig 2A). However, the animals in the OBStr group showed reduced values, with no difference to CTL group (Fig 2A). Consequently, the AUC during the test was higher for sedentary obese group, returning to similar values to lean group after strength training (Fig 2B). When we evaluated the hepatic lipid content using Oil Red O staining, we observed high fat accumulation in obese animals; however, the short term strength training reverser this excess (Fig 2C).

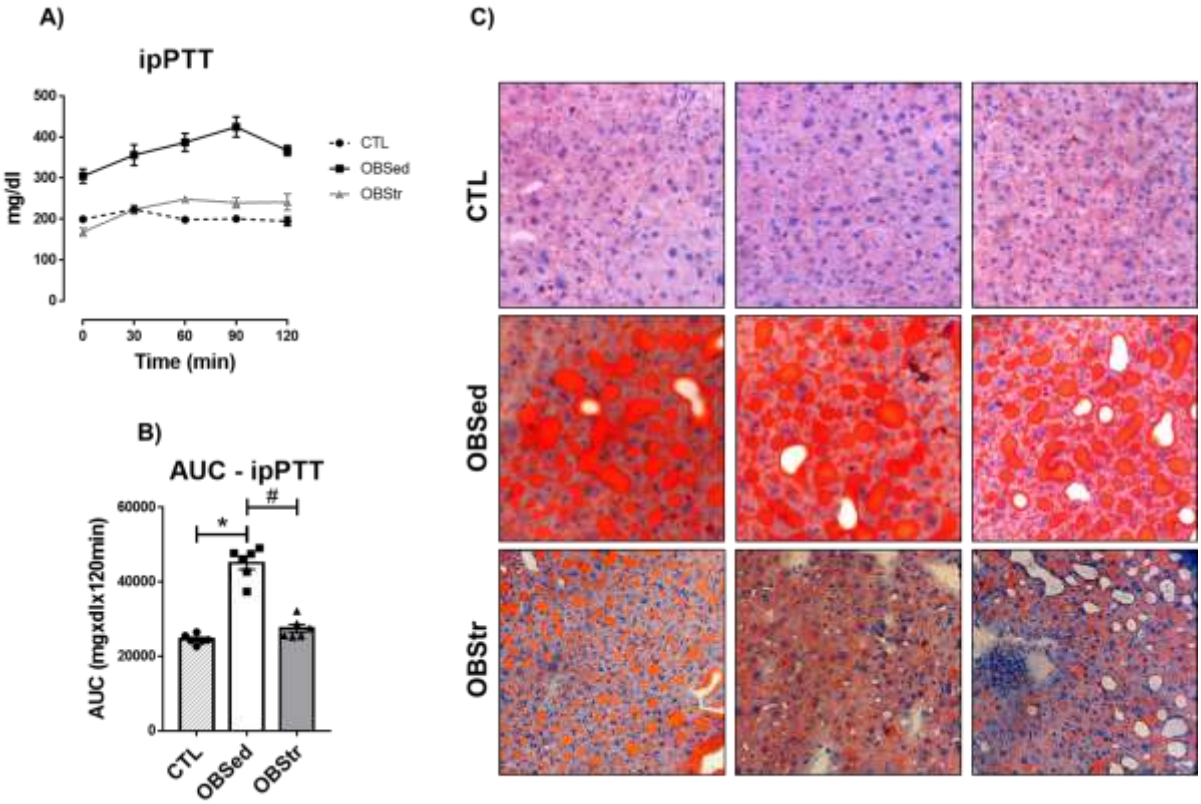

Figure 2: ipPTT and hepatic fat content of CTL, OBSed and OBStr groups. A) Glycemic curve during ipPTT. B) The area under the curve during ipPTT. C) Oil red O staining of the right lobe from three experimental groups. *P < 0.05 vs CTL; #P < 0.05 vs OBSed (n = 5–6 per group).

Trained obese animals have elevated levels of hepatic clusterin:

Finally, we investigated if short-term strength training changes hepatic clusterin levels. Initially, we observed that the animals in the OBSed group showed high clusterin levels when compared to CTL group (Fig 3A and B). However, after short-term strength training, the animals in the OBStr group showed even higher levels when compared to OBSed group. (Fig 3A and C).

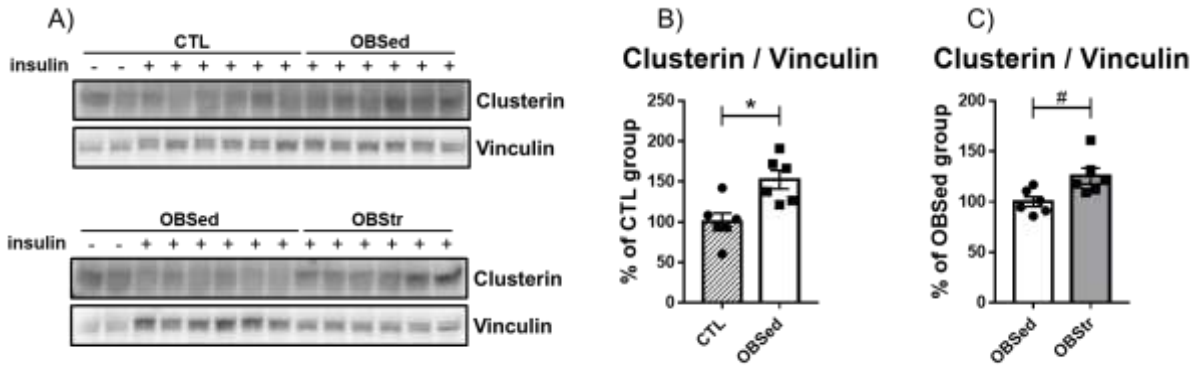

Figure 3: Hepatic clusterin levels of CTL, OBSed and OBStr groups. A) Bands of clusterin in the liver of mice from CTL and OBSed groups (upper) and from OBSed and OBStr groups (lower). B) Quantification of hepatic clusterin / vinculin of CTL and OBSed groups. C) Quantification of hepatic clusterin / vinculin of OBSed and OBStr groups. Only bands of the animals stimulated with insulin were quantified. *P < 0.05 vs CTL; #P < 0.05 vs OBSed (n = 6 per group).

DISCUSSION

Despite great advances in medicine, we still need an efficient pharmacological treatment to NAFLD management. The use of peroxisome proliferator-activated receptor (PPAR)γ agonists as thiazolidinediones (PHIELIX; SZENDROEDI; RODEN, 2011), vitamin E (SANYAL et al., 2010) and Incretin-based therapies as glucagon-like peptide 1 (GLP-1) receptor agonists (ARMSTRONG et al., 2016) show promising results in patients with NAFLD and T2DM, such as increased insulin action, reduced liver fat and serum liver damage markers. However, these treatments seem just enhance the results obtained by reducing body adiposity. Therefore, non-pharmacological strategies such as reducing food consumption and increasing energy expenditure continue to be primary interventions against NAFLD

(TILG; MOSCHEN; RODEN, 2017). Important studies provided evidences about strength training reducing NAFLD and increasing hepatic insulin action, being efficient to hyperglycemia reduction (MEDRANO et al., 2018; PEREIRA et al., 2017a). However, the biomolecular mechanisms involved in this process remain poorly understood. In the present study, we demonstrated that short-term strength training increased clusterin levels in the liver of obese mice, increasing HGP control and reducing liver fat content. Thus, the glycemic values of trained animals returned to baseline values presented by lean animals. Interestingly, these results were observed independently of changes in body composition, showing that this is a direct effect provided by training, and not a secondary effect to adiposity reduction.

Despite the growing number of studies showing that strength training is efficient to improving hepatic insulin action and HGP control, the invasive methods used for analyzing the mechanisms involved in these phenomena turns difficult this analyzes. Recently, it has been shown that one mechanisms by which strength training increases HGP control is related to the reduction of Pyruvate Carboxylase hepatic protein content (PEREIRA et al., 2020), a gluconeogenic protein acting in the pyruvate to oxaloacetate conversion and described as an important therapeutic target against obese-related hyperglycemia (KUMASHIRO et al., 2013). Strength training also reduces hepatic inflammation induced by obese state. Tumor Necrosis Factor-α (TNF-α) and Interleukin-1β (IL-1β) proteins are known for their pro-inflammatory activity, reducing insulin action (GREGOR; HOTAMISLIGIL, 2011). In a previous study, it was demonstrated that short-term strength training was efficient providing reduction in TNF-α and IL-1β hepatic content in obese mice submitted to the same training protocol performed in this study, providing increase in hepatic insulin sensitivity and reducing fasting hyperglycemia (PEREIRA et al., 2019). These anti-inflammatory and anti-gluconeogenic effects provided by strength training support our findings in the present study, since that trained animals here presented reduction in hyperglycemia and better control of HGP after injection of pyruvate.

In the present study, we also observed reduction in hepatic lipid content in trained animals, confirming previously published data (PEREIRA et al., 2019). Important studies have already revealed that humans with NAFLD performing strength training reduces hepatic lipid content (BACCHI et al., 2013; SHAMSODDINI et al., 2015). And recently, the biomolecular mechanisms involved in this process have started to be investigated. It has been shown that lipogenic proteins Fatty Acid

Synthase (FAS) and Acetyl CoA carboxylase (ACC) was reduced after short-term strength training in the liver of obese mice, with increased expression of oxidative genes *Cpt1a* and *Ppara* (PEREIRA et al., 2019). Similarly, obese rats that performed strength training for 12 weeks also reduced Sterol regulatory element-binding protein-1c (SREBP-1c) content (DOS SANTOS et al., 2019), protein known for its important role in lipogenic genes transcription (RUI, 2014). Therefore, the reduction in hepatic lipid content in obese mice is in line with data previously published in the literature.

Finally, the main finding in our study was that strength training increased hepatic clusterin content in obese mice. As previously described, hepatic clusterin has a consistent antilipogenic effect in this tissue (PARK et al., 2018; SEO et al., 2013). It has been shown that hepatic clusterin reduces liver lipogenesis inhibiting SREBP-1 activity, thereby reducing FAS, ACC and SCD1 levels (SEO et al., 2013). In that same study, obese rats were fed HFD and received adenovirus for clusterin overexpression. As expected, obese animals that received adenovirus for overexpression of GFP showed high liver fat accumulation, but the animals that overexpressed clusterin were protected from HFD-induced NAFLD (SEO et al., 2013). Here, we found that obese animals have incresead levels of hepatic clusterin, confirming previous data (KWON et al., 2014). This phenomena is not fully understood, but we believe that this is a counter-regulatory mechanism against the metabolic adverse condition during obesity. However, after 15 sessions of strength training, obese animals showed even more elevations in hepatic clusterin. Thus, this training-induced elevation can affect the lipogenic machinery, culminating a reduction in SREBP-1 transcripts levels such FAS, ACC and SCD-1, as previously demonstrated (PEREIRA et al., 2019). This is a new mechanism by which this modality counteracts NAFLD and hyperglycemia.

CONCLUSION

In summary, short-term strength training increases hepatic clusterin levels in obese mice. Thus, excessive hepatic fat accumulation is reduced, increasing HGP control and reducing fasting hyperglycemia. This is a new mechanism by which strength training is an efficient strategy against NAFLD and hyperglycemia in obese state.

4.7 ARTIGO 7

Apesar dos grandes avanços da medicina, estratégias que proporcionam o aumento do gasto energético e a redução do consumo alimentar continuam sendo a principal intervenção para o tratamento da NAFLD. Nesse contexto, é sabido que o treinamento aeróbio é eficiente em aumentar a razão gasto / consumo energético, reduzir vias lipogênicas e estimular vias de quebra e oxidação de lipídios no fígado com NAFLD. Entretanto, ainda não se sabe os efeitos do treinamento aeróbio sobre as níveis de clusterina hepática. Dessa forma, nosso próximo trabalho teve como objetivo investigar se os efeitos anti-lipogênicos proporcionados pelo treinamento aeróbio de curta duração no fígado de camundongos obesos estão associados a alterações nos níveis hepáticos de clusterina.

Artigo em construção:

Aerobic training increases hepatic clusterin levels and reverses NAFLD in obese mice

Aerobic training increases hepatic clusterin levels and reverses NAFLD in obese mice

INTRODUCTION

It is estimated that around the world more than 400 million people between 20 and 79 years have type 2 diabetes *mellitus* (T2DM), and this number could increase to 700 million in 2045 (SAEEDI et al., 2019). Between the T2DM complications, the non-alcoholic fatty liverdisease (NAFLD) deserves attention, present in 55% of diabetic subjects (YOUNOSSI et al., 2019). Several studies reveal that NAFLD is closely related to reduced hepatic insulin sensitivity and general impairment in glycemic homeostasis (PETERSEN; SHULMAN, 2017; SMITH et al., 2020). However, despite the advances in medicine, interventions about changes in lifestyle remain the primary and most efficient recommendation (FRANCO et al., 2019; TILG; MOSCHEN; RODEN, 2017). Therefore, the study of new mechanisms involved with the treatment and prevention of NAFLD is of great relevance.

In this context, clusterin (or Apolipoprotein J) is pointed as a possible functional link with insulin action. It is a disulfide-linked heterodimeric glycoprotein expressed in most tissues and found in almost all body fluids, found in secreted, cytoplasmic and nuclear isoforms (TROUGAKOS; GONOS, 2002). Recently It has been shown that serum clusterin levels are closely correlated with the magnitude of insulin resistance, and that pharmacological treatment to increase insulin action provides reduction in Clusterin circulating levels (SEO et al., 2018). These results reinforce the previous discussion that secreted clusterin may have a protective effect in metabolically adverse conditions such as obesity and T2DM (KUJIRAOKA et al., 2006). However, cytoplasmic and nuclear isoforms remain poorly explored. The cytoplasmic isoform seems to have an important anti-apoptotic action in different tissues, pointed as an important target in the treatment of different types of cancer (CHUN, 2014; TROUGAKOS et al., 2009; WANG et al., 2015a), On the other hand, the nuclear isoform have the opposite effect (BETTUZZI; RIZZI, 2009). However, there is an increasing number of evidence showing a new function of cytoplasmic clusterin in regulating the liver fat accumulation in different experimental models.

One of the first studies investigating the role of hepatic clusterin on lipogenic mechanisms was conducted by Seo and colleagues (SEO et al., 2013). The

authors observed that hepatocytes AML-12 overexpressing clusterin had a lower Sterol regulatory element-binding protein-1c (SREBP-1c) activity, reducing Fatty Acid Synthase (FAS), Acetyl-CoA carboxylase (ACC) and Stearoyl-CoA desaturase (SCD) levels. Coherently, mice overexpressing clusterin specifically in hepatocytes were protected from diet-induced hepatic fat accumulation, with reductions in liver triglyceride levels and inflammation markers such as macrophage infiltration, Toll-like Receptor-4 (TLR-4) expression and Tumor Necrosis Factor-α (TNF-α) levels (PARK et al., 2018). Finally, hepatocytes from clusterin knockout mice showed increase in the Interleukin-1β (IL-1β) and TNF-α expression after palmitate treatment, reflecting lower Akt phosphorylation after insulin stimulus and increased gluconeogenic genes expression as Fructose-1,6-bisphosphatase (FBPase) and Glucose 6-phosphatase (G6Pase) (KWON et al., 2014). Combined, these data reveal hepatic clusterin as an important therapeutic target for the treatment and prevention of NAFLD and whole hepatic metabolism.

As described above, strategies promoting lifestyle changes remain the most effective in NAFLD prevention and treatment (TILG; MOSCHEN; RODEN, 2017). In this context, aerobic training is an efficient strategy in the management of NAFLD and its associated complications (CARNEROS; LÓPEZ-LLUCH; BUSTOS, 2020). Recently, overweight women with NAFLD and T2DM showed reduction in liver lipids after 10 weeks of different aerobic training modalities (BANITALEBI et al., 2019). Similarly, 8 weeks of aerobic training reduced liver fat in inactive and overweight/obese adults, even when performed at low volume and low intensity (KEATING et al., 2015). Finally, an important meta-analysis revealed that the reduction in intrahepatic triglyceride provided by aerobic training is associated with an increase in the hepatic action of insulin, reinforcing the therapeutic potential of aerobic training in the treatment of NAFLD and T2DM (SARGEANT et al., 2018a). However, the biomolecular mechanisms by which aerobic training contributes to reduction in NAFLD are still poorly understood, and there are no studies investigating the effects of aerobic training on hepatic clusterin levels. Thus, the aim of this study was to investigate whether short-duration aerobic training alters hepatic clusterin levels in obese mice.

MATERIAL AND METHODS

Animals and diet

All procedures were made in accordance with Brazilian law, and accepted by the Ethics Committee on Animal Use – UNICAMP (#4773-1/2018). Male Swiss mice were allocated in individual cages with the enriched environment (PVC pipes were sawed in the middle generating a shelter of 10x10 cm of the base and 5 cm of height) and under controlled conditions of the light-dark cycle (12 / 12h), food and water *ad libitum*, temperature controlled at 22 ± 2 ° C, relative humidity maintained at 45-55%, and on-site noises below 85 decibels. Moreover, at 8 weeks old the animals were divided into two groups: the control (CTL) group kept eating chow diet and the obese group started a high-fat diet (HFD) treatment to induce obesity. After 8 weeks of HFD treatment the obese animals were subdivided into sedentary obese (OBSed) and aerobic trained (OBAer) with no differences in body weight and fasting glycemia between them.

Short-term aerobic training

Obese trained mice were adapted to the treadmill for 5 days and then submitted to the maximal power test to determine animals' aerobic capacity. Briefly, maximal power is an incremental test in which the animals performed an initial running at 6 m/min and every 3 minutes the velocity increases 3 m/min until the animal could no longer run. The stage in which the animal stopped running was considered its maximal power. After determining maximal power, the short-term aerobic training protocol started and consisted in 1 hour per day of treadmill running at 75% of maximum power in the dark cycle and lasted 7 days. Moreover, short-term aerobic training was chosen to evaluate the effects of physical training without body weight change.

Intraperitoneal Pyruvate Tolerance Test (ipPTT)

Eight hours after the end of 5th exercise session, the animals were submitted to ipPTT, 8 hours fasting. The animals were submitted to an intraperitoneal injection of pyruvate (2 g/kg of sodium pyruvate Azis Científica®, Cotia, SP) and the blood samples of the animals were drawn from the tail before the pyruvate injection, which was considered the time 0, and after 30, 60, 90, and 120 min of the injection to determine the blood glucose concentration. The results of ITT and ipPTT were evaluated using the areas under the serum glucose curves (AUC) during the test by

the trapezoidal method (MATTHEWS et al., 1990) by Microsoft Excel (2013), Microsoft Corporation, Redmond, WA,USA.

Tissue extraction and immunoblotting analysis

The euthanasia was performed 8 hours after the 7th exercise session, and the animals also were fasted for 8 hours. Ten minutes before the euthanasia, the animals received either human insulin (8 U/kg body wt Humulin-R; Lilly, Indianapolis, IN) or saline via i.p. The animals were anesthetized via i.p. by the injection of chloral hydrate of ketamine (100 mg/kg, Parke-Davis, Ann Arbor, MI) and xylazine (10 mg/kg, Rompun, Bayer, Leverkusen), and after the verification and assurance of the lack of corneal reflexes, the liver was collected and rapidly snap-frozen in liquid nitrogen and stored at -80 °C until analysis. The epididymal and retroperitoneal adipose tissue (right side) were removed and weighted to measure the fat depots. The liver was homogenized in extraction buffer [1% Triton-X 100, 100 mM Tris (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 mg of aprotinin/mL] at 4° C with a TissueLyser II (QUIAGEN®) operated at maximum speed for 120 s. The lysates were centrifuged (Eppendorf 5804R) at 12.851 × *g* at 4 °C for 15 min to remove insoluble material, and the supernatant was used for the assay. The protein content was determined by the bicinchoninic acid method (WALKER, 1994). The samples containing 60 µg of total protein were applied to a polyacrylamide gel for separation by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk at room temperature for 1 h and incubated with primary antibodies against the protein of interest. After that, a specific secondary antibody was used. The specific bands were labeled by chemiluminescence, and visualization was performed by photo documentation system in G: box (Syngene). The bands were quantified using the software UN-SCAN-IT gel 6.1.

Statistical analysis

The results were shown as the mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to evaluate the Gaussian distribution of the data, and Student's *t*-test was used to compare two groups with parametric data when it was necessary. Furthermore, to compare more than two groups, the one-way Analysis of Variance (ANOVA) test followed by Bonferroni's post-hoc test was performed. To

analyze the points of ipPTT, the Two-way ANOVA (with repeated measures when appropriate), with Bonferroni's correction for multiple comparisons was used. The statistical significance level considered was P<0.05. The construction of the graphics and the statistical analysis were performed using Prism (7.00) GraphPad Software San Diego, CA, USA.

RESULTS

Short-term aerobic training reduces hyperglycemia in obese mice, even without changes in body composition:

Initially, we observed that HFD provided weight and adiposity gains, inducing fasting hyperglycemia (Fig 1A-D). However, after the short-term aerobic training protocol, trained animals reversed obesity-induced hyperglycemia, even without changes in body weight and adiposity. (Fig 2A-D).

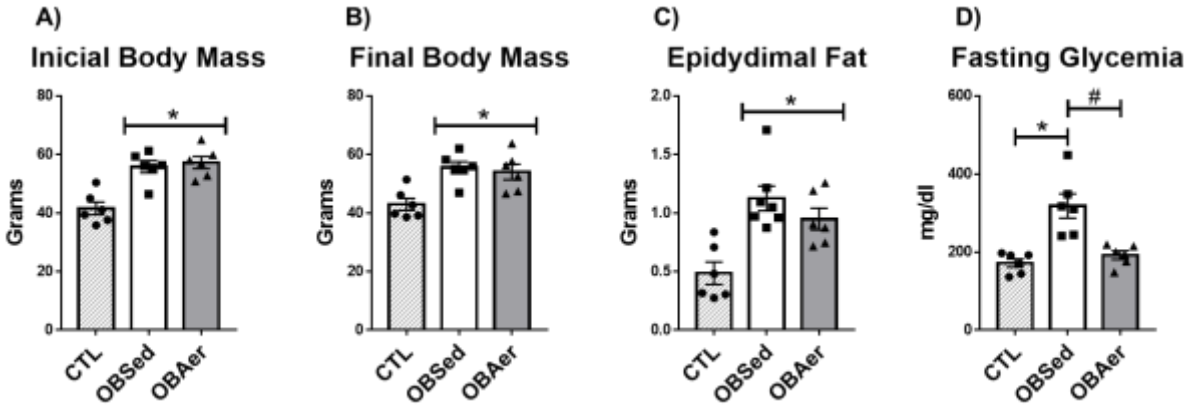

Figure 1: Physiological parameters of CTL, OBSed and OBAer groups. A and B) Body mass of the animals at the beginning and at the end of the experiment. C) Adipose tissue weight of the epididymal region. D) Fasting glycemia (after 8 h of fasting). *P < 0.05 vs CTL; #P < 0.05 vs OBSed (n = 6 per group).

Short-term aerobic training reduces NAFLD and increases HGP control and liver insulin sensitivity:

Our next step was to check the effects of short-term aerobic training in liver fat accumulation and hepatic insulin action. Using Oil Red O staining, we observed a robust liver fat accumulation in OBSed group (Fig 2A). However, short-term aerobic

training was effective reversing this scenario (Fig 2A). During ipPTT, obese animals showed a large increase in glycemia after pyruvate injection, with glycemic values remaining high during all the points during the test (Fig 2B). On the other hand, trained animals had reduced glycemic values compared to sedentary obese animals (Fig 2B). Thus, the AUC during the test was higher for OBSed group, and reduced with aerobic training (Fig 2C). Coherently, after insulin injection we observed that Akt activation was decreased in OBSed group compared to CTL group, however aerobic training increased the hepatic insulin action in obese mice (Fig 2D-F).

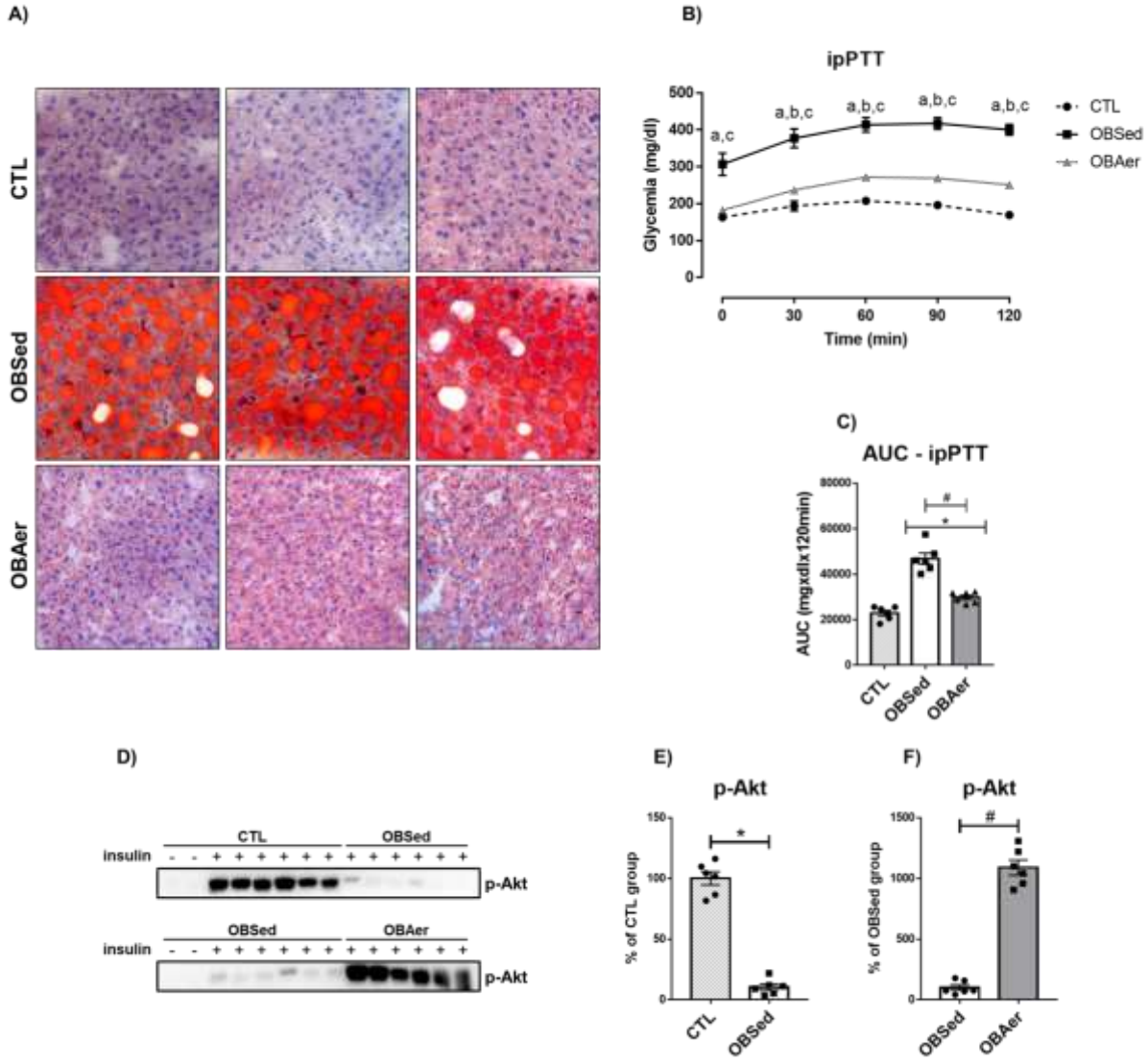

Figure 2: Liver fat content, ipPTT and hepatic insulin sensivity of CTL, OBSed and OBAer groups. A) Oil red O staining of the right lobe from three experimental groups. B) Glycemic curve during ipPTT. C) The area under the curve during ipPTT. D) Bands of p-Akt in the liver of mice from CTL and OBSed groups (upper) and from OBSed and OBAer groups (lower). E) Quantification of hepatic p-Akt of CTL and

OBSed groups. F) Quantification of hepatic p-Akt of OBSed and OBAer groups. Only bands of the animals stimulated with insulin were quantified. *P < 0.05 vs CTL; #P < 0.05 vs OBSed (n = 6 per group).

Trained animals have less lipogenesis and liver inflammation:

Next, we evaluated the effects of short-term aerobic training in liver lipogenesis, fat oxidation and inflammation. As shown in figure 4, trained animals showed reduction in lipogenic genes expression *Fans* and *Scd1*, with an increase in the oxidative gene *Cpt1a* (Fig 3A-C). We also observed increasement in phosphorylation and reduction in ACC total content, demonstrating a lower activity of this protein (Fig 3E-G). Similarly, we also found reduction in FAS, TNF-α and IL-1β protein content (Fig 3A, I and J).

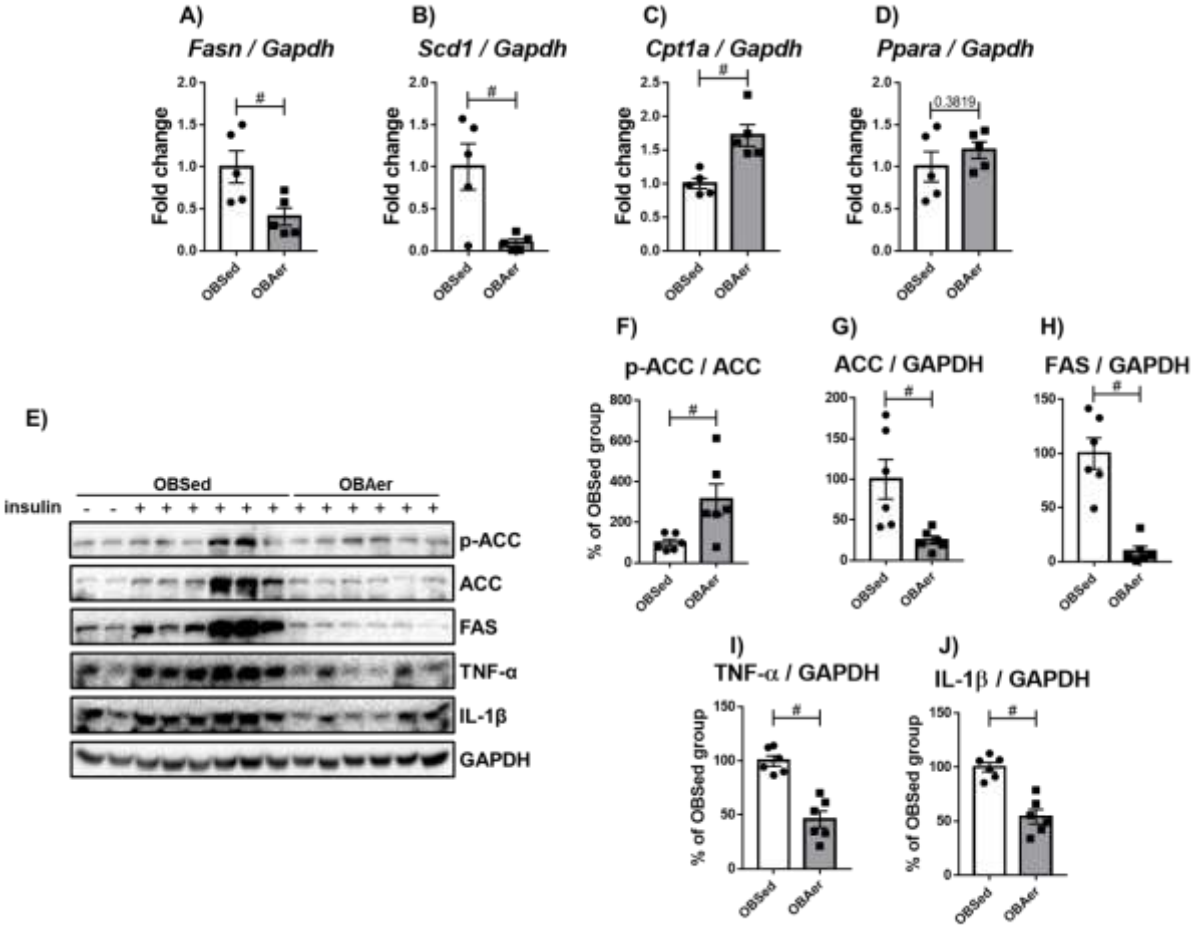

Figure 3: Parameters of hepatic lipogenesis, fat oxidation, and inflammation profile. A-D) Levels of mRNA genes related to lipogenesis (*Fasn* and *Scd1*) and oxidation (*Cpt1a* and *Ppara*) of OBSed and OBStr groups. E) Bands of the lipogenic and inflammatory proteins in the liver of mice from OBSed and OBAer groups after

insulin stimulus. F) Quantification of hepatic p-ACC^{ser79}/ACC of OBSed and OBAer groups. G and H) Quantification of hepatic ACC and FAS content, respectively, of OBSed and OBAer groups. I and J) Quantification of hepatic TNF-α and IL-1β content, respectively, of OBSed and OBAer groups. Only the bands of the animals stimulated with insulin were quantified. *P < 0.05 vs CT; #P < 0.05 vs OBSed (n = 5-6 per group).

Short-term aerobic training increases clusterin hepatic levels in obese mice:

Finally, we check the effects of short-term aerobic training in hepatic clusterin levels. After the experimental period, we observed a tendency to increase hepatic clusterin levels in OBSed group (P=0.0830) (Fig 5A and B). However, trained animals showed higher levels when compared to sedentary obese animals (Fig 5A and C).

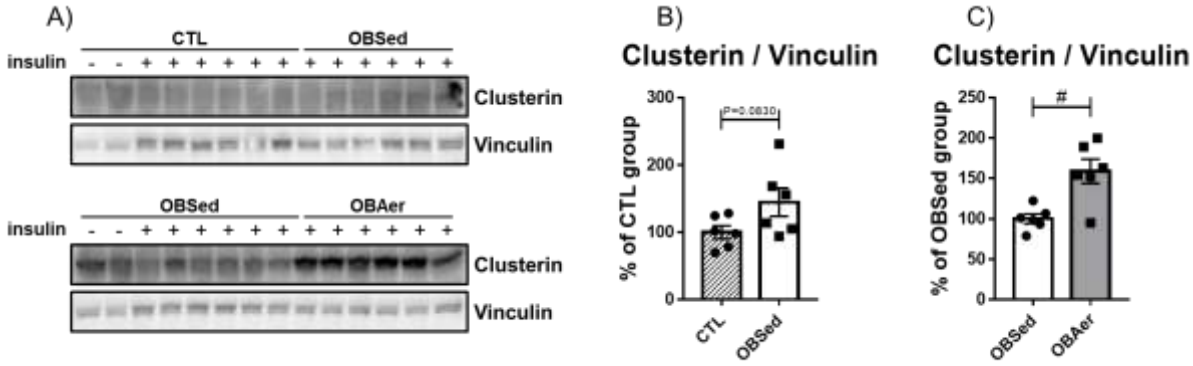

Figure 4: Hepatic clusterin levels of CTL, OBSed and OBAer groups. A) Bands of clusterin in the liver of mice from CTL and OBSed groups (upper) and from OBSed and OBAer groups (lower). B) Quantification of hepatic clusterin / vinculin of CTL and OBSed groups. C) Quantification of hepatic clusterin / vinculin of OBSed and OBAer groups. Only bands of the animals stimulated with insulin were quantified. *P < 0.05 vs CTL; #P < 0.05 vs OBSed (n = 6 per group).

CONCLUSIONS

In summary, we found that short-term aerobic training increases hepatic clusterin levels in obese mice, independently of body weight and adiposity changes. Thus, lipogenesis and liver inflammation are reduced, increasing insulin action and

HGP control and reducing NAFLD. This is a new mechanism by which aerobic training is an efficient strategy against NAFLD and hyperglycemia obese-state.

5. CONCLUSÃO

A presente obra traz importantes avanços a respeito das compreensão dos mecanismos pelos quais a prática de exercícios físicos contribui na redução do acúmulo de gordura hepática e melhora a sinalização da insulina no fígado, contribuindo para um melhor controle da PHG e redução da hiperglicemia associada à obesidade. Pela primeira vez, demonstramos que diferentes protocolos de treinamento de curta duração são capazes de reduzir o acúmulo de gordura hepática por reduzir a ativação da maquinaria biomolecular de síntese de lipídios, e proporcionar aumento na expressão de genes oxidativos. Ainda, assim como previamente descrito em relação ao treinamento aeróbio, o treinamento de força contribui para o melhor controle da PHG por aumentar a ação da insulina no fígado e proporcionar maior inativação da proteína FOXO1, reduzindo assim o conteúdo de proteínas gliconeogênicas. Um possível mecanismo proposto pelo qual as diferentes modalidades de treinamento contribuem para a melhora no quadro de resistência hepática seletiva à insulina (aumentando a ação da insulina promovendo controle da PHG e simultaneamente reduzindo a lipogênese hepática) se dá por meio do aumento do conteúdo proteico da isoforma citoplasmática de clusterina no fígado de camundongos obesos.

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7. APÊNDICES

Por fim, incluo como apêndice a lista de demais artigos que elaborei e executei, porém que não tem íntima relação com os objetivos da minha tese, e também os artigos que colaborei como coautor:

Artigos publicados como primeiro autor:

PEREIRA, R. M. et al. Fructose Consumption in the Development of Obesity and the Effects of Different Protocols of Physical Exercise on the Hepatic Metabolism. **Nutrients**, v. 9, n. 4, p. 405, 20 abr. 2017.

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RODRIGUES, K. C. DA C. et al. The Role of Physical Exercise to Improve the Browning of White Adipose Tissue via POMC Neurons. **Frontiers in Cellular Neuroscience**, v. 12, n. March, p. 88, 28 mar. 2018.

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8. ANEXOS

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CERTIFICADO

Certificamos que a proposta intitulada **EFEITOS DO EXERCÍCIO DE FORÇA NO METABOLISMO DA CLUSTERINA E SUA INTERFERÊNCIA NA SINALIZAÇÃO DA INSULINA EM ROEDORES**, registrada com o nº **4406-1**, sob a responsabilidade de **Prof. Dr. Leandro Pereira de Moura e Rodrigo Martins Pereira**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em reunião de **03 de novembro de 2016**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**04/11/2016-04/11/2018**
Vigência da autorização para manipulação animal:	**04/11/2016-04/11/2018**
Espécie / linhagem/ raça:	**Camundongo isogênico / C57BL/6J**
No. de animais:	**240**
Peso / Idade:	**04 semanas / 15g**
Sexo:	**machos**
Origem:	**CEMIB/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 03 de novembro de 2016.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos

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CERTIFICADO

Certificamos que a proposta intitulada **EFEITOS DO EXERCÍCIO NA INFILTRAÇÃO DE MACRÓFAGOS E MUDANÇA DE M1 PARA M2 EM CAMUNDONGOS OBESOS**, registrada com o nº **4773-1/2018**, sob a responsabilidade de **Prof. Dr. Leandro Pereira de Moura e Rodrigo Martins Pereira**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal** (**CONCEA**), **tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **13 de março de 2018**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**23/02/2018-23/02/2021**
Vigência da autorização para manipulação animal:	**13/03/2018-23/02/2021**
Espécie / linhagem/ raça:	**Camundongo heterogênico / Unib:SW (Swiss)**
No. de animais:	**448**
Idade/Peso:	**04 semanas / 40g**
Sexo:	**machos**
Origem:	**CEMIB/UNICAMP**
Biotério onde serão mantidos os animais:	**Biotério da Faculdade de Ciências Aplicadas, FCA/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 13 de março de 2018.

Prof. Dr. Wagner José Fávaro
Coordenador

Fátima Alonso
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

Profa. Dra. Nancy Lopes Garcia
Presidente
Comissão Central de Pós-Graduação
Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **EFEITOS DE DIFERENTES PROTOCOLOS DE TREINAMENTO FÍSICO EM VIAS BIOMOLECULARES DE CONTROLE DE PRODUÇÃO HEPÁTICA DE GLICOSE E METABOLISMO LIPÍDICO NO FÍGADO DE ROEDORES OBESOS**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Limeira, 20/12/2021

Autor Rodrigo Martins Pereira

RG n.° 46.032.867-0

Orientador Leandro Pereira de Moura

RG n.° 60.571.997-4