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PROGRAMA DE PÓS-GRADUAÇÃO EM NUTRIÇÃO

EFEITO DA RESTRIÇÃO ALIMENTAR INTERMITENTE APÓS CONSUMO DE DIETA HIPERENERGÉTICA SOBRE A DINÂMICA MORFOLÓGICA DOS DEPÓSITOS DE GORDURA E A VIA INFLAMATÓRIA HIPOTALÂMICA EM RATAS

MARIANA ALEJANDRA ROSAS FERNÁNDEZ

RIO DE JANEIRO
FEVEREIRO 2021

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MARIANA ALEJANDRA ROSAS FERNÁNDEZ

Tese apresentada ao Programa de Pós-graduação em Nutrição (PPGN), do Instituto de Nutrição Josué de Castro da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de **doutor em Ciências Nutricionais**.

Orientadoras: Prof[a]. Dr[a]. Kelse Tibau de Albuquerque e Prof[a]. Dr[a]. Maria das Graças Tavares do Carmo.

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TESE SUBMETIDA AO CORPO DOCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM NUTRIÇÃO DO INSTITUTO DE NUTRIÇÃO JOSUÉ DE CASTRO DA UNIVERSIDADE FEDERAL DO RIO DE JANEIRO COMO PARTE DOS REQUISITOS NECESSÁRIOS PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS NUTRICIONAIS.

Examinada por:

_____

Profª. Drª. Kelse Tibau de Albuquerque

_____

Profª. Drª. Maria das Graças Tavares do Carmo

_____

Profª. Drª. Fátima Lúcia de Carvalho Sardinha

_____

Profª. Drª. Tatiana El-Bacha Porto

_____

Profª. Drª. Monica Marques Telles

_____

Profª. Drª. Lila Missae Oyama

_____

Profª. Drª. Marta Citelli dos Reis

RIO DE JANEIRO, RJ - BRASIL

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RESUMO

Introdução: O aumento global da prevalência de obesidade tem motivado a investigação de diferentes protocolos de restrição energética, incluindo a restrição alimentar intermitente (RAI), caracterizada tanto pela flexibilidade, como por induzir perda de peso em curto prazo, ganhando número crescente de adeptos. Apesar da literatura apontar benefícios sobre marcadores bioquímicos e antropométricos, ainda não há consenso sobre estes resultados, além disso, poucas são as evidências das repercussões deste tratamento sobre o sistema nervoso central. **Objetivo:** Avaliar em fêmeas Wistar o efeito da RAI alternada com dieta que induz a obesidade (DIO) na dinâmica morfológica do tecido adiposo branco (TAB) e marrom (TAM) e sobre a resposta inflamatória hipotalâmica. **Métodos:** Fêmeas jovens (45 dias de vida) foram divididas aleatoriamente em 4 grupos: *Standard Control* (ST-C) tratado com ração padrão e água filtrada *ad libitum*; DIO *Control* (DIO-C) tratado com dieta hiperenergética durante os primeiros e últimos 15 dias da intervenção e ração padrão entre o 16° e 45° dia; *Standard Restricted* (ST-R) tratado com ração padrão nos primeiros e últimos 15 dias da intervenção, seguido de RAI a 50% do ST-C entre o 16° e 45° dia; e DIO *Restricted* (DIO-R) tratado com dieta hiperenergética durante os primeiros e últimos 15 dias da intervenção e foi submetido a RAI nas mesmas condições que o ST-R. **Resultados:** Artigo 1: O consumo alimentar, a eficiência energética, os depósitos viscerais e de TAM foram maiores nos grupos com RAI comparados aos seus respectivos controles. Os grupos submetidos a RAI apresentaram hipertrofia do TAB e do TAM e observou-se fibrose no TAM. Artigo 2: Os grupos ST-R e DIO-R, comparados ao ST-C, apresentaram maior expressão dos genes hipotalâmicos PPARα, JNK, SOCS3 e NFκB, enquanto a expressão do gene CCL5 foi

maior no grupo DIO-R, comparado a todos os grupos. **Conclusão:** a RAI pode estabelecer resistência prospectiva à perda de massa corporal por favorecer alterações na morfologia do tecido adiposo, incremento no consumo e na eficiência energética, indicando que a RAI foi capaz de exacerbar os prejuízos causados pela restrição, enquanto que no hipotálamo, a RAI intercalada ou não com DIO, agravou o perfil inflamatório hipotalâmico, associado a prejuízo na resposta anorexígena causada por acentuada expressão de SOCS3 com possível repercussão na resposta central da insulina e leptina. As alterações verificadas nos mecanismos morfológicos adipocitário e termogênico no TAB e TAM sugerem que a RAI não promove perda saudável de peso e prejudica a resposta inflamatória hipotalâmica em ratas Wistar.

Palavras chave: jejum intermitente, tecido adiposo, eficiência energética, hipotálamo, inflamação, homeostase energética.

EFFECT OF INTERMITTENT FOOD RESTRICTION AFTER HYPERENERGETIC DIET INTAKE ON THE MORPHOLOGICAL DYNAMICS OF FAT DEPOTS AND THE HYPOTHALAMIC INFLAMMATORY RESPONSE IN RATS

MARIANA ALEJANDRA ROSAS FERNÁNDEZ

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Advisors: Prof[a]. Dr[a]. Kelse Tibau de Albuquerque

Prof[a]. Dr[a]. Maria das Graças Tavares do Carmo.

ABSTRACT.

Introduction: The global increase in obesity prevalence has motivated the investigation of different energy restriction protocols, including intermittent food restriction (IFR), characterized by both flexibility and inducing short-term weight loss, gaining an increasing number of adepts. Although the literature shows benefits on biochemical and anthropometric markers, there is still no consensus on these results, and there is little evidence of the repercussions of this treatment on the central nervous system. **Objective:** To evaluate in Wistar females the effect of IFR alternated with obesity-inducing diet (DIO) on the morphological dynamics of white (WAT) and brown (BAT) adipose tissue and on the hypothalamic inflammatory response. **Methods:** Young females (45 days old) were randomly divided into 4 groups: Standard Control (ST-C) treated with standard feed and filtered water *ad libitum*; DIO Control (DIO-C) treated with a hyperenergetic diet during the first and last 15 days of the intervention and standard ration between the 16th and 45th days; Standard Restricted (ST-R) treated with standard feed in the first and last 15 days of the intervention, followed by IFR at 50% of the ST-C between the 16th and 45th days; and DIO Restricted (DIO-R) treated with a hyperenergetic diet during the first and last 15 days of the intervention and was submitted to RAI under the same conditions as the ST-R. **Results:** Article 1: Food consumption, energy efficiency, visceral, and BAT deposits were higher in groups with IFR compared to their respective controls. Groups with IFR showed hypertrophy of WAT and BAT and fibrosis was observed in BAT. Article 2: The groups ST-R and DIO-R, compared to ST-C, showed greater expression of the PPARα, JNK, SOCS3 and NFκB genes, while the expression of the CCL5 gene was higher in the DIO-R group, compared to all groups. **Conclusion:** IFR can establish prospective resistance to loss of body mass by favoring changes in adipose tissue morphology, increased consumption and energy efficiency, indicating that IFR was able

to exacerbate the damage caused by the restriction, whereas in the hypothalamus, the intercalated IFR or not with DIO, it worsened the hypothalamic inflammatory profile, associated with impaired anorectic response caused by marked SOCS3 expression with possible repercussions on the central response of insulin and leptin. The changes observed in the adipocyte and thermogenic morphological mechanisms in WAT and BAT suggest that IFR does not promote healthy weight loss and impairs the hypothalamic inflammatory response in Wistar rats.

Keywords: intermittent fasting, adipose tissue, energy efficiency, hypothalamus, inflammation, energy homeostasis.

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ANEXO B – Artigo publicado na revista British Journal of Nutrition

LISTA DE ABREVIATURAS

AgRP	Proteína relacionada ao gene *agouti*, do inglês *Agouti-Related Protein*
AKT	Proteína quinase B, do inglês *threonine kinase Akt*
AMPK	Proteína quinase ativada por AMP, do inglês *Adenosine monophosphate-activated protein kinase*
CART	Transcrito regulado por cocaína e anfetamina, do inglês *Cocaine- and Amphetamine-Regulated Transcript*
CCL5	Ligante 5 de quimiocina, do inglês *Chemokine (C-C motif) ligand 5)*
DIO	Dieta que induz obesidade, do inglês Diet-induced Obesity
DIO-C	DIO controle, do inglês *DIO control*
DIO-R	DIO restrito, do inglês *DIO restricted*
EE	Eficiência energética
GABA	ácido ɣ-aminobutírico, do inglês *Gamma-AminoButyric Acid*
IKKβ	Fator nuclear kappa-B quinase subunidade beta, do inglês *inhibitor of nuclear factor kappa-B kinase subunit beta*
IL-6	Interleucina 6
IR	Receptor de insulina, do inglês *Insulin receptor*
IRS-1	Substrato do receptor de insulina 1, do inglês *Insulin receptor substrate 1*
JNK	c-Jun quinase N-terminal, do inglês *c-Jun N-terminal kinase*
LPS	Lipopolisacariídeos
NFκ-B	Fator nuclear κB, do inglês *Nuclear factor kB*
NK	do inglês Natural Killer Cell)
NPY	Neuropeptídeo Y, do inglês *Neuropeptide Y*
ObRb	Isoforma longa do receptor de leptina, do inglês *Long leptin receptor signal transduction*
PAMPs	Padrão de reconhecimento de patógenos, do inglês *Pathogen-associated molecular patterns*
PI3K	Fosfatidilinositol 3-quinase, do inglês *Phosphoinositide 3-kinase*
PPARα	Receptor ativado por proliferadores peroxissomais tipo alfa, do inglês *Peroxisome proliferator-activated receptor alpha*
POMC	Proopiomelanocortina, do inglês *Proopiomelanocortin*

qPCR	Reação em cadeia da polimerase em tempo real, do inglês *real time quantitative Polymerase Chain Reaction*
RAI	Restrição alimentar intermitente
SNC	Sistema nervoso central
ST-C	Controle padrão, do inglês *Standard Control*
ST-R	Controle restrito, do inglês *Standard Restricted*
SOCS3	Supressor da sinalização de citocinas 3, do inglês *Suppressor of cytokine signaling 3*
TAB	Tecido adiposo branco
TAM	Tecido adiposo marrom
TLR	Receptores Toll-Like, do inglês *Toll-Like receptor*
TNF- α	Fator de necrose tumoral α, do inglês *Tumor necrosis factor alpha*
α-MSH	Hormônio estimulante de melanócitos, do inglês *alpha-Melanocyte-stimulating hormone*

1. INTRODUÇÃO

O aumento da prevalência de obesidade no mundo (CHOOI; DING; MAGKOS, 2019), assim como no Brasil (GOMES et al., 2019), tem motivado a recomendação e utilização de diversos protocolos de restrição energética, incluindo a restrição alimentar intermitente (RAI) (OBERT et al., 2017). Existe ampla variação de protocolos para RAI, porém o mais usual refere-se aos períodos de restrição alimentar total ou parcial, com consumo de 25 a 75% dos requerimentos energéticos totais diários, podendo-se prolongar por vários dias, seguidos ou alternados. O período pós-restrição caracteriza-se pelo retorno à livre alimentação, repetindo-se estes ciclos de restrição-realimentação durante intervalo definido de tempo (TINSLEY; LA BOUNTY, 2015). Os estudos vêm mostrando benefícios periféricos frente a sua utilização em curto prazo, principalmente no que se refere à perda de massa corporal, diminuição dos riscos cardiovasculares e aumento da sensibilidade à insulina (DONG et al., 2020; HODDY et al., 2020). Contudo, não há consenso quanto aos efeitos desta intervenção em longo prazo, não sendo promissores no sistema nervoso central (SNC), pois indicam prejuízos nas respostas anorexígenas relacionadas a esse sistema (CHAUSSE et al., 2014; 2015; ROSAS FERNÁNDEZ et al., 2018).

O hipotálamo é considerado o centro regulador da homeostase energética, onde são encontradas populações neuronais que têm como função principal a regulação do consumo alimentar. Neurônios que expressam neuropeptídeo Y (NPY) e proteína relacionada ao gene agouti (AgRP) são sensíveis à redução de energia, sinalizando a resposta de fome e diminuindo o gasto energético, enquanto neurônios que expressam proopiomelanocortina (POMC) e transcrito regulado por cocaína e anfetamina (CART) diminuem o estado de fome e aumentam o gasto energético. A ação coordenada destes neurônios hipotalâmicos no controle homeostático da fome e do gasto energético é necessária para a manutenção da massa corporal, no entanto, dietas restritivas assim como ricas em ácidos graxos podem promover efeitos capazes de romper este equilíbrio e conduzindo ao ganho de massa corporal (TIMPER; BRÜNING, 2017).

A inflamação faz parte da resposta do sistema imune, inata e adquirida, ativando processos que têm como finalidade a reparação dos tecidos lesionados ou a eliminação de patógenos (COOKE, 2019). O consumo elevado de energia, principalmente proveniente de ácidos graxos saturados e açúcar refinado, desencadeia resposta metabólica

inflamatória. A presença destes componentes da dieta no cérebro estimula resposta inflamatória aguda imediata que, inicialmente, não produz aumento de adipocinas circulantes. No entanto, a manutenção de padrão alimentar, caracterizado por elevado consumo destes constituintes dietéticos, por período prolongado, produz, no hipotálamo, inflamação crônica de baixo grau, causando desequilíbrio na resposta alimentar, que pode resultar, em longo prazo, no aumento da massa corporal (DE SOUZA et al., 2005). Enquanto que no sistema periférico, este tipo de padrão alimentar pode potencializar aumento da adiposidade, levando a modificações morfológicas do tecido adiposo (LONGO et al., 2019).

A RAI é praticada, principalmente, por mulheres que sustentam alimentação hipercalórica por períodos prolongados de tempo e utilizam este protocolo de restrição energética, com o objetivo de emagrecer rapidamente, ainda que interpondo períodos de consumo de dieta obesogênica (CRANE; JEFFERY; SHERWOOD, 2017; KASHUBECK-WEST; MINTZ; WEIGOLD, 2005; LEAL; PHILIPPI; ALVARENGA, 2020). Apesar de se conhecer os efeitos das dietas hiperenergéticas, promotoras de aumento dos depósitos de gordura e de prejuízos na homeostase energética (CHRIST; LAUTERBACH; LATZ, 2019), pouco se sabe sobre as consequências metabólicas da RAI, intercalada ou não com dieta obesogênica. Foi hipótese deste trabalho que a sinergia entre a realização da RAI e a dieta que induz a obesidade (DIO) pode trazer prejuízos a saúde tanto a nível periférico como a nível central, inclusive mais significantes que o consumo de DIO de forma isolada. Assim, foi objetivo deste estudo avaliar a dinâmica morfológica dos depósitos de gordura branco e marrom, assim como a resposta inflamatória hipotalâmica após RAI, intercalada com dieta hiperenergética, promotora de obesidade, em fêmeas Wistar.

2. REVISÃO BIBLIOGRÁFICA

O hipotálamo é o centro regulador da homeostase energética, capaz de receber informações provenientes dos tecidos periféricos como fígado, pâncreas, tecido adiposo, intestino e músculo, promovendo ajustes e inicializando respostas orexígenas ou anorexígenas. Duas populações de neurônios, localizadas no núcleo arqueado hipotalâmico, têm como função principal a regulação do consumo alimentar. Neurônios que co-expressam NPY/AgRP, sensíveis aos diferentes sinais de alteração da homeostase energética, sinalizam resposta de fome e redução do gasto energético, mediante condições indicativas de baixo estoque de energia. De outro modo, a ativação de neurônios que co-expressam POMC/CART resulta na redução da ingestão alimentar e aumento do gasto energético (TIMPER; BRÜNING, 2017).

No período pós-prandial as concentrações circulantes de leptina, provenientes do tecido adiposo, e de insulina, liberada em resposta às concentrações séricas de glicose, se encontram elevadas no sangue. Ambos os hormônios atravessam a barreira hematoencefálica e se ligam aos receptores ObRb e IR respectivamente, iniciando a cascata de sinalização, por meio da ativação da via PI3K-Akt, que leva ao aumento da expressão de POMC e CART. Neurônios POMC se projetam para neurônios de segunda ordem, localizados no hipotálamo lateral e ventromedial, promovendo a liberação do hormônio estimulante de melanócitos (α-MSH), com conseguinte ativação de vias catabólicas, o que resulta na redução da ingestão alimentar e aumento do gasto energético (ROH; SONG; KIM, 2016).

Em contrapartida, durante o período de jejum, concentrações séricas elevadas de glucagon e grelina, aumento na expressão de AMPK intracelular, assim como teores basais de hormônios anorexígenos, conduzem à ativação do NPY/AgRP desencadeando sensação de fome e consequente resposta alimentar. Além disso, NPY reduz o gasto de energia pela diminuição sináptica em direção ao tecido adiposo marrom (TAM), enquanto o AgRP atua como agonista inverso, evitando o efeito anorexígeno do α-MSH em neurônios de segunda ordem. Tanto NPY como AgRP inibem indiretamente os neurônios POMC por meio do ácido ɣ-aminobutírico (GABA) (WEN et al., 2019).

Em condições fisiológicas ideais a resposta “fome – saciedade” permanece sob controle por meio de processos coordenados pelo hipotálamo, capazes de manter a massa corporal estável. No entanto, o consumo de dietas hiperenergéticas pode romper a

homeostase energética e conduz ao aumento da massa corporal. A perda da capacidade do controle homeostático do balanço de energia parece ser decorrente, principalmente, da inflamação hipotalâmica resultante do consumo elevado de ácidos graxos saturados e açúcar refinado. Estes constituintes dietéticos, no tecido cerebral, são capazes de estimular resposta inflamatória aguda local, independente, inicialmente, do aumento de adipocinas circulantes, que se estabelece em mais longo prazo. A manutenção deste padrão de ingestão alimentar, por período prolongado, tem sido associada ao quadro de inflamação crônica de baixo grau hipotalâmica (CHRIST; LAUTERBACH; LATZ, 2019).

Nessa perspectiva, os receptores Toll-like (TLR) são uma família de proteínas transmembrana que desempenham papel essencial no sistema imunológico inato. Estes receptores reconhecem patógenos utilizando uma estratégia evolutiva conhecida como padrão de reconhecimento de patógenos (PAMPs), respondendo a lipopolissacarídeos (LPS), endotoxinas encontradas em abundância na membrana externa da maior parte das bactérias Gram-negativas. No entanto, vários estudos *in vitro* e *in vivo* (CAESAR et al., 2015; HUANG et al., 2012; LEE et al., 2001; NGUYEN et al., 2007; PAL et al., 2012; SHI et al., 2006), indicaram que o receptor do tipo Toll 4 (TLR4) também pode ser ativado por ácidos graxos saturados, da mesma forma como seria por um patógeno, dada sua natureza comum à porção do lipídio A do LPS, iniciando resposta inflamatória por meio da ativação da via do fator nuclear κB (NF-κB). Esta família de proteínas atua como fator de transcrição regulando a expressão de diversos genes relacionados à resposta imune, com processo desencadeado pela ativação do inibidor do fator nuclear kappa-B quinase subunidade beta (IKKβ) (DOUGLASS et al., 2017; BENOMAR; TAOUIS, 2019).

Recentemente, a teoria de que os ácidos graxos atuariam como agonistas do TLR4 vem sendo questionada. O TLR4 é o principal receptor dos LPS, contudo, o fator de dimerização mieloide 2 (MD2) é um correceptor, principal responsável pela união dos LPS. Lancaster et al (2018), utilizando simulações de dinâmica molecular, mostraram que a presença de 3 a 5 moléculas de palmitato dentro do complexo TLR4 / MD2 conduz a desestabilização do complexo receptor como seria com os LPS, indicando que os ácidos graxos de cadeia longa não são agonistas diretos de TLR4. Também, a dimerização dos heterodímeros TLR4 / MD2 permite a formação do heterotetrâmero do receptor ativo ($TLR4_2$ / $MD2_2$), que é o evento mais próximo ao início da ativação do TLR4. Em experimento *in vitro* utilizando macrófagos primários derivados de medula óssea, foi

observado que o tratamento com LPS conduz a dimerização de 80% do TLR4 em 15 minutos após tratamento, assim como a endocitose do $TLR4_2$ / $MD2_2$. No entanto, o tratamento com palmitato não foi capaz de causar dimerização do receptor, nem endocitose do heterotetrâmero durante o transcurso do experimento. Estes resultados demonstram que o palmitato não é um ligante agonista para o TLR4 (LANCASTER et al., 2018).

No mesmo estudo foi observado que a ativação da cascata de sinalização NF-kB e c-Jun quinase N-terminal (JNK) ocorreu de forma imediata com a ligação dos LPS ao receptor. No entanto, após tratamento com palmitato, foi evidenciado aumento da fosforilação do JNK, liberação de TNF 20 vezes menos do que com os LPS, assim como, estresse do reticulo endoplasmático, o que não foi evidenciado com outros ácidos graxos de cadeia longa saturado (miristato) e insaturado (palmitoleato). A diferença da ativação da via por LPS, com estes resultados utilizando palmitato, foram evidentes apenas após 4h do tratamento. Outro dado interessante é que a inibição farmacológica aguda de TLR4, tanto com o antagonista LPS penta-acilado de *Rhodobacter sphaeroides* (LPS-Rs) como com TAK-242, não levou a ativação de JNK após estimulação com LPS *E. coli*, o que já era o esperado. Contudo, os LPS-Rs não puderam prevenir a fosforilação da JNK induzida por palmitato, demostrando assim que o bloqueio do TLR4 não evita a ativação de JNK induzida pelo palmitato. Dessa forma, parece que o palmitato não ativa JNK diretamente pela ligação com TLR4, mas sim de outra via não convencional (LANCASTER et al., 2018).

O hipotálamo apresenta expressão significativa de IKKβ e NF-κB, que são mantidas inativas. Contudo, o consumo crônico e excessivo de lipídios, que promove aumento de LPS circulante, afeta a atividade destas proteínas (ZHANG et al., 2008). A ligação do lipídio A da endotoxina ao TLR4 desencadeia a via de sinalização IKKβ / NF-κB, com consequente produção de citocinas inflamatórias, como interleucina 6 (IL-6) e o fator de necrose tumoral α (TNF-α) (DALVI et al., 2017; CAVALIERE et al., 2018). Adicionalmente, o ligante 5 de quimiocina (CCL5) ou RANTES (do inglês Regulated on Activation, Normal T cell Expressed and Secreted), implicado no recrutamento de leucócitos, medeia a acumulação local de células T e macrófagos, envolvendo-se também na gênese da inflamação crônica (LE THUC et al., 2017).

A produção elevada de citocinas inflamatórias conduz a ativação do supressor de sinalização de citocinas 3 (SOCS3), que se liga à proteína *janus kinase 2* (JAK2) e aos

substratos do receptor de insulina (IRS), inibindo, assim, as vias anorexígenas dos hormônios leptina e insulina, promovendo consequente descontrole da resposta alimentar (PEDROSO; RAMOS-LOBO; DONATO, 2019). Ademais, o aumento da inflamação e de sinais de estresse, principalmente do retículo endoplasmático, promovem ativação da proteína JNK, que resulta, também, na inibição de ambas as vias (ZHANG et al., 2020).

O PPARα é um fator de transcrição nuclear que responde de forma evolutiva à inanição e pode ser ativado por várias moléculas endógenas, principalmente ácidos graxos de cadeia longa, de forma a regular positivamente genes de vias que são ativadas em jejum, como a gliconeogênese, a cetogênese, a captação e oxidação de ácidos graxos. Em condições fisiológicas, a função do PPARα é ativada principalmente durante o jejum, entretanto a obesidade e o jejum parecem representar dois extremos do espectro metabólico, ambos associados à ressíntese de ácidos graxos hepáticos e a diminuição da sinalização da insulina (PATSOURIS et al., 2006). Nos tecidos periféricos a função principal de PPARα é a regulação do catabolismo lipídico, enquanto no sistema nervoso central (SNC) o PPARα regula o metabolismo energético (CHAKRAVARTHY et al., 2007). Contudo, são escassos os estudos que avaliam o comportamento de PPARα no hipotálamo, até mesmo em razão de não constituir tecido com expressão significativa deste fator de transcrição.

Adicionalmente à clássica função do tecido adiposo branco (TAB) como armazenador de gordura, são reconhecidas outras propriedades funcionais que caracterizam este tecido como órgão endócrino, na medida em que produz e secreta citocinas com diversas funções fisiológicas. Consumo excessivo de energia promove hiperplasia e hipertrofia do TAB de modo a permitir maior estoque de gordura, ao passo que a expansão do tecido causa lipotoxicidade e aumento da secreção de citocinas pro-inflamatórias. Por outro lado, a manutenção desse padrão de consumo por períodos prolongados, também causa acúmulo de gordura em órgãos não especializados no estoque de lipídios, levando à disfunção do tecido (SEARS; PERRY, 2015).

Os adipócitos estão implicados na imunidade inata por apresentar receptores TLR. A ativação destes receptores conduz a translocação do NF-κB ao núcleo celular e aumento da secreção de citocinas inflamatórias como IL-6 e do TNF-α. O incremento de estresse metabólico também resulta na disfunção dos orgânulos, particularmente de mitocôndrias e do retículo endoplasmático, além de ativar a JNK, que inativa o IRS. O aumento dos ácidos graxos não esterificados, disfunção do tecido adiposo e estado hiperlipidêmico,

amplificam e perpetuam a inflamação crônica de baixo grau no tecido e o mantêm em produção constante de citocinas pró-inflamatórias (SUN et al., 2012).

Outra complicação relacionada à disfunção dos adipócitos é a infiltração de células imunes que incluem macrófagos, células T efetoras, células NK (do inglês *Natural Killer Cell*) e granulócitos. Estas células imunes produzem citocinas inflamatórias, metaloproteinases, espécies reativas de oxigênio e quimiocinas, que remodelam a arquitetura tissular e afetam a sinalização dos adipócitos. Em conjunto, estas alterações promovem maior disfunção metabólica, alteram a homeostase sistêmica, manejo da glicose e sensibilidade à insulina (JIANG et al., 2020).

Diferente do TAB, o TAM não tem como função principal o estoque de energia, estando relacionado à termogênese. O excesso de energia ingerida é oxidado nas mitocôndrias do TAM por meio de processo que utiliza a proteína desacopladora 1 (UCP 1, do inglês Uncoupling Protein 1), que interrompe o fluxo de elétrons da cadeia respiratória, produzindo calor ao invés de ATP (TRAYHURN, 2018). Contudo, a manutenção de padrão alimentar hiperenergético, por períodos prolongados, pode causar prejuízos neste tecido especializado, conduzindo a diminuição da termogênese, branqueamento dos adipócitos marrons e acúmulo de ácidos graxos (KUIPERS et al., 2019; MERCER; TRAYHURN, 1984).

Dados nacionais (GOMES et al., 2019) e globais (CHOOI; DING; MAGKOS, 2019) apontam para o elevado consumo de energia da população, existindo estreita relação com o aumento da prevalência de sobrepeso e obesidade, assim como das comorbidades associadas a esta doença. Neste contexto, diversas estratégias destinadas à perda de peso têm sido propostas, inclusive gerando a expectativa popular de rápida redução da massa corporal, de modo que a RAI tem ganhado destaque (OBERT et al., 2017).

A restrição alimentar intermitente teve origem na prática religiosa do Ramadan, quando muçulmanos praticantes, durante um mês (sagrado), jejuam entre o nascer e o pôr do sol, sendo permitida a alimentação fora deste período. Os estudos realizados com este grupo mostraram que a prática do jejum resultou em benefícios para a saúde, principalmente relacionados à prevenção de doenças cardiovasculares (MO’EZ AL-ISLAM’ et al., 2019)

Entre os diferentes protocolos para RAI, identifica-se como o mais empregado aquele que estabelece períodos de restrição alimentar total ou parcial, com consumo de 25 a 75% da energia total diária requerida, podendo perdurar por vários dias, seguidos ou alternados. O retorno à livre alimentação configura o período pós-restrição. O protocolo ainda institui a repetição destes ciclos de restrição-realimentação durante intervalo definido de tempo (RYNDERS et al., 2019; TINSLEY; LA BOUNTY, 2015).

Como modelos de estudo, os protocolos para RAI mais utilizados incluem diferentes propostas. O "Jejum em dias alternados" (ADF, do inglês *Alternate Day Fasting*), consiste em reduzido consumo energético em dias alternados da semana e realimentação *ad libitum* nos dias restantes. O "Regime de jejum modificado" (MFR, do inglês *Modified Fasting Regimens*), também conhecido como "Restrição energética intermitente" (IER, do inglês *Intermittent Energy Restriction*), estabelece consumo de energia limitado em 20 a 25% das necessidades energéticas totais nos dias programados de jejum. O "Regime 5:2", bastante popular nos últimos anos, representa a prática de cinco dias de alimentação *ad libitum* seguida de dois dias, não consecutivos, de restrição alimentar. A "Alimentação com restrição de tempo" (TRF, do inglês *Time-Restricted Feeding*), também observada mais recentemente, varia entre 12 e 21 horas de jejum diário, destacando-se a importância de sincronizar os regimes de jejum intermitente com o ciclo circadiano (PATTERSON; SEARS, 2017).

Estudos realizados tanto em humanos como em animais concordam que o principal objetivo da RAI consiste na redução da massa corporal total (SANTOS; MACEDO, 2018; STOCKMAN et al., 2018). Ainda que permaneçam controversos os efeitos periféricos deste tipo de intervenção sobre a massa adiposa corporal, a massa livre de gordura, as concentrações séricas de glicose, triacilgliceróis e marcadores cardiovasculares (HARVIE; HOWELL, 2017), há evidências de efeitos deletérios, em modelos animais (HAN et al., 2014; KLIEWER et al., 2015; PARKES et al., 2017; ROSAS FERNÁNDEZ et al., 2018). Apesar deste panorama, são escassas as evidências apontando os efeitos centrais da RAI no longo prazo.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o efeito da restrição alimentar intermitente alternada com o consumo de dieta hiperenergética sobre a dinâmica morfológica dos depósitos de gordura branca e marrom e a resposta inflamatória hipotalâmica de ratas.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar o comportamento alimentar e o ganho da massa corporal durante os períodos de restrição alimentar e realimentação;
- Quantificar os depósitos de gordura visceral;
- Avaliar os teores de triacilgliceróis e colesterol séricos;
- Analisar a morfologia do tecido adiposo branco subcutâneo e retroperitoneal;
- Analisar a morfologia do tecido adiposo marrom interescapular;
- Quantificar a expressão dos genes Leprot, PPARα, SOCS3, JNK, TLR4, IKKβ, NFκB e CCL5 no hipotálamo.

4. MATERIAL E MÉTODOS

4.1 CUIDADO ANIMAL E ÉTICA

Foram utilizadas quarenta fêmeas Wistar adultas jovens (quarenta e cinco dias de vida), obtidas e mantidas no biotério de roedores do Polo Universitário da (UFRJ), Campus Macaé. As ratas permaneceram alojadas em caixas individuais durante todo o período experimental, em ambiente controlado [12 h de ciclos claro / escuro (7:30-19:30), temperatura média de 22 ± 2ºC e umidade de 50% ± 10%]. A oferta de alimento e água respeitou protocolo específico estabelecido para cada grupo experimental.

O estudo foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFRJ, Campus Macaé (protocolo número MAC030, ANEXO A) e os procedimentos experimentais realizados obedeceram às normas do Conselho Nacional de Controle de Experimentação Animal - CONCEA.

4.2 DESENHO EXPERIMENTAL, PROTOCOLO DIETÉTICO E COMPOSIÇÃO DAS RAÇÕES

As ratas, distribuídas aletoriamente, constituíram quatro grupos experimentais, cada um composto por 10 animais, designados conforme descrição seguir: ST-C (grupo controle padrão); DIO-C (grupo controle DIO); ST-R (grupo padrão restrito) e DIO-R (grupo DIO restrito), submetidos a 60 dias de tratamento.

Os grupos ST-C e ST-R tiveram acesso à água filtrada e dieta padrão (Nuvilab, São Paulo, Brasil) ao longo de todo o protocolo experimental. Os grupos DIO-C e DIO-R receberam dieta que induz a obesidade (DIO) durante os primeiros e últimos 15 dias de tratamento, e o DIO-C consumiu dieta padrão e água filtrada *ad libitum* entre o 16° e 45° dia. Os animais ST-R e DIO-R foram submetidos ao protocolo de RAI entre o 16° e 45° dia de tratamento (Figura 1).

O protocolo RAI, aplicado aos grupos ST-R e DIO-R consistiu em dois dias de restrição alimentar a 50% do consumo alimentar do grupo ST-C, seguido de três dias de realimentação *ad libitum*, completando seis ciclos durante 30 dias (Figura 1). Durante o período de RAI foi ofertada ração padrão e água filtrada.

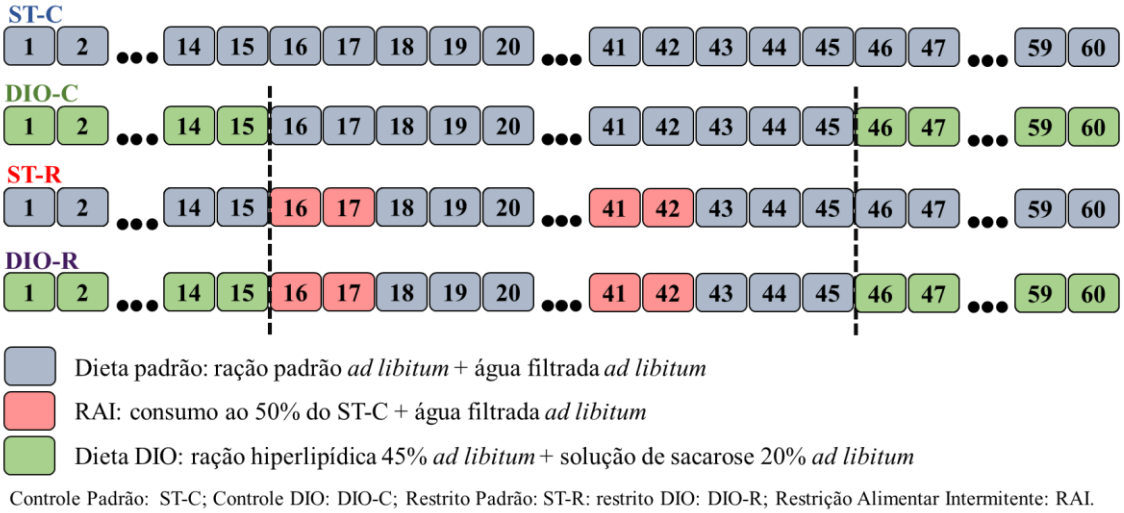

Figura 1. Desenho Experimental. Linha de tempo do tratamento dos grupos Controle Padrão (ST-C), DIO Controle (DIO-C), Restrito Padrão (ST-R), DIO Restrito (DIO-R). Os ratos ST-C consumiram ração padrão durante todo o período experimental. Ratos DIO-C consumiram dieta DIO nos primeiros e últimos 15 dias de tratamento, mas entre o 16º e 45º dias do período experimental alimentados com dieta padrão e água filtrada *ad libitum*. Durante os primeiros e últimos 15 dias, ST-R e DIO-R foram alimentados com ração padrão comercial ou dieta que induz a obesidade (DIO), respectivamente, e foram submetidos a ciclos de restrição alimentar intermitente (IFR) entre o 16° e 45° dias, recebendo ração comercial e água filtrada.

A composição da ração padrão, estimada pelo fornecedor (Nuvilab, São Paulo, Brasil), compreendeu 4% de gordura, 22% de proteína total, 42% de carboidratos, 10% de minerais, 1% de vitaminas; 8% de fibras e 12,5% de umidade, com densidade de energia igual a 2,22 Kcal / g.

Na elaboração da ração empregada no protocolo DIO, preparada no Laboratório de Nutrição Experimental – LABNEX da UFRJ, campus Macaé, onde foi realizado o estudo, foi utilizado 60% de ração padrão, 2% de óleo de soja, 18% de banha, 13% de açúcar, 7% de caseína (Synth) e 2% de BHT, resultando em distribuição de energia nas seguintes proporções: 45% de gordura, 19% de proteína e 35% de carboidratos e oferta estimada de 4,45 Kcal / g. Assim, a intervenção DIO consistiu na oferta *ad libitum* da ração hiperlipídica, conjugada a oferta de solução de sacarose a 20%, em substituição a água pura filtrada.

4.3 AVALIAÇÃO DO CONSUMO ALIMENTAR, MASSA CORPORAL, EFICIÊNCIA ENERGÉTICA E GORDURA VISCERAL

O consumo alimentar e a massa corporal foram monitorados diariamente, entre 8:00 – 9:00 h, ao longo dos 60 dias de tratamento. A massa corporal foi avaliada no exato momento em que o animal se encontrava apoiado nas quatro patas sem movimentar-se durante três segundos. O ganho diário de massa corporal foi obtido pela diminuição do peso mensurado menos o peso nas 24 h anteriores. O ganho da massa final foi obtido pela diferença entre o peso final e o peso inicial do animal, enquanto para consumo alimentar, uma quantidade conhecida de ração foi oferecida aos animais e após 24 h o restante foi mensurado, o consumo foi obtido então, pela diferencia entre essas duas medidas. O consumo da água de bebida, seja filtrada ou solução de sacarose foi mensurado da mesma forma.

A eficiência energética (EE) foi obtida a partir da equação: EE = [(média do ganho de massa corporal (g)/ média no consumo energético (kJ) x 100]. (CHAUSSE et al., 2014)

A massa de gordura visceral total foi estimada por meio do somatório dos depósitos de gordura retroperitoneal, mesentérico e gonadal (periovário), cujos valores foram expressos como g/100 g de massa corporal.

4.4 COLETA DE SANGUE E TECIDOS

Finalizados os 60 dias de tratamento, as ratas, aos 105 dias de vida, foram submetidas à eutanásia por decapitação com guilhotina, entre 08:00 e 10:00 h, após jejum de 12h. Para separação do soro, o sangue coletado foi imediatamente centrifugado a 14.000 rpm durante 15 min a 4° C. Os depósitos viscerais de gordura [retroperitoneal, mesentérico e gonadal (periovário)] foram dissecados, retirados e pesados. Também foram coletados, em cassetes histológicos, TAB subcutâneo (área da coxa) e visceral (retroperitoneal) assim como TAM interescapular. Adicionalmente, o crânio de cada animal foi dissecado para separação e rápida retirada do hipotálamo, que foi mantido em Trizol para os ensaios de qPCR.

4.5 QUANTIFICAÇÃO DE TRIACILGLICEROIS E COLESTEROL TOTAL SÉRICOS

As análises de triacilgliceróis e colesterol total foram realizadas por microensaio, empregando-se kit colorimétrico (Triglycerides Liquiform/Colesterol, Analisa, MG, Brasil). O protocolo seguiu as instruções do fabricante, com leitura em espectrofotômetro em comprimento de onda de 505 nm (Asys Expert 96, Biochrom, Cambridge, UK) e resultados expressos em mg/dL.

4.6 ANÁLISE HISTOLÓGICA DE TECIDOS ADIPOSOS BRANCO E MARROM

Os tecidos coletados em cassetes histológicos (TAB subcutâneo e visceral, da área da coxa e retroperitoneal, respectivamente, e TAM interescapular) permaneceram em tampão fosfato (Formaldeído10%, metano 15% e PBS 85%) durante 48 h. Após esse período, foram lavados em água corrente e mantidos em álcool 70% até análise. Antes do emblocamento em parafina, foram desidratados em concentrações crescentes de álcool e diafanizado em xilol. Os blocos foram cortados em micrótomo (Lupetec MRP2015, SP, Brasil) com 5 µm de espessura e dispostos em lâminas, que foram imersas em xilol, seguido de concentrações decrescentes de álcool para hidratação. Posteriormente, as lâminas de TAB e TAM foram coradas com hematoxilina – eosina (H&E). Lâminas de TAM também foram coradas com *Picrosirius red* (Sigma-Aldrich, Diadema, Brasil). Todas as lamínulas foram montadas rapidamente após administração dos corantes. As imagens do TAB e TAM, coradas com H&E, foram registradas com aumento de 20x e 40x respectivamente. Para as imagens de TAM coradas com *Picrosirius red* o registro correspondeu ao aumento de 20x. Todas as imagens foram obtidas em microscópio (Olympus-BX61, SP, Brasil). Para cada animal (n=10 por grupo) foram registrados oito campos histológicos, de forma randomizada. As medidas morfométricas foram realizadas no programa ImageJ 1.50i (National Institutes of Health, USA). Todos os adipócitos registrados por campo, brancos ou marrons, foram contados manualmente (DANKEL et al., 2014; LI et al., 2017). O diâmetro de todos os adipócitos subcutâneos e retroperitoneais registrados por campo, e as áreas de pelo menos cinquenta adipócitos marrons, por campo registrado, foram medidos usando as ferramentas “direto” e “à mão livre” do *software* Image J, respectivamente. O número de pixels obtido em cada medida foi convertido automaticamente para micrômetros por meio de calibração de software

com lâmina em escala micrométrica. Além disso, todos os vacúolos lipídicos por adipócito marrom medidos foram contados manualmente. Para a quantificação da deposição de colágeno no TAM, a porcentagem de área positiva para coloração com *Picrosirius red* foi calculada nas seções do TAM, determinando o limite específico para a cor rosa / vermelha que representa fibras de colágeno, e um quadrado foi aplicado nesta cor para registro, usando o *software* Image J. Áreas *Picrossírius red* positivas (vermelhas) foram obtidas automaticamente por meio da conversão do número total de pixels / intensidades em micrômetros. Os dados foram expressos como a porcentagem da área com coloração positiva / área total examinada do tecido (ROJAS-RODRIGUEZ et al., 2015).

4.7 EXPRESSÃO DE GENES HIPOTALÂMICOS POR PCR EM TEMPO REAL (qPCR)

Para a extração do RNA o tecido hipotalâmico foi colocado em tubo *Falcon* de 1.5 mL contendo 1 mL do reagente Trizol, logo foi congelado a -20° C até a realização das análises. Para extração do RNA foi utilizado o método fenol/clorofórmio, para isso, o tecido hipotalâmico foi descongelado e transladado para tubo de ensaio para ser homogeneizado no Trizol. Após disso o homogeneizado foi transferido para tudo *Falcon* de 1.5 mL e levado para centrifugação à 11450 rpm durante 15 min a 4° C, o sobrenadante foi transferido novamente para tubo *Falcon* e foi adicionado 200 uL de clorofórmio (100%) por mL de Trizol, os tubos foram agitados fortemente durante 15 seg e incubados a temperatura ambiente por 3 min, para ser centrifugado à 11450 rpm durante 15 min a 4° C. Após centrifugação a fase aquosa foi coletada em novo tubo *Falcon* e adicionado 500 μL de isopropanol e incubado a temperatura ambiente durante 10 min, se agitou brevemente e centrifugou à 11450 rpm durante 10 min a 4° C. Finalizada essa etapa, o sobrenadante foi removido por inversão e o pellet formado na fase anterior foi lavado com 1 mL de etanol 75%, o tubo foi agitado no vortex por 25 seg e em seguida foi centrifugado à 9050 rpm durante 5 min a 4° C. Em seguida, o sobrenadante foi novamente removido por inversão e o tubo virado para abaixo secou durante 5 min. O RNA obtido (pellet), foi resuspendido em 30 μL de H2O ultra pura (RNAse-free). Para quantificação do RNA o tubo foi agitado no vortex durante 25 seg e a leitura foi realizada utilizando NanoDrop 2000 (Thermofisher Scientific, Brasil). Foram utilizadas unicamente amostras cuja razão entre as absorbâncias, medidas a 260 e 280 nm, se manteve entre 1.9-2.1

(PEIRSON; BUTLER, 2007). Para a amplificação do RNA foi utilizado o kit GoTaq® Probe 1-Step RT-qPCR System (Promega, Brasil). A placa foi montada com 10 μL de Master Mix, 0,4 μL de RT Mix, 3,6 μL de H_2O Ultrapura, 5 μL de RNA e 1 μL do probe Leprot, PPARα, SOCS3, JNK, TLR4, IKKβ, NF-κB e CCL5 (Thermofisher Scientific, Brasil) para determinar o teor de RNAm dos genes hipotalâmicos de interesse, completando 20 μL em cada poço. Foi utilizado o equipamento StepOne Plus (Thermofisher Scientific, Brasil) cujas reações obedeceram a seguinte padronização: a 45°C durante 15 min para a incubação, seguido de 95°C durante 2 min para ativação da polimerase e PCR (40 ciclos) a 95°C por 15 seg para desnaturar e 60°C por 1 min para extensão. O gene 18S (ribosomal) foi utilizado como gene de referência de todos os genes hipotalâmicos de interesse. Para a quantificação do RNA, foi utilizado o método ΔΔCt, no qual se calcula as mudanças na expressão genica como uma mudança relativa (número de vezes) entre a amostra do gene de interesse e o gene endógeno (LIVAK; SCHMITTGEN, 2001).

4.8 ANÁLISE ESTATÍSTICA

O programa The G*Power 3.1.9.2 foi utilizado para calcular o tamanho da amostra a *priori* para o artigo 1. O tamanho da amostra foi calculado para obter um poder de 0,080 e um nível de significância de 0,05. O tamanho do efeito *f* foi definido como 0,56 (η2 p = 0,24) com base em nosso estudo anterior (ROSAS FERNÁNDEZ et al., 2018) e na literatura (AMENGUAL-CLADERA et al., 2013; KIM et al., 2017; TUNG et al., 2019). O tamanho da amostra obtido foi de dez ratos por grupo. Os grupos ST-C e DIO-C foram considerados controles, respectivamente, dos grupos ST-R e DIO-R. Foram empregados os testes *Kolmogorov-Smirnov* e *Levene* para identificação da normalidade e homogeneidade dos dados, respectivamente. Posteriormente, os resultados foram submetidos à análise de variância (ANOVA) de uma via, seguida do teste *post hoc* de comparações múltiplas de Bonferroni, utilizando-se o s*oftware GraphPad Prism* versão 6.0. Foi adotado nível de significância de 5% ($p < 0,05$) e os resultados estão apresentados como média e erro padrão da média. O cálculo do tamanho do efeito foi realizado *post hoc* e utilizado o s*oftware* Jamovi versão 1.1.9.0 e apresentado como *Eta Squared* (η2).

5. RESULTADOS E DISCUSSÃO

Parte dos resultados desta tese foram publicados na revista *British Journal of Nutrition* (ANEXO B), constituindo o artigo 1, reproduzido na presente seção, abrangendo dados relativos à análise periférica após o tratamento com RAI alternado com DIO. O segundo artigo, a ser submetido para publicação, compreende resultados relativos aos marcadores de inflamação hipotalâmica avaliados após a intervenção, alternada ou não com DIO, e complementa os achados da presente tese.

Artigo 1

Fasting and refeeding cycles alter subcutaneous white depot growth dynamics and the morphology of brown adipose tissue in female rat

FASTING AND REFEEDING CYCLES ALTER SUBCUTANEOUS WHITE DEPOT GROWTH DYNAMICS AND THE MORPHOLOGY OF BROWN ADIPOSE TISSUE IN FEMALE RATS

Abstract

Intermittent food restriction (IFR) is used mainly for weight loss; however, its effects on adipose tissue are not known when alternating with an obesogenic diet. To demonstrate its effects on morphological dynamics of fat deposits, female Wistar rats were distributed into groups: standard control (ST-C), with commercial diet; DIO control (DIO-C), with a diet that induces obesity (DIO) during the first and last 15 d, replaced by a standard diet for thirty intermediate days; standard restricted (ST-R), with standard diet during the first and last 15 d, with six cycles of IFR at 50 % of ST-C; and DIO restricted (DIO-R), in DIO during the first and last 15 d, with six cycles of IFR at 50 % of DIO-C. At 105 d of life, white adipose tissue (WAT) and brown adipose tissue (BAT) deposits were collected, weighed and histology performed. The DIO-R group showed higher total food intake (DIO-R 10 768·0 (SEM 357·52) kJ/g v. DIO-C 8868·6 (SEM 249·25) kJ/g, P < 0·0001), energy efficiency during RAI (DIO-R 2·26 (SEM 0·05) g/kJ v. DIO-C 0·70 (SEM 0·03) g/kJ, P < 0·0001) and WAT (DIO-R 5·65 (SEM 0·30) g/100 g v. DIO-C 4·56 (SEM 0·30) g/100 g) than their respective control. Furthermore, IFR groups presented hypertrophy of WAT and BAT, as well as fibrosis in BAT. Thus, IFR can establish prospective resistance to weight loss by favouring changes in adipose tissue morphology, increased energy intake and efficiency. Finally, the DIO diet before and after IFR aggravates the damages caused by the restriction.

Keywords: Intermittent fasting: Fat tissue: Brown adipose tissue fibrosis: Energy efficiency: Diet-induced obesity model

Introduction

The adipose tissue derives from different cell lines and is divided into two compartments: subcutaneous (SUB) and visceral, which present functional and morphological differences [(1)]. It is essentially distinguished by white adipose tissue (WAT) and brown adipose tissue (BAT), exercising functions of energy storage and thermogenesis, respectively [(2,3)]. Additionally, both tissues exert an endocrine function, secreting a set of substances, such as hormones, adipokines and cytokines, which regulate energy homoeostasis, glucose, lipids and inflammation [(4,5)]. The principal difference

between WAT and BAT compartments can be explained by different gene expressions and different progenitor cells [6]. BAT's colour stems from the density of mitochondria and rich vascularization [7], its adipocytes in response to sympathetic nervous system action and other hormones oxidise their stored lipids, to produce heat, contributing to thermogenesis [8].

WAT has aroused clinical interest due to its expansion potential. The excessive accumulation of this tissue promotes the release of macrophages and the production of inflammatory cytokines, characterising the inflammatory process, observed in obesity, and determining other metabolic disorders [9,10]. Thus, the main objective of restrictive diets should be the loss of body weight accompanied by the reduction of WAT in order to promote a reduction in metabolic risks [11].

Some dietary patterns may increase adiposity, including saturated fat and refined sugar consumption [12–15]. On the other hand, the high prevalence of obesity in the world has led to the practice of several dietary strategies suggested as inducing loss of body mass, which can result in physiological losses and establish a pro-obesogenic cycle in the body, with repercussions on adipogenesis [16]. From an observational point of view, in Brazil, these practices are often developed by individuals without compromise with healthier eating practices and deal with temporary behaviours and subsequent return to consumption of palatable diets, rich in energy, fat and sugar [17,18].

Rapid weight loss protocols are more practised by women than by men, due to the desire to lose weight resulting from greater body dissatisfaction [19–21]. Intermittent food restriction (IFR), also called intermittent fasting or fasting–refeeding (RF) cycles, is a strategy that became popular with the promise of loss of body mass. It consists of total or partial food restriction for hours in the day, on alternate days or on selected days during the week, followed by periods of RF, without limiting the amount of food eaten [22]. The proposal meets the expectations of some individuals who seek to lose weight, but with freedom in food consumption outside the short periods of food restriction, that is, after the restriction, they return to ingesting palatable foods, establishing eating behaviour that alternates between food restriction and hyperenergetic consumption.

In humans and experimental obese rodents, IFR seems to produce positive results in the loss of body mass, as well as in the improvement of markers related to cardiometabolic diseases [23,24]. These findings, however, are controversial, with evidence

that IFR also has a negative effect on health, due to the increased activity of adipogenic enzymes and the consequent gain in fat mass [25–30].

Therefore, herein, we tried to represent human behaviour, in which women consume a hyperenergetic diet pattern during their youth; then in an attempt to lose weight quickly, they carry out restrictive diets, a period in which they strive to consume a healthy diet; however, they are unable to maintain this pattern of restriction and end up abandoning it, returning to the habitual consumption of a hyperenergetic diet. Considering the few investigations on the relationship of IFR on morphology and function of SUB and visceral adiposity, this study aimed to evaluate, in female rats, the effect of the alternation between periods of diet that induces obesity (DIO) and IFR in the morphological dynamics of white and brown fat deposits.

Materials and methods

Animal care and ethics

Wistar female rats were provided by our breeding facility and were housed in individual cages, under a 12 h light–12 h dark cycle with lights off at 20.00 hours, at 22 ± 1°C room temperature. The handling of rats and experimental procedures were performed according to ARRIVE guidelines, the rules of the National Council of Animal Experiments Control, and all experimentation received approval from the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro, Campus Macaé, under reference number MAC030. The same rats were used for all experiments, following the 3Rs principle reduction.

Experimental model

Forty-five-day-old Wistar females rats (n 40, 10 per group; 153·34 (SEM 1·57) g) were randomly distributed into four groups: standard control (ST-C; n 10), DIO control (DIO-C; n 10), standard restricted (ST-R; n 10) and DIO restricted (DIO-R; n 10), during 60 d of treatment. Groups ST-C and ST-R had access to filtered water and a standard diet (Nuvilab). The DIO-C and DIO-R groups received an obesity-induced diet (DIO) during the first 15 d, as well as, the last 15 d of treatment and consumed a standard diet and filtered water ad libitum between the 16th and 45th d of treatment. ST-R and DIO-R rats were submitted to IFR between the 16th and 45th d of treatment. IFR consisted of 2 d of food restriction in which the restricted groups received 50 % of the amount of chow

consumed by the ST-C group, followed by 3 d of ad libitum feeding, completing six restriction cycles for 30 d (Fig. 1 shows the representation of diet treatments and IFR of experimental groups).

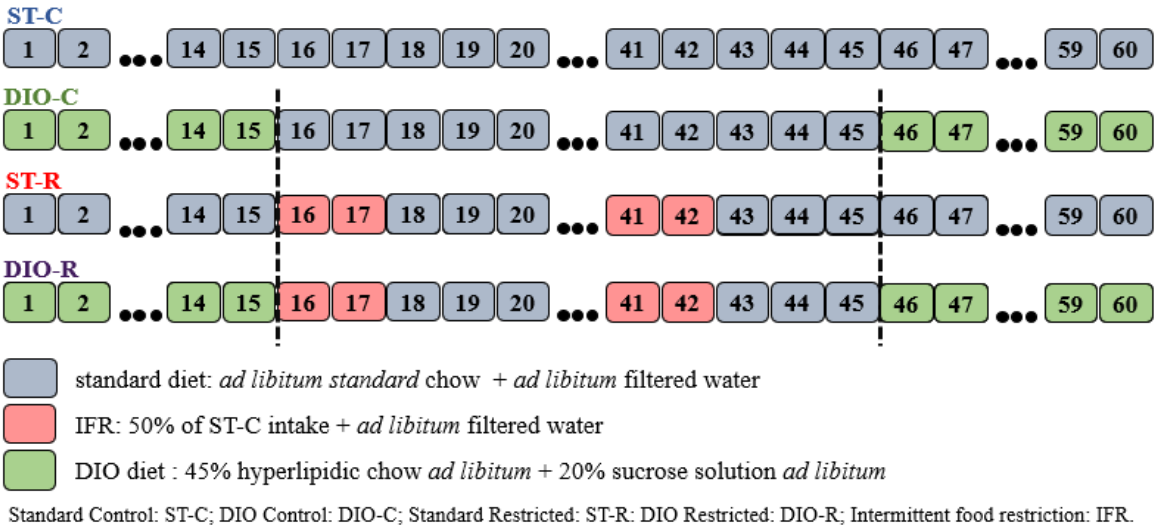

Fig. 1. Experimental design. Treatment timeline of standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R), DIO restricted (DIO-R) groups. ST-C rats consumed standard chow during all experimental period. DIO-C rats consumed DIO diet in the first and last 15 d of treatment, but between the 16th and 45th day of the experimental period fed on a standard diet and filtered water ad libitum. During the first and last 15 d, ST-R and DIO-R were fed with commercial standard chow or diet induced obesity (DIO), respectively, and were submitted to intermittent food restriction (IFR) cycles between 16th and 45th day, receiving commercial chow and filtered water.

The standard diet consisted of commercial rodent chow (Nuvilab) and filtered water, both ad libitum. The chow composition was 4 % fat, 22 % protein, 42 % carbohydrates, 10 % minerals, 0·8 % P, 1 % vitamins; 8 % fibres and 12·5 % humidity, with an energy density of 12·2 kJ/g. The DIO diet consisted of high-fat chow (46 % fat of total energy) and 20 % sucrose in its drinking water, both ad libitum. The DIO diet was prepared in our laboratory and consists of 60 % of the standard chow, 2 % soya oil, 18 % lard, 13 % sugar and 7 % casein (Synth). The energy distribution was 45 % fat, 19 % protein and 35 % carbohydrates with an estimated supply of 18·2 kJ/g.

Assessment of food intake, body mass energy efficiency and visceral fat

Food and drinking water consumption and body mass were registered daily, between 08.00 and 09.00 hours, throughout the 60 d of treatment using an electronic balance (TOLEDO DO BRASIL). Body weight was assessed at the exact moment when the rat was resting on all four paws without moving for 3 s. Energy efficiency (EE) was obtained from the equation: EE = ((average body mass gain (g)/average energy consumption (kJ)) × (100)). At the end of the experimental period, after euthanasia of all rat groups, the visceral fat deposits (retroperitoneal (RET), mesenteric and gonadal) were removed and weighed and the visceral fat content (TOTAL) was estimated.

Serum biochemical analysis

After 60 d of DIO and/or IFR experimental period, all rat groups were food-deprived for 12 h and were euthanised by beheading with guillotine, between 08.00 and 10.00 hours. The blood was collected for analysis of TAG and total cholesterol using a colorimetric kit according to the manufacturer's instructions (TAG Liquiform/total cholesterol; Analisa) and was performed with a 505 nm wavelength spectrophotometer reading (Asys Expert 96; Biochrom).

Histological analysis

At the end of the experimental period, SUB and visceral WAT (from the thigh and RET areas, respectively) and the interscapular BAT were collected on histological cassettes, remaining in phosphate buffer (formaldehyde 10 %, methane 1·5 % and PBS 88·5 %) for 48 h. Afterwards, tissues were washed under running water and kept in 70 % alcohol until analysis. Before paraffin embedding, the tissue was dehydrated in increasing concentrations of alcohol and diaphanised in xylol. The samples were cut in sections of 5 μm in thickness, using a microtome (Lupetec MRP2015), and arranged in sheets, which were immersed in xylol, followed by decreasing concentrations of alcohol for hydration. Subsequently, the WAT and BAT sections were stained with haematoxylin and eosin, and BAT was also stained with picrosirius red (Sigma-Aldrich, Diadema), and the coverslips were quickly assembled. Images from WAT and BAT sections stained with haematoxylin and eosin or picrosirius red were captured using a digital camera (Olympus DP71; Olympus Optical) coupled with an optical microscope (Olympus BX51; Olympus Optical). For each female rat (n 10 per experimental group), eight histological random fields from each haematoxylin and eosin stained section obtained from the SUB or RET

WAT, and BAT were registered. All morphometric measurements were performed with the aid of the Image J software (https://imagej.nih.gov/ij/download.html). All white or brown adipocyte per field registered were manually counting. The diameter of all SUB and RET adipocytes per field registered and the areas of at least fifty brown adipocytes per field registered were measured using the 'straight' and 'freehand' tools of Image J software, respectively. The pixel number obtained in each measure was automatically converted to micrometres through software calibration with a micrometre scale slide. In addition, all lipid vacuoles per brown adipocyte measured were manually counted [31,32]. For the quantification of BAT collagen deposition, the percentage area that was positive for picrosirius red staining was calculated in the BAT sections by determining the specific threshold for pink/red colour that represents stained collagen fibres, and a square was applied on this colour for recording, using Image J Software. Positive picrosirius red areas were automatically obtained through the conversion of total pixel numbers/intensities to micrometres. Data are expressed as the percentage of the positive-stained area/total tissue area examined [33].

Statistical analysis

The G*Power 3.1.9.2 [34] software was used to design sample size in this study; the power analysis was carried out a priori during the design stage of the study. We calculated the sample size needed in each group to obtain a power of 0·80 and a significance level of 0·05, the effect size f was defined as 0·56 ($\eta 2$ p = 0·24) based on our previous study [28] and in the literature [35–37]. The sample size obtained was of ten rats per group.

For multiple comparisons, one-way ANOVA was used, followed by the Bonferroni post-test, and a significance level of $P < 0{\cdot}05$ was adopted. The ST-C and DIO-C groups were considered controls, respectively, of the ST-R and DIO-R groups. The analyses were performed using the GraphPad Prism 6.0 Software, and the results are presented as mean values with their standard errors. The effect size was calculated using the statistical package Jamovi version 1.1.9.0 (https://www.jamovi.org/download.html). Eta-squared ($\eta 2$; small effect = 0·01, medium effect = 0·06, large effect = 0·14) and Cohen's d (small effect = 0·2, medium effect = 0·5, large effect = 0. 8) are reported when analyses were performed by ANOVA and in post hoc tests, respectively [38].

Results

Body mass behaviour

When observed per week, the difference in weight between the groups was not detected (Fig. 2(a)). No differences were observed before (PRE) and during the IFR; however, ANOVA revealed a main effect of diet in this period on the groups ($P < 0{\cdot}001$, $\eta 2 = 0{\cdot}505$). DIO-C and DIO-R groups showed significant body weight gain (1·16 (SEM 0·19) g, $P = 0{\cdot}0073$ and 1·83 (SEM 0·18) g, $P < 0{\cdot}0001$, respectively) than ST-C (0·63 (SEM 0·07) g). In addition, body weight gain, in the period following IFR (POS), was higher in DIO-R rats than that observed for DIO-C ($P = 0{\cdot}0008$). In this same period, the ST-R group showed significantly less body mass gain ($P < 0{\cdot}0001$), compared with the DIO-R group (Fig. 2(b)). The final body weight gain was significantly different among groups ($P = 0{\cdot}019$, $\eta 2 = 0{\cdot}195$). The DIO-R group showed greater final body weight gain (102·69 (SEM 4·40) g, $P = 0{\cdot}0254$, Cohen's d = 0·134) compared with the ST-C (87·64 (SEM 3·22) g) (Fig. 2(c)).

Throughout the six feed restriction cycles, the ST-R and DIOR groups showed similar reductions of body mass, except in the first IFR cycle, in which DIO-R rats (−1·33 (SEM 0·59) g, $P < 0{\cdot}0001$) displayed a minor reduction when compared with that observed for ST-R (−5·55 (SEM 0·40) g) (Fig. 2(d)). Fig. 2(e) shows the six RF cycles of the IFR period; it is possible to observe that body mass gain was significantly higher in the ST-R and DIOR groups, compared with the ST-C in all cycles. In the first two RF cycles (RF1 and RF2), the DIO-R rats showed a lower body mass gain (RF1 3·38 (SEM 0·34) g, $P < 0{\cdot}0001$ and RF2 5·33 (SEM 0·23) g, $P = 0{\cdot}0242$), than the ST-R group (RF1 5·87 (SEM 0·42) g and RF2 6·74 (SEM 0·38) g). But, at RF3, RF4 and RF5, DIO-R and ST-R groups maintained a similar body mass gain; however, in the last RF cycle (RF6), a significant difference was observed among these groups ($P = 0{\cdot}019$, $\eta 2 = 0{\cdot}195$), in which the body mass gain of the DIO-R group was significantly higher (7·38 (SEM 0·33) g, $P = 0{\cdot}0283$, Cohen's d = 0·148) than that observed for the ST-R group (6·00 (SEM 0·42) g) (Fig. 2(e)).

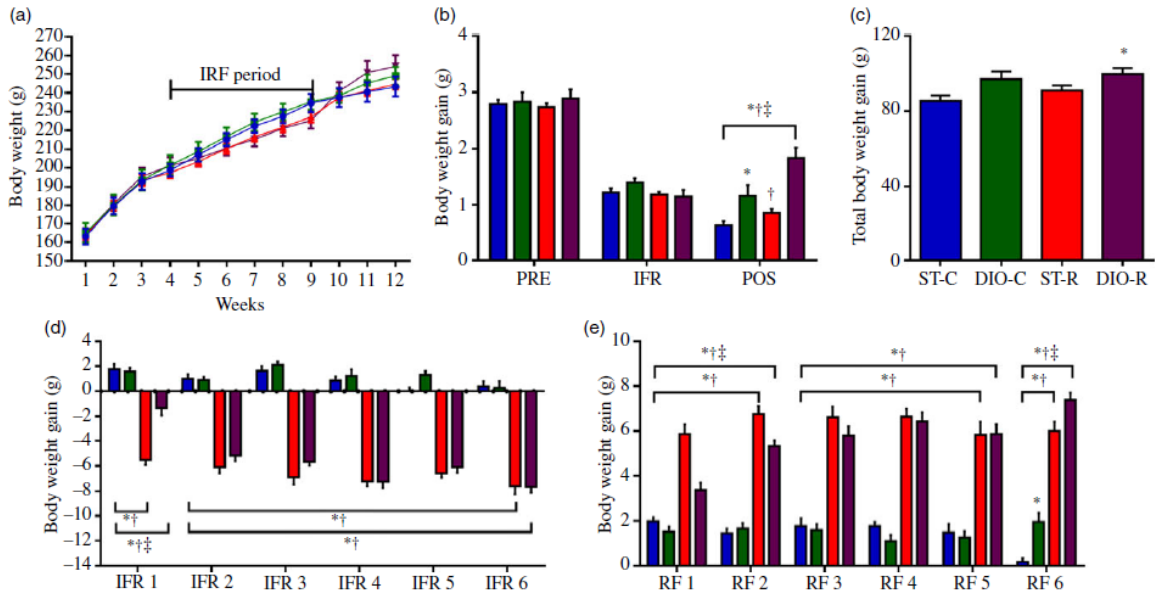

Fig. 2. Diet that induces obesity (DIO) consumption after intermittent food restriction (IFR) exacerbates body mass gain in female rats. (a) Timeline of body weight changes. ●, ST-C; ■, DIO-C; ▲, ST-R; ▼, DIO-R. (b) Body weight gain, before (PRE), during (IFR) and after (POS) IFR. (c) Total body weight gain at the end of the trial period. (d) Body weight gain on each effective restraint cycle during IFR. (e) Body weight gain in each refeeding (RF) cycle during IFR. ■, ST-C; ■, DIO-C; ■, ST-R; ■, DIO-R. Experimental groups: standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R), DIO restricted (DIO-R). Values are mean values with their standard errors (n 10). Data were analysed using one-way ANOVA followed by Bonferroni post-test. P < 0·05 * v. ST-C, † v. DIO-C, ‡ v. ST-R.

Intermittent food restriction increases intake and energy efficiency

DIO-C and DIO-R rats maintained a higher energy intake than the ST-C group, before (2820·96 (SEM 106·01) kJ/g, P = 0·0015, Cohen's d = 1·021 and 2872·81 (SEM 93·51) kJ/g, P = 0·0003, Cohen's d = 1·074 v. 1787·59 (SEM 43·9) kJ/g) (PRE), during (3978·65 (SEM 124·21) kJ/g, P = 0·036, Cohen's d = 0·797 and 4322·17 (SEM 260·37) kJ/g, P < 0·0001, Cohen's d = 1·034 v. 2784·17 (SEM 61·02) kJ/g) (IFR) and after (2537·50 (SEM 109·76) kJ/g, P < 0·0001, Cohen's d = 1·726 and 2806·70 (SEM 133·30) kJ/g, P < 0·0001, Cohen's d = 1·978 v. 1109·47 (SEM 63·55) kJ/g) (POS) IFR. ANOVA revealed a main effect of diet on the total amount of energy consumed throughout the whole treatment (P < 0·0001, η2 = 0·829); all groups were significantly different from ST-C, even DIO-R (10 768·00 (SEM 357·52) kJ/g, P = 0·0006, Cohen's d = 0·722) group

differed significantly from its own control (DIO-C) (8868·60 (SEM 249·25) kJ/g) (Fig. 3(a)).

EE was significantly lower in the DIO-C and DIO-R groups (0·86 (SEM 0·04) g/kJ, P < 0·0001, Cohen's d = 0·05 and 0·87 (SEM 0·03) g/kJ, P < 0·0001, Cohen's d = 0·05 v. ST-C 1·28 (SEM 0·03) g/kJ) during the first 15 d of treatment, when these rats received a DIO. However, over the IFR period, ANOVA revealed a main effect of diet on EE (P < 0·0001, η2 = 0·957), in which EE was significantly higher in both ST-R and DIO-R groups, regardless of diet, indicating a greater anabolic capacity of these rodents in the feeding periods (2·35 (SEM 0·06) g/kJ, P < 0·0001, Cohen's d = 0·139 and 2·26 (SEM 0·05) g/kJ, P < 0·0001, Cohen's d = 0·132 v. ST-C 0·83 (SEM 0·04) g/kJ). In the 15 d subsequent of IFR, only ST-R group (0·41 (SEM 0·02) g/kJ, P = 0·044, Cohen's d = 0·014) maintained a significantly higher EE than the ST-C (0·25 (SEM 0·03) g/kJ; Fig. 3(b)).

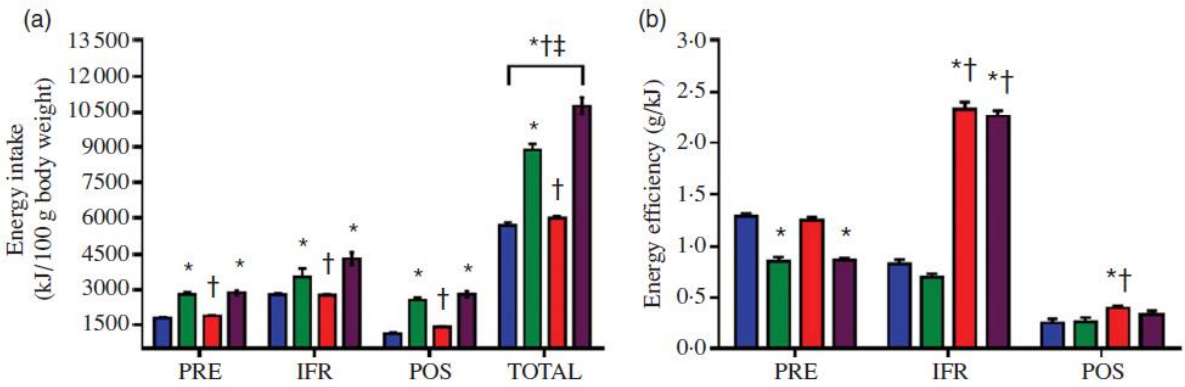

Fig. 3. Intermittent food restriction (IFR) increased energy intake and feed efficiency in female rats fed on a standard diet or diet that induces obesity (DIO). (a) Energy intake (in kJ) before (PRE), during (IFR) and after (POS) IFR and total (accumulated at the end of the experimental period). (b) Energy efficiency in the 15 d before IFR (PRE), in IFR and in the last 15 d of treatment (POS). Experimental groups: standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R). ■, ST-C; ■, DIO-C; ■, ST-R; ■, DIO-R. Values are mean values with their standard errors (n 10). Data were analysed by one-way ANOVA followed by Bonferroni post-test. P < 0·05 * v. ST-C, † v. DIO-C, ‡ v. ST-R.

Analysis of serum lipids

Fasted DIO-C rats exhibited significantly higher serum TAG levels than ST-C and ST-R groups ($P < 0{\cdot}0001$, $\eta 2 = 0{\cdot}667$; Table 1). The total cholesterol levels were not different between the groups (Table 1).

Table 1. Serum content of TAG and total cholesterol (TC) at the end of treatment in standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R) female rats (n 8) ‡
(Mean values with their standard errors)

	ST-C		DIO-C		ST-R		DIO-R	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
TAG (mg/dl)	137·47	2·84	152·52*	3·81	126·86†	1·65	142·68	2·53
TC (mg/dl)	142·52	6·26	154·57	4·71	161·44	6·47	159·23	3·35

‡ Data analysed using one-way ANOVA followed by Bonferroni post-test. $P < 0{\cdot}05$ * v. ST-C, † v. DIO-C.

Increase of adipose tissue after intermittent food restriction

At the end of the treatment, ANOVA revealed a significant effect of diet on WAT depots when evaluating RET ($P < 0{\cdot}0001$, $\eta 2 = 0{\cdot}477$), mesenteric ($P < 0{\cdot}0001$, $\eta 2 = 0{\cdot}408$), gonadal ($P < 0{\cdot}0001$, $\eta 2 = 0{\cdot}509$) and total white visceral fat ($P < 0{\cdot}0001$, $\eta 2 = 0{\cdot}529$). DIO-C (2·19 (SEM 0·21) g/100 g) and DIO-R rats (2·88 (SEM 0·12) g/100 g) had a significantly higher content of gonadal fat ($P < 0{\cdot}0027$ and $P < 0{\cdot}0001$, respectively), than the ST-C (1·26 (SEM 0·21) g/100 g). As for the total visceral white fat, DIO-C, DIO-R and ST-R groups (4·56 (SEM 0·30) g/100 g, $P < 0{\cdot}0001$, Cohen's d = 0·054; 5·65 (SEM 0·30) g/100 g, $P < 0{\cdot}0001$, Cohen's d = 0·089 and 3·64 (SEM 0·23) g/100 g, $P = 0{\cdot}0110$, Cohen's d = 0·025) showed significantly higher deposits than ST-C (2·87 (SEM 0·31) g/100 g). Furthermore, DIOR group displayed higher WAT deposits ($P = 0{\cdot}0006$, Cohen's d = 0·034), when compared with its respective control (DIO-C) (Fig. 4(a)).The DIO diet also had a significant effect on interscapular BAT ($P < 0{\cdot}0001$, $\eta 2 = 0{\cdot}735$), since DIO-C and DIO-R (0·13 (SEM 0·005) g/100 g, $P < 0{\cdot}0001$ and 0·13 (SEM 0·004) g/100 g, $P < 0{\cdot}0001$) groups exhibited significantly higher BAT amounts, than that observed for ST-C rats (0·08 (SEM 0·005) g/100 g). But, in the ST-R rats, BAT deposits were significantly lower (0·0958 (SEM 0·014) g/100 g, $P < 0{\cdot}0001$), when compared with the DIO-R group (Fig. 4(b)).

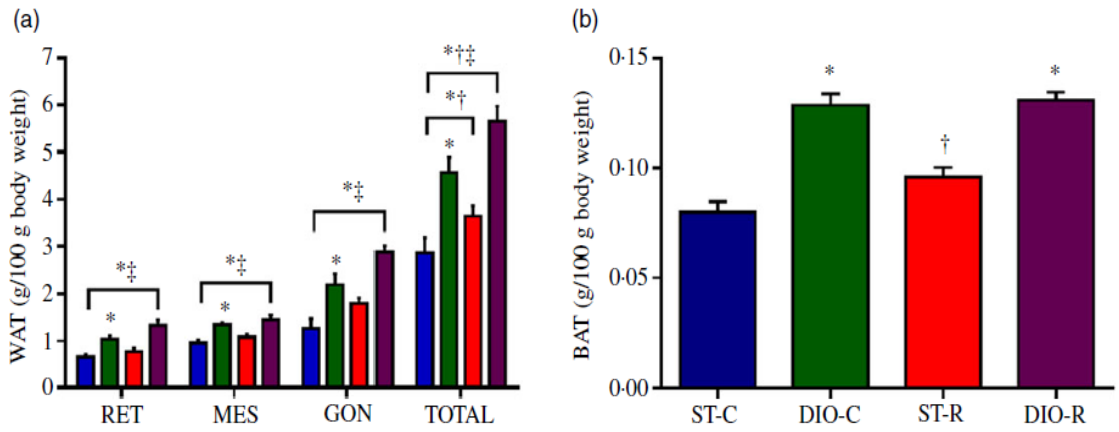

Fig. 4. Increase of adipose tissue after intermittent food restriction (IFR). (a) Mean values with their standard errors (n 10) of retroperitoneal (RET), mesenteric (MES), gonadal (GON) white fat pad weights, total white visceral fat stores (TOTAL), and in (b) interscapular brown adipose tissue weight in standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R) female rats at the end of the experimental period. ■, ST-C; ■, DIO-C; ■, ST-R; ■, DIO-R. Data were analysed by one-way ANOVA followed by Bonferroni post-test. P < 0·05 * v. ST-C, † v. DIO-C, ‡ v. ST-R.

Hyperplasia and hypertrophy of white subcutaneous adipose tissue after intermittent food restriction

The treatment showed significant differences on the size (P < 0·0001, η2 = 0·427) and number (P < 0·0001, η2 = 0·854) of the SUB white adipocytes among the groups. Adipocyte hypertrophy in SUB WAT was observed in all groups, when compared with ST-C rats (DIO-C 47·39 (SEM 0·90) µm, P < 0·0001; ST-R 48·88 (SEM 1·68) µm, P < 0·0001, and DIO-R 67·55 (SEM 0·70) µm, P < 0·0001 v. ST-C 36·82 (SEM 0·60) µm). But, DIO-R rats displayed higher hypertrophic adipocytes in SUB WAT (P < 0·0001), when compared with DIO-C (Fig. 5(b)). Also, adipocyte hyperplasia was observed in the SUB WAT of DIO-R (104·00 (SEM 3·75)) and ST-R groups (32·18 (SEM 3·44)), when compared with their controls (P < 0·0001, Cohen's d = 0·544). Notably, adipocyte hyperplasia in this WAT store was 223 % higher in DIO-R rats, when compared with the ST-R group (P < 0·0001, Cohen's d = 0·629; Fig. 5(c)).

Visceral adipose tissue hypoplasia and hypertrophy after intermittent food restriction

At the end of the treatment, significant differences on the size ($P < 0{\cdot}0001$, $\eta2 = 0{\cdot}543$) and number ($P < 0{\cdot}0001$, $\eta2 = 0{\cdot}121$) of adipocytes from the visceral WAT were observed between the groups. ST-R (254·12 (SEM 3·94) μm, $P < 0{\cdot}0001$, Cohen's d = 0·29) and DIO-R groups (276·23 (SEM 9·83) μm, $P < 0{\cdot}0001$, Cohen's d = 0·36) showed adipocyte hypertrophy ($P < 0{\cdot}0001$, Cohen's d = 0·30), when compared with the ST-C (187·59 (SEM 4·14) μm) and DIO-C rats (202·17 (SEM 10·88) μm), respectively (Fig. 5(d)). Hypoplasia was observed only in DIO-R rats (51·71 (SEM 1·45), $P = 0{\cdot}0133$), when compared with ST-C (67·22 (SEM 3·86)) (Fig. 5(e)).

Intermittent food restriction induces hypertrophy and fibrosis of brown adipose tissue

ANOVA revealed a significant effect of diet on the adipocytes size ($P < 0{\cdot}0001$, $\eta2 = 0{\cdot}616$), lipid inclusions per field analysed ($P < 0{\cdot}0001$, $\eta2 = 0{\cdot}346$) and adipocytes number ($P < 0{\cdot}0001$, $\eta2 = 0{\cdot}671$) of BAT among the groups. BAT histology showed hypertrophy in all groups compared with ST-C (DIO-C 1244·82 (SEM 43·73) μm, $P < 0{\cdot}0001$; ST-R 1198·29 (SEM 36·64) μm, $P < 0{\cdot}0001$ and DIO-R 1259·65 (SEM 40·48) μm, $P < 0{\cdot}0001$ v. ST-C 440·01 (SEM 17·69) μm; Fig. 5(f)). On the other hand, the number of lipid inclusions per brown adipocyte was significantly lower in ST-R (6·83 (SEM 0·20), $P < 0{\cdot}0001$) and DIO-R rats (8·63 (SEM 0·29), $P = 0{\cdot}0007$), when compared with the ST-C (10·06 (SEM 0·25)). But, DIO-C rats (13·16 (SEM 0·42), $P < 0{\cdot}0001$) exhibited higher number of lipid inclusions per brown adipocyte (Fig. 5(g)). All groups showed BAT hypoplasia (DIO-C 61·36 (SEM 2·57), $P < 0{\cdot}0001$; ST-R 59·86 (SEM 1·63), $P < 0{\cdot}0001$ and DIO-R 59·56 (SEM 3·12), $P < 0{\cdot}0001$; respectively), when compared with ST-C (172·79 (SEM 18·39); Fig. 5(h)).

The percentage of positive-picrosirius area that indicates collagen fibres deposition in BAT of all rat groups can be observed in Fig. 5(a) and (i). Both, DIO diet and IFR increased BAT fibrosis ($P < 0{\cdot}0001$, $\eta2 = 0{\cdot}655$) in DIO-C (0·60 (SEM 0·02) %, $P < 0{\cdot}0001$) or in ST-R (0·47 (SEM 0·03) %, $P < 0{\cdot}0001$) and DIO-R rats (0·61 (SEM 0·03) %, $P < 0{\cdot}0001$), when compared with ST-C (0·15 (SEM 0·02) %). Notably, collagen deposition in BAT of DIO-C and DIO-R groups was higher than that observed in ST-R (Fig. 5(i)).

(a)

ST-C DIO-C ST-R DIO-R

Subcutaneous WAT

Visceral WAT

BAT

BAT

100 µm

50 µm

(b) Subcutaneous adipocyte diameter (µm)

(c) Adipocyte number / field assessed

(d) Visceral adipocyte diameter (µm)

(e) Adipocyte number / field assessed

(f) Adipocyte area (µm²)

(g) Lipid inclusions / adipocyte

(h) Adipocytes number / field assessed

(i) Collagen fibres (% area)

ST-C DIO-C ST-R DIO-R

Fig. 5. Morphological alterations of white adipose tissue (WAT) and brown adipose tissue (BAT). (a) Representative images of histological sections (5 μm in thickness) of subcutaneous (thigh region) and visceral (retroperitoneal) WAT (scale bars 100 μm), and interscapular BAT (scale bars 50 μm) stained with haematoxylin and eosin. The lower panel shows representative histological sections of the intrascapular BAT stained with picrosirius red, for collagen deposition measurement in BAT of standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R) female rats. Mean values with their standard errors (n 10) of diameter or number of white adipocytes from subcutaneous (b and c, respectively) or visceral fat pads (d and e, respectively), and the areas and number of brown adipocyte (f and g) and the number of lipid inclusions per brown adipocyte (h). Percentage of the picrosirius-positive area in BAT (i) of ST-C, DIO-C, ST-R and DIO-R female rats. Data were analysed by one-way ANOVA followed by Bonferroni post-test. $P < 0{\cdot}05$ * v. ST-C, † v. DIO-C, ‡ v. ST-R.

Discussion

Here, for the first time, we demonstrated that obesity-inducing diet (DIO) consumption, rich in saturated fat and refined sugar, after an IFR period (IFR), characterising popular cyclical behaviour to get rapid loss of body mass, promoted hyperplasia and hypertrophy of SUB WAT in female rats, as well as brown adipocyte hyperplasia and fibrosis. These adiposity changes appear to be linked to a compensatory increase in energy intake and feed efficiency as a result of periods of IFR. Highlighting that at the contrary of which is expected with the reduction of food intake, herein we observed that IFR did not promote the effective reduction of body mass, and when DIO was provided in rats submitted to IFR, a higher body mass gain and adiposity were induced in DIO-R group, suggesting that IFR changes lipid metabolism at least in adipocytes, causing an economic phenotype which exacerbates body fat storage.

Rodents treated with DIO and submitted to IFR (DIO-R group) showed higher body mass gain at the end of the treatment, which may be due to the fact that in the last 15 d of treatment, a period in which the groups received DIO again (groups DIO-C and DIO-R), there was an increase in weight gain, compared with the corresponding controls (groups ST-C and ST-R). Despite this mass gain, the circulating levels of TAG and total cholesterol were not affected by IFR. These findings indicate that the performance of IFR resulted in damage to the DIO-R group, which was able to compensate for the loss of body mass, throughout the RF cycles, ending the treatment with body mass higher than

the ST-C group. Additionally, food restriction, which occurred after the consumption of DIO, resulted in excessive energy consumption. Studies carried out in both experimental rodents and humans consider IFR to be an efficient strategy for a loss of body mass and improvement in the markers of metabolic diseases [39,40]. In the present study, the absence of differences in body mass between the ST-R and DIO-R groups, at the end of the treatment, can be partly explained by the energy compensation in the RF periods, which may lead to greater mass regain in the long term.

The consumption of DIO during the first 15 d of treatment reduced EE. In this sense, it was shown that rats fed exclusively with foods rich in saturated fat showed less EE, increasing the oxidation of fatty acids, in order to disadvantage the accumulation of lipids [41]. Here, during the IFR period, ST-R and DIO-R rats tripled the EE compared with their respective controls, demonstrating the body's effectiveness in recovering the loss of body mass during the IFR periods, a fact that justifies the absence of difference in the final body mass between the groups. However, in the last 15 d of treatment, the ST-R group was the most affected by IFR, since even returning to ad libitum consumption of a standard diet, it maintained high EE. As for DIO-R rats, the difference was not significant, but it seems to remain greater during this period. Similarly, but in male mice, Dankel et al. [32] also observed an increase in body mass gain and EE in animals that intercalated IFR with a hyperenergetic diet, resulting in greater recruitment of adipocytes in epididymal WAT.

The improvement in EE may also explain the increase in the total white visceral fat deposits of the ST-R and DIO-R rats in relation to their respective controls, allowing greater energy storage in the WAT. In addition, increased deposits of interscapular BAT were identified in the DIO-C and DIO-R groups, indicating that the obesogenic diet negatively affects BAT. The high energy consumption results in higher TAG storage in the visceral and SUB WAT; however, with the maintenance of this dietary pattern, there is a storage of fatty acids in other tissues, characterizing ectopic fat [10]. It is recognised that through excessive consumption of fatty acids, WAT adipocytes suffer from hyperplasia and hypertrophy; however, this growth is limited [9]. Thus, despite is not addressed in our study, it is possible that the IFR protocol performed here could also increase fat storage ectopically, which will be considered in future investigations.

Changes in the morphology of SUB WAT of DIO-R rats demonstrated that IFR aggravated the increase in adiposity induced by DIO. The growth dynamics of the tissue

was impaired by both hypertrophy and hyperplasia of the cells. These findings diverge from other studies carried out with male mice, which indicated that IFR improves the damage caused by the consumption of an obesogenic diet. A decrease in inguinal and epididymal tissue has been demonstrated due to the transdifferentiation process of white adipocytes, known as browning. This process leads to increased expression of uncoupling protein 1 [31,35], which increases thermogenesis in the tissues that store energy and reduces the overload in BAT [42]. However, in our study, ST-R and DIO-R rats showed hypertrophy in visceral WAT, whereas hypoplasia was observed only in DIO-R, which could be the cause of lipolysis of this tissue due to IFR treatment. Studies using experimental rodents and in humans have shown that sex can determine differences in the energy storage, so that in females there is greater hypertrophy and hyperplasia of SUB WAT compared with visceral, as a way of protecting against insulin resistance [36,43]. The difference in fat deposition is mainly due to hormonal differences, such as the estrogen that favours lipolysis; hormone receptors of the β type present in greater quantity in the abdominal area or of the α type in greater proportion in the gluteus femoral area; enzyme concentrations and increased insulin sensitivity in females [44].

Obesity establishes a profile of adipose tissue hyperplasia, mainly to protect the body from damage caused by the circulation of high amounts of NEFA. When the adipose tissue loses its expansion capacity, becoming more lipolytic, thus, the lipotoxicity process begins, with consequent ectopic adipogenesis [45,46]. Okuno et al. [47] showed that adipose tissue presents healthy expansion, featuring protective properties, and that oxidative stress inhibits this expansion, modifying the tissue architecture and exacerbating inflammation. Studies showed that prolonged IFR caused increased stress and elevated serum corticosterone concentration in rats [48], as well as increased net ROS production, leading to oxidative imbalance [49]. In our study, although the content of reactive oxygen and corticosterone species was not assessed, hypertrophy was identified in BAT and WAT, as well as fibrosis in BAT, which could be resulted from the increase in the number of reactive oxygen species.

We also verified hypertrophy of BAT in DIO rats, which may constitute a physiological response aimed at minimising the damage caused by the diet. It was described that palatable diets cause an increase in thermogenesis in BAT, as a mechanism to dissipate excess energy in the form of heat [50]. Kuipers et al. [42] showed that 1 d of exposure to a high-fat dietmay be enough to promote the first signs of BAT bleaching. In

fact, we verified that the BAT of the DIO-C and DIO-R groups presented hypoplasia, while the restricted groups (ST-R and DIO-R) lost the multilocularity characteristic of this tissue. The presence of small lipid vacuoles in BAT increases the area of contact with the lipase, promoting effective lipolysis and consequent thermogenesis [51]. Therefore, the decrease in multilocularity, which was an effect evidenced in both ST-R and DIO-R rats, is an important indicative of loss of the main function of BAT in these rodents, leading the BAT to storage than dissipate energy as heat.

In addition, intense thermogenesis in BAT also causes tissue injury through the depletion of the mitochondrial complex, leading to loss of main function and tissue atrophy [52]. In our study, DIO-C as well as restricted groups (ST-R and DIO-R) exhibited BAT fibrosis, indicative of BAT impaired function. The expansion of WAT causes hypoxia, leading to increased expression of hypoxia-inducible factor 1-alpha, stimulating changes in the extracellular structure, and fibrillar collagen deposition in the tissue [53].

Both hypertrophy in SUB and visceral WAT as well as hypertrophy, hypoplasia and decreased lipid inclusions in BAT observed in the ST-R and DIO-R groups can result from the frequency of restriction cycles. It has been shown that in humans, the 24-h fast increases lipolysis and fatty acid re-esterification by 2·8 and 2·5 times, respectively [54,55]. Repeated restraint and RF cycles lead to an increase in lipolysis during the fasting period, an increase in circulating fatty acids and re-esterification, generating an increase in abdominal and hepatic fat deposits [25,56]. Thus, the increase in EE and compensatory energy consumption in rats may have concomitantly promoted the increase in WAT and BAT.

In summary, IFR, when associated with DIO, results in synergistic effects, favouring long-term gain in body mass, with increased deposits of total visceral fat and BAT content, as well as promoting hypertrophy in WAT and BAT. These results indicate that IFR, in female rats, in this experimental design that tried to mimic the human eating behaviour, in the pursuit of weight loss, does not favour effective prolonged body mass loss, since it induces greater energy consumption, increased EE and adiposity effects aggravated by alternating periods of consumption of DIO and IFR.

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The authors declare that there are no conflicts of interest.

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Artigo 2

A restrição alimentar intermitente conduz a aumento da expressão de genes relacionados com inflamação hipotalâmica induzida por dieta hiperenergética

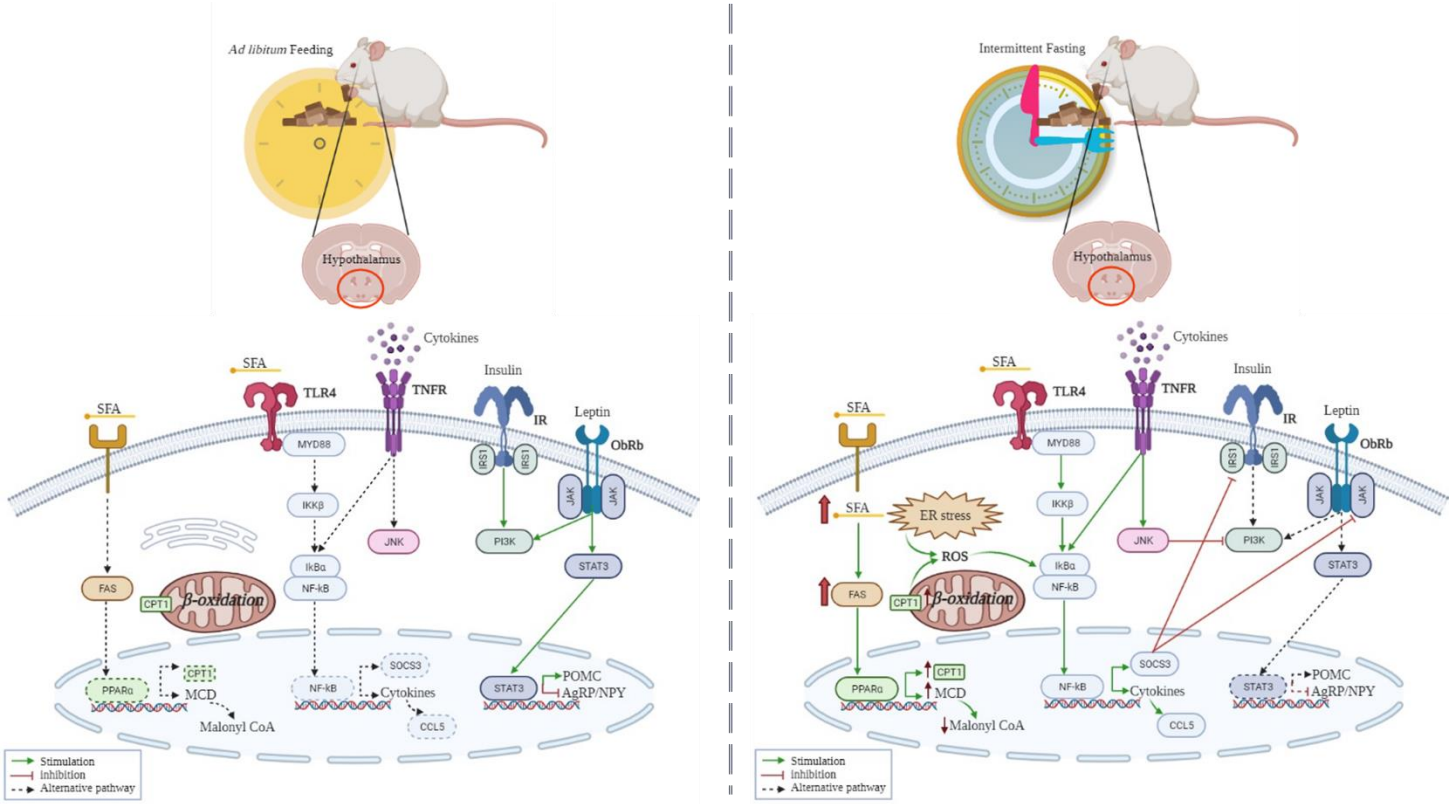
A RESTRIÇÃO ALIMENTAR INTERMITENTE CONDUZ A AUMENTO DA EXPRESSÃO DE GENES RELACIONADOS COM INFLAMAÇÃO HIPOTALÂMICA INDUZIDA POR DIETA HIPERENERGÉTICA

Resumo

Introdução: Padrões alimentares hiperenergéticos provocam inflamação hipotalâmica e geram alterações na resposta alimentar favorecendo maior ingestão de alimentos e obesidade. A restrição alimentar intermitente (RAI) é uma estratégia de perda de peso amplamente utilizada, especialmente por mulheres. Contudo, não são claros seus efeitos no sistema nervoso central (SNC) quando alternada com dieta que induz obesidade (DIO). **Objetivo:** Avaliar marcadores de inflamação hipotalâmica após RAI alternada ou não com DIO em ratas. **Metodologia:** Fêmeas *Wistar* de 45 dias de vida foram divididas nos grupos: *Standard Control* (ST-C), tratado com dieta padrão e água filtrada *ad libitum*; DIO *Control* (DIO-C), tratado com DIO nos primeiros e últimos 15 dias de intervenção e dieta padrão no período seguinte entre o 16º e 45º dia; *Standard Restricted* (ST-R), tratado com dieta padrão nos primeiros e últimos 15 dias de intervenção, seguido de RAI a 50% do ST-C entre o 16º e 45º dias seguintes; e DIO *Restricted* (DIO-R), tratado com DIO nos primeiros e últimos 15 dias de intervenção, seguido de dieta padrão entre o 16º e 45º dias, sendo submetido a RAI nas mesmas condições que ST-R. Aos 105 dias de vida os animais foram eutanasiados e o hipotálamo foi retirado para as análises por qPCR. Para análise estatística foi utilizado ANOVA de uma via, seguido de teste *post hoc* Bonferroni e o tamanho do efeito foi calculado *post hoc.* **Resultados:** os grupos ST-R e DIO-R apresentaram maior expressão do gene PPARα quando comparados ao ST-C ($p<0{,}001$). O gene SOCS3 também foi mais expresso nos grupos ST-R e DIO-R comparados ao ST-C (DIO-C: $p=0{,}002$; ST-R: $p=0{,}020$; DIO-R: $p=0{,}002$). Além disso, os genes IKKβ e NF-κB apresentaram maiores teores no grupo ST-R em relação ao ST-C ($p=0{,}045$ e $p=0{,}047$ respetivamente). O gene CCL5 foi mais elevado no DIO-R do que nos demais grupos. **Conclusão:** RAI intercalada ou não com DIO, incrementa a expressão de genes inflamatórios no hipotálamo, associado a possível prejuízo na resposta anorexígena relacionada à acentuada expressão de SOCS3 e efeito sobre as vias da insulina e leptina.

Palavras chave: Jejum intermitente, inflamação hipotalâmica, SOCS3, IKKβ, NF-κB, DIO.

Resumo gráfico



Introdução

A obesidade é uma doença metabólica que atinge milhões de pessoas no mundo e tem estreita relação com o início e manutenção da inflamação sistêmica de baixo grau, favorecendo o desenvolvimento de comorbidades associadas (Engin, 2017). A resposta inflamatória é ativada com a finalidade de reparação dos tecidos afetados, e tanto a resposta imune inata como a adquirida participam da resolução deste processo (Cooke, 2019). A inflamação aguda cursa com modificações hemodinâmicas, alterações na permeabilidade vascular e recrutamento leucocitário, entretanto, se esta resposta inicial falhar, a inflamação crônica se estabelece, caracterizando-se pela ativação prolongada das vias de sinalização inflamatórias, com o aumento nas concentrações de diversos biomarcadores e células, conduzindo a tolerância e posterior dano tecidual (Rocha & Folco, 2011).

As células conservaram, evolutivamente, receptores capazes de identificar características presentes na membrana dos patógenos, um deles é o receptor Toll-Like 4 (TLR4), que reconhece patógenos por meio da resposta a lipopolisacarídeos (LPS), comuns nas bactérias Gram-negativas. No entanto, TLR4 também pode ser ativado por

ácidos graxos saturados que apresentam componente comum com as endotoxinas bacterianas, a porção do lipídio A, iniciando a cascata inflamatória com ativação do inibidor do fator nuclear kappa-B quinase subunidade beta (IKKβ) e do fator nuclear kappa B (NF-κB) e consequente síntese de citocinas inflamatórias. A expressão destes fatores pode ser também ativada por outras vias que estão relacionadas ao excesso de nutrientes e produção periférica de citocinas inflamatórias (Rogero & Calder, 2018). Neste contexto, a resposta inflamatória iniciada indevidamente, pode levar à desorientação na sinalização deste complexo mecanismo.

No hipotálamo são encontradas duas populações de neurônios que atuam principalmente na regulação da ingestão de alimentos e manutenção da homeostase energética. Neurônios que expressam neuropeptídeo Y (NPY) e proteína relacionada ao gene agouti (AgRP), sensibilizados nas situações de reduzida disponibilidade de energia, sinalizam respostas que induzem o estado de fome e redução do gasto energético. Em contrapartida, a ativação dos neurônios que expressam proopiomelanocortina (POMC) e transcrito regulado por cocaína e anfetamina (CART) resulta na diminuição da fome e aumento da termogênese. A regulação hipotalâmica da homeostase energética tem papel crucial na manutenção do peso corporal, no entanto, dietas ricas em ácidos graxos promovem inflamação hipotalâmica, rompendo este equilíbrio, resultando em sobrepeso e, possivelmente, obesidade (Timper & Brüning, 2017).

O elevado consumo de energia, principalmente de ácidos graxos saturados e açúcares refinados estimulam a resposta inflamatória aguda imediata no tecido cerebral, primeiramente, a despeito do aumento de adipocinas circulantes, que se desenvolve em mais longo prazo (De Souza et al., 2005). A conservação deste padrão de ingestão alimentar estabelece quadro de inflamação crônica de baixo grau hipotalâmica, causando desequilíbrio na resposta alimentar e resultando em maior ganho de massa corporal (Macedo et al., 2019).

A busca por estratégias capazes de conduzir a perda acelerada de massa corporal tem motivado a adesão a diferentes condutas terapêuticas, incluindo a restrição alimentar intermitente (RAI), caracterizada tanto pela flexibilidade, como por induzir perda de peso em curto prazo (Freire, 2020). Além da redução da massa corporal, melhora na sensibilidade à insulina e diminuição do risco cardiovascular, também são apontados como benefícios associados a RAI (Tinsley & La Bounty, 2015). Contudo, estudos em animais indicam efeitos deletérios que poderiam resultar, em longo prazo, no desequilíbrio da homeostase energética (Han et al., 2014; Kliewer et al., 2015; Parkes et

al., 2017; Rosas Fernández et al., 2018). Além disso, existe grande dificuldade na manutenção de dietas restritivas, que resulta em baixa adesão e retorno ao consumo de alimentação rica em gordura e açúcares refinados (Heilbronn et al., 2005; Hoddy et al., 2015; Harvie & Howell, 2017).

Praticada principalmente por mulheres com comportamento alimentar obesogênico de longa data, a RAI tem atraído número crescente de adeptos, considerando seu rápido efeito sobre a redução da massa corporal. Apesar de se conhecer os prejuízos da dieta hipercalórica na perda da homeostase energética, não estão claros os efeitos da RAI, intercalada ou não com dieta obesogênica. Dessa forma, foi objetivo deste estudo avaliar, em fêmeas Wistar, a resposta inflamatória hipotalâmica após a ingestão de dieta hiperenergética, promotora de obesidade, e intercalada com RAI.

Metodologia

Animais e ética

Quarenta fêmeas Wistar com 45 dias de vida foram utilizadas neste estudo. As ratas foram criadas e mantidas, em caixas individuais, no biotério do Polo Universitário da Universidade Federal do Rio de Janeiro (UFRJ), Campus Macaé, Brasil, sob condições controladas de luz, com 12 h de ciclos claro / escuro (7:30-19:30), temperatura média de 22 ± 2ºC e umidade de 50% ± 10%. O acesso à água e alimento obedeceu a protocolo específico desenhado para cada grupo experimental. Todos os procedimentos experimentais foram realizados de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal - CONCEA, Brasil e o estudo foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFRJ, Campus Macaé (protocolo número MAC030).

Desenho experimental

As ratas foram distribuídas aleatoriamente nos grupos: *Standard Control* (ST-C), mantido em consumo de ração padrão e água filtrada *ad libitum* durante todo o período de tratamento; *Diet-Induced Obesity Control* (DIO-C), mantido com dieta hiperenergética durante os primeiros e os últimos 15 dias do tratamento, e consumo *ad libitum* de dieta padrão, .entre o 16º e 45º dia de tratamento; *Standard Restricted* (ST-R), mantido com dieta padrão durante os primeiros e os últimos 15 dias do tratamento, e submetido à protocolo de restrição alimentar intermitente (RAI), entre o 16º e 45º dia de tratamento e

DIO *Restricted* (DIO-R), mantido com dieta hiperenergética nos primeiros e nos últimos 15 dias do tratamento e submetido à RAI da mesma forma que ST-R (Figura 1).

Protocolo de RAI e DIO

O protocolo RAI consistiu em 2 dias de restrição alimentar a 50% do consumo alimentar do grupo ST-C, seguido de 3 dias de realimentação *ad libitum*, completando 6 ciclos durante 30 dias. Foi oferecida ração padrão e água filtrada durante o período correspondente a RAI. A intervenção DIO envolveu oferta de ração hiperlipídica, contendo 45% de gordura, conjugada com a oferta de solução de sacarose 20% em substituição a água pura filtrada. A ração hiperlipídica e a solução de sacarose foram preparadas no Laboratório de Nutrição Experimental – LABNEX, onde foi realizado o estudo, a partir da ração padrão, com adição de banha, óleo vegetal e açúcar (Figura 1).

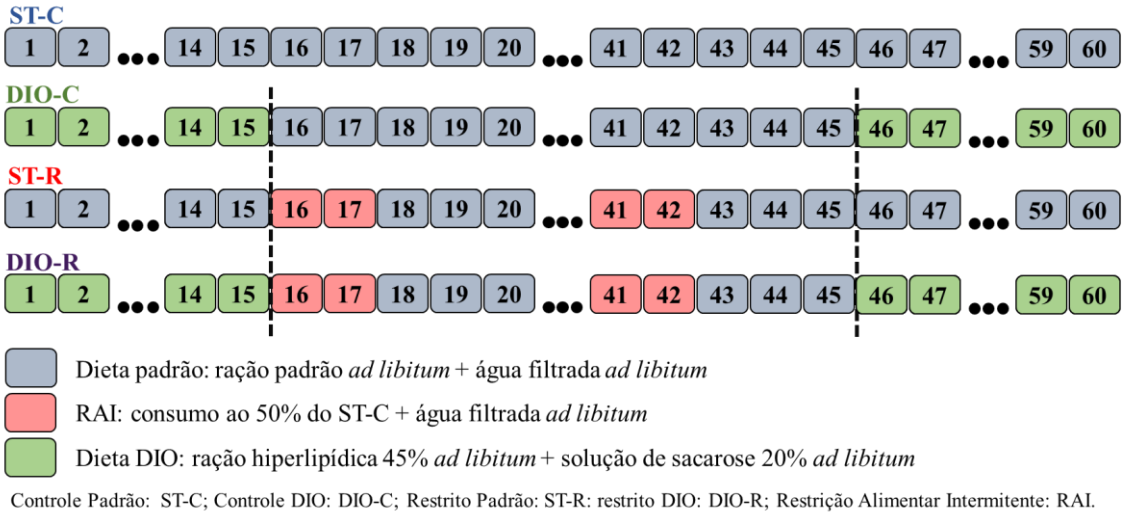


Figura 1. Desenho Experimental. Linha de tempo do tratamento dos grupos Controle Padrão (ST-C), DIO Controle (DIO-C), Restrito Padrão (ST-R), DIO Restrito (DIO-R). Os ratos ST-C consumiram ração padrão durante todo o período experimental. Ratos DIO-C consumiram dieta DIO nos primeiros e últimos 15 dias de tratamento, mas entre o 16º e 45º dias do período experimental alimentados com dieta padrão e água filtrada *ad libitum*. Durante os primeiros e últimos 15 dias, ST-R e DIO-R foram alimentados com ração padrão comercial ou dieta que induz a obesidade (DIO), respectivamente, e foram submetidos a ciclos de restrição alimentar intermitente (IFR) entre o 16° e 45° dias, recebendo ração comercial e água filtrada.

Composição das dietas

A composição estimada pelo fornecedor da ração padrão (Nuvilab, São Paulo, Brasil) consistiu em: 4% de gordura, 22% de proteína total, 42% de carboidratos, 10% de minerais, 1% de vitaminas; 8% de fibras e 12,5% de umidade, com densidade de energia de 2,22 Kcal / g. A DIO foi composta por ração hiperlipídica 45% e solução de sacarose a 20%. A ração rica em gordura foi preparada no LABNEX e consistiu em 60% de ração padrão, 2% de óleo de soja, 18% de banha, 13% de açúcar, 7% de caseína (Synth) e 2% de BHT. A distribuição de energia foi de 45% de gordura, 19% de proteína e 35% de carboidratos com uma oferta estimada de 4,45 Kcal / g.

Real Time PCR (qPCR)

Aos 105 dias de vida, os animais foram eutanasiados por guilhotina, o hipotálamo foi retirado rapidamente e colocado em reagente Trizol para a extração do RNA. Somente amostras com razão A260 / A280entre 1.9-2.1 foram utilizadas para quantificação do RNA em equipamento NanoDrop 2000 (Thermofisher Scientific, Brasil). O protocolo para a amplificação do RNA seguiu as instruções do fabricante do kit empregado (GoTaq® Probe 1-Step RT-qPCR System, Promega, Brasil). Para determinação dos teores hipotalâmicos de RNAm dos genes de interesse foi montada placa contendo 10 µL de Master Mix, 0,4 µL de RT Mix, 3,6 µL de H2O Ultrapura, 5 µL de RNA e 1 µL do probe Leprot, PPARα, SOCS3, JNK, TLR4, IKKβ, NF-κB e CCL5 (Thermofisher Scientific, Brasil), completando 20 µL em cada poço. Foi utilizado o equipamento StepOne Plus (Thermofisher Scientific, Brasil) cujas condições estabelecidas incluíram manutenção de 45°C durante 15 minutos para a incubação, 95°C durante 2 minutos para ativação da polimerase, 40 ciclos da reação em cadeia da polimerase (PCR) a 95°C por 15 segundos para desnaturar e 60°C por 1 minuto para extensão. O gene de referência utilizado foi o 18S.

Análise estatística

Posteriormente aos testes *Kolmogorov-Smirnov* e *Levene*, aplicados para identificar a normalidade e a homogeneidade dos dados, respectivamente, os resultados foram submetidos à ANOVA de uma via, seguida do teste *post hoc* de Bonferroni, utilizando-se o *software GraphPad Prism* versão 6.0. Foi adotado o nível de significância $p < 0,05$. O tamanho do efeito foi realizado *post hoc*, e apresentado como *Eta-squared* (η2) (η2; efeito pequeno = 0,01, efeito médio = 0,06, efeito grande = 0,14), utilizando-se

o *software* Jamovi versão 1.1.9.0 (https://www.jamovi.org/download.html). Os grupos ST-C e DIO-C foram considerados controles, respectivamente, dos grupos ST-R e DIO-R e os resultados estão apresentados como média e erro padrão da média.

Resultados

Regulação do metabolismo de ácidos graxos

Para o gene PPARα, o tratamento provocou efeito expressivo (η2=0,809), mostrando que os grupos ST-R e DIO-R apresentaram maior expressão do gene quando comparados ao ST-C (ambos com $p<0,001$). Também foi possível observar maior teor do gene no grupo DIO-R, quando comparado ao DIO-C ($p<0,001$) (Figura 2B).

Inibição da via da insulina e leptina

O tratamento promoveu efeito entre os grupos (η2=0,509) para o gene SOCS3, e todos os grupos mostraram maior expressão do gene comparados ao ST-C (DIO-C: $p=0,002$; ST-R: $p=0,020$; DIO-R: $p=0,002$) (Figura 2D). Quanto ao gene da proteína JNK, o tratamento causou efeito médio (η2=0,588), ocorrendo que os grupos ST-R e DIO-R expressaram maior teor do gene comparados ao ST-C ($p=0,001$ e $p=0,003$ respectivamente) (Figura 2C). Não foi observada diferença no gene Leprot entre os grupos (Figura 2A).

Sinalização inflamatória

O tratamento causou diferença entre os grupos, resultando em efeito médio (η2=0,393). O grupo ST-R mostrou menor expressão do gene TLR4 ($p=0,020$) comparado com o ST-C (Figura 3A). No entanto, para o gene IKKβ, cujo efeito do tratamento também foi médio (η2=0,323) e o grupo ST-R exibiu maior expressão comparado ao ST-C ($p=0,045$) (Figura 3B). Quando avaliado o gene NF-κB, o tratamento também resultou em efeito médio (η2=0,420), e os grupos ST-R e DIO-R apresentaram maior expressão do gene quando comparados ao ST-C ($p=0,047$ e $p=0,022$ respetivamente) (Figura 3C).

Inflamação crônica

O tratamento causou efeito no gene CCL5 (η2=0,677), sendo identificado maior teor no grupo DIO-R quando comparado ao ST-C ($p=0,004$) e ao DIO-C ($p<0,001$) (Figura 3D).

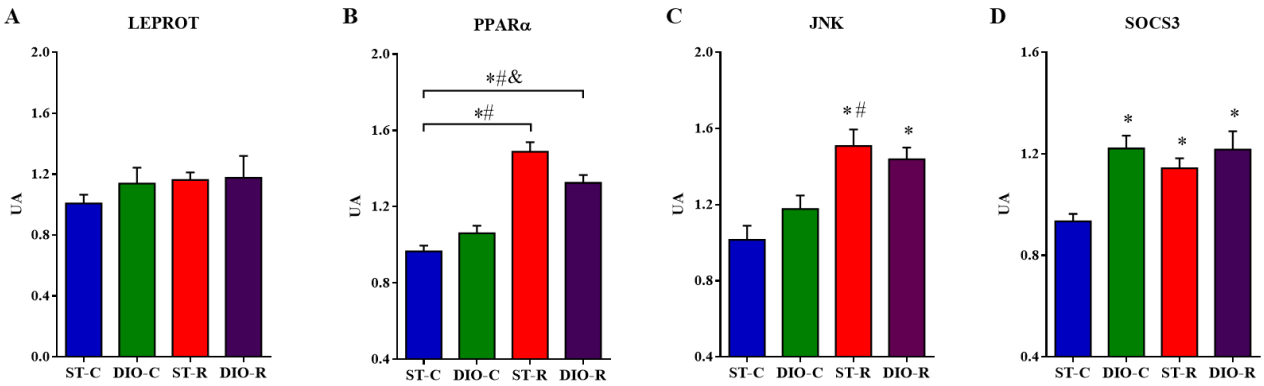


Figura 2. Genes relacionados com a inibição das vias anorexígenas insulina e leptina. Transcrição de sobreposição de receptor de leptina (LEPROT) **(A)**. Receptor ativado por proliferadores peroxissomais tipo alfa (PPARα) **(B)**. c-Jun quinase N-terminal (JNK) **(C)**. O supressor da sinalização de citocinas 3 (SOCS3) **(D)**. *Standard Control* (ST-C); DIO *Control* (DIO-C); *Standard Restricted* (ST-R); DIO *Restricted* (DIO-R). *One-way* ANOVA, Bonferroni pos hoc, $p<0.05$. *vs. ST-C, # vs. DIO-C; & vs. ST-R, n=5-8.

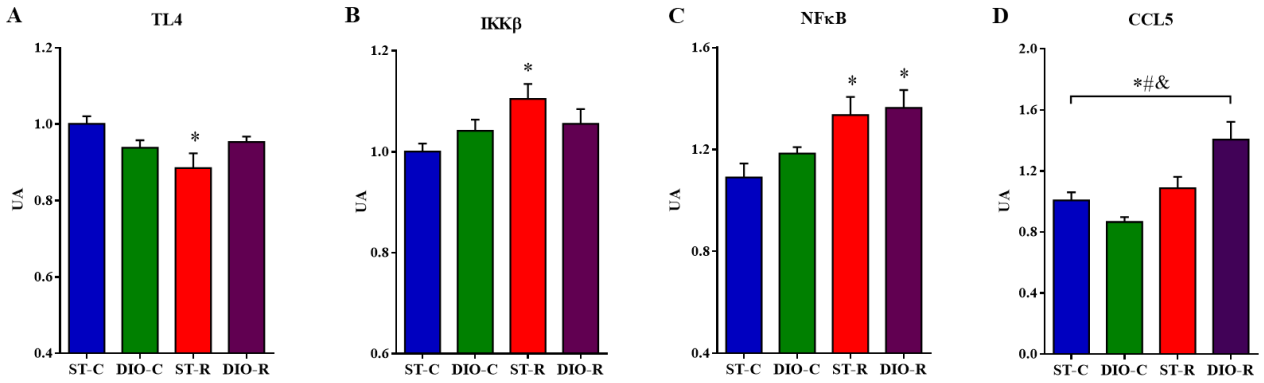


Figura 3. Genes inflamatórios hipotalâmicos. Receptor Toll Like tipo 4 (TLR4) **(A)**. Inibidor do fator nuclear kappa-B quinase subunidade beta (IKKβ) **(B)**. Fator nuclear kappa B (NF-κB) **(C)**. Ligante 5 de quimiocina (CCL5). *Standard Control* (ST-C); DIO *Control* (DIO-C); *Standard Restricted* (ST-R); DIO *Restricted* (DIO-R). *One-way* ANOVA, Bonferroni pos hoc, $p<0.05$. *vs. ST-C, # vs. DIO-C; & vs. ST-R, n=5-8.

Discussão

Demonstramos que a RAI, intercalada ou não com consumo de dieta obesogênica rica em gordura e açúcar, foi capaz de alterar a expressão do gene PPARα, relacionado com o metabolismo lipídico. Além disso, verificamos que tanto a ingestão intercalada de dieta obesogênica quanto a RAI causaram aumento na expressão do gene SOCS3, porém somente os grupos RAI apresentaram maior expressão do gene JNK, inclusive comparado ao grupo DIO-C.

A hipótese inicial deste estudo foi baseada em trabalho prévio do grupo no qual a alternância entre RAI e DIO, representado pelo grupo DIO-R, levou à alteração no metabolismo e morfologia do tecido adiposo branco e marrom (Rosas Fernández et al., 2020). No entanto, na presente investigação, identificamos relevantes alterações de expressão gênica em resposta a RAI, com ou sem DIO. Os sinais apontam para aumento da atividade lipolítica, com possível falha na ativação das vias da insulina e da leptina, acompanhada de aumento da atividade inflamatória, o que pode significar prejuízo à homeostase energética, quando no uso prolongado da RAI.

Avaliando marcadores de resposta inflamatória, observamos que a RAI promoveu aumento na expressão dos genes IKKβ e NF-κB, porém sugerimos que a composição da dieta obesogênica parece modular a expressão do IKKβ, posto que não houve diferença entre os grupos DIO em relação a esse gene. Tendo em vista os dados da literatura de que obesidade cursa com inflamação crônica de baixo grau investigamos o gene CCL5, relacionado a este perfil inflamatório e verificamos aumento de sua expressão no DIO-R em relação a todos os grupos.

PPARα pode ser ativado por várias moléculas endógenas, principalmente ácidos graxos de cadeia longa, de forma a regular positivamente genes de vias que são ativadas, em condições fisiológicas, especialmente durante o jejum, entretanto a obesidade e o jejum parecem representar dois extremos do espectro metabólico, ambos associados a ressíntese de ácidos graxos hepáticos e a diminuição da sinalização da insulina (Patsouris et al., 2006). Nos tecidos periféricos a função principal de PPARα é a regulação do catabolismo lipídico, enquanto no sistema nervoso central (SNC) este fator de transcrição regula o metabolismo energético (Chakravarthy et al., 2007).

Já foi descrito que no hipotálamo de camundongos alimentados com dieta hiperlipídica que a ativação de PPARα parece ocorrer pela enzima ácido graxo sintase (FAS), em reposta aos ácidos graxos sintetizados via lipogênese *de novo*, e não

necessariamente pelos ácidos graxos provenientes da dieta, concomitante com a ativação das enzimas carnitina palmitoiltransferase-1 (CPT-1), acil-CoA oxidase (ACO) e malonil-CoA descarboxilase (MCD) necessárias para a β-oxidação e diminuição dos teores de malonil-CoA (Chakravarthy et al., 2007). Nosso trabalho apontou aumento da expressão do PPARα com RAI, mesmo após retorno ao consumo da dieta *ad libitum*, sinalizando prejuízo do controle energético. Apesar de não existir diferença entre os grupos restritos, o grupo ST-R, do ponto de vista fisiológico, apresentou tendência a ser maior que o DIO-R, indicando que a lipogênese *de novo per se*, tem efeito prejudicial, inclusive maior do que quando intercalado com dieta hiperlipídica, criando-se assim, um círculo vicioso entre a ativação da PPARα e manutenção de baixo teor de malonil-CoA, como indicador da baixa de energia, podendo resultar no aumento da excitabilidade dos neurônios NPY/AgRP e consequente comportamento orexígeno e redução do gasto energético (Gao et al., 2013).

Evidências indicam que o PPARα pode estar envolvido na resposta anti-inflamatória, contudo, também pode induzir genes alvo mais consistentes com perfil pró-inflamatório, num processo de sensibilização na cascata de sinalização. De qualquer forma, o contexto inflamatório e celular específico que desencadeia estas respostas ainda não é totalmente conhecido (Bougarne et al., 2018). Estudo realizado com camundongos machos que receberam dieta hiperlipídica durante 20 semanas, identificou inflamação na substância negra e na área ventromedial hipotalâmica, porém, acompanhada de menor expressão de PPARα nas mesmas áreas cerebrais (Kao et al., 2020). Nossos resultados apontam aumento na expressão do PPARα nos grupos submetidos a RAI parecendo estar mais relacionado com resposta inflamatória, o que poderia ser resultado de maior estresse nesses grupos, decorrente da restrição alimentar, consistente com o incremento nos genes IKKβ, NF-κB e JNK.

O mecanismo atribuído ao papel anti-inflamatório de PPARα indica a inativação direta de NF-κB, assim como aumento na expressão de IκBα, sirtuína 1, e a fosfatidilinositol-3,4,5-trifosfato 3-fosfatase, além da promoção das enzimas antioxidantes catalase, superóxido dismutase e heme oxigenasse 1 (Korbecki et al., 2019). Contudo, trabalho realizado *in vitro* com células do tecido adiposo visceral mostrou que o caminho de PPARα em direção à via inflamatória ou anti-inflamatória reside na taxa de lipólise do tecido, mediado pelo aumento das espécies reativas de oxigênio causada pela elevada oxidação de ácidos graxos, conduzindo à ativação de NF-κB, com consequente aumento das citocinas inflamatórias (Sun et al., 2019). Os grupos submetidos a RAI no

presente trabalho mostraram maior expressão do PPARα, corroborando com este mecanismo, pois dietas restritivas mostraram conduzir ao aumento do estresse oxidativo (Chausse et al., 2015; Lenglos et al., 2013; Munhoz et al., 2020).

O estresse metabólico causado por alimentação hiperlipídica em longo prazo pode tornar-se prejudicial à saúde (Kopp, 2019; Tan & Norhaizan, 2019). A importância do PPARα foi observada em animais deletados do gene que mostraram alterações na capacidade de responder a grandes teores energéticos, resultando em acúmulo de gordura e esteatose hepática (Chen et al., 2020; Montagner et al., 2016). Os estudos no SNC são escassos, enquanto que os periféricos são discordantes indicando aumento, diminuição, ou manutenção dos teores de PPARα no fígado, tecido adiposo marrom e aorta, após 12 e 10 semanas de tratamento (Gao et al., 2013; Miranda et al., 2020; Zhuang et al., 2017). Já foi descrito que o consumo de dieta hiperlipídica alternada com ciclos de dieta padrão *ad libitum*, foi capaz de diminuir os prejuízos da dieta hiperlipídica e aumentar os teores de PPARα no fígado de camundongos macho (Ma et al., 2016). No nosso trabalho, o grupo DIO-C não mostrou diferença na expressão de PPARα quando comparado com o ST-C, isso pode ser devido ao fato de que os animais foram mantidos com ração padrão no período intermediário do tratamento.

As citocinas cumprem diversas funções no organismo, como crescimento e diferenciação celular, ligando-se a receptores e ativando e inativando vias de transdução de sinais intracelulares como a via JAK-STAT. O fator de transcrição SOCS3 é ativado principalmente em resposta ao aumento de citocinas, atua por feedback negativo, regulando indiretamente esta via ao inibir a sinalização da JAK2 evitando a ativação de STAT3. No entanto, a sobrerregulação de SOCS3 demonstrou causar resistência à leptina e insulina por interferir na cascata de sinalização, inibindo a fosforilação dos resíduos de tirosina e fosfotirosina, o que finalmente resulta na falta de resposta aos sinais de saciedade (Howard & Flier, 2006; Carow & Rottenberg, 2014; Pedroso et al., 2019 Wunderlich et al., 2013).

No nosso trabalho observamos maior expressão do gene SOCS3 em todos os grupos tratados quando comparados ao ST-C, indicando que tanto a dieta hiperlipídica, como sua alternância com RAI, são fatores igualmente prejudiciais da via. Há descrição de aumento na expressão de SOCS3 no hipotálamo de camundongos alimentados com dieta hiperlipídica em curto e longo prazo (Dalby et al., 2018). Também já foi mostrado que o jejum de 24 ou 48 horas é capaz de aumentar a expressão do gene no hipotálamo de camundongos machos, o que foi relacionado com a diminuição da leptina durante esse

período (Pedroso et al., 2016). Nos nossos resultados, o grupo ST-R, submetido à RAI, também apresentou aumento na expressão de SOCS3. Recentemente foi descrito aumento hipotalâmico de SOCS3 em camundongos machos submetidos ao jejum intermitente (JI) durante 4 semanas, assim como à dieta hiperlipídica no mesmo período, além disso os animais que realizaram JI tanto com dieta controle, como com dieta hiperlipídica apresentaram maiores teores das citocinas inflamatórias IL6, IL1b, TNFα (Spezani et al., 2020). Ao que tudo indica, a RAI não atenua o estado de inflamação promovido pela DIO, pelo contrário, a superexpressão do SOCS3 pode causar prejuízos por sua relação com a resistência à leptina e insulina.

A cascata de sinalização inflamatória TLR4 / IKKβ / NF-κB é ativada na presença de patógenos, após reconhecimento dos PAMPs, no entanto, dietas hiperlipídicas também são capazes de ativar esta via por meio da porção do lipídio A, comum em ambos os eventos (Kleinridders et al., 2009; Milanski et al., 2009). Em condições fisiológicas ideais, IKKβ / NF-κB se encontram altamente expressos no hipotálamo, porém permanecem suprimidos. Zhang et al. (2008) mostraram que o consumo de dieta hiperlipídica dobrou a atividade de IKKβ / NF-κB no hipotálamo de camundongos machos. A ingestão prolongada de dieta hiperlipídica também promove aumento nos lipopolissacarídeos (LPS), que podem se ligar ao TLR4 e ativar a via IKKβ / NF-κB (Mohammad & Thiemermann, 2021). No entanto, contrariando a hipótese de que o gene TLR4 poderia estar mais expresso nos grupos tratados, nossos resultados apontaram menor expressão de TLR4 e maior do gene IKKβ no grupo ST-R. Em relação ao NF-κB, ambos os grupos submetidos a RAI mostraram maior expressão do gene.

Em vista dos nossos resultados, consideramos que o aumento na expressão de NF-κB nos grupos RAI independe do aumento na expressão do TLR4. O grupo ST-R apresentou menor expressão do TLR4, corroborando com o estudo de Vasconcelos et al. (2014) que apontou menor expressão do gene após 30 dias de RAI em ratos. Dalby et al. (2018) citaram ocorrer fraca expressão de TLR4 no hipotálamo de ratos, indicando baixa probabilidade de que LPS atue diretamente por esta via neste tecido. Adicionalmente, foi mostrado que dieta rica em gordura produz endotoxemia metabólica (aumento crônico de LPS) 10 a 50 vezes menor do que o choque séptico (Cani et al., 2007). Dalby et al. (2018) também verificaram, em camundongos machos com deleção do gene TLR4, alimentados com dieta hiperlipídica, elevação significativa da proteína de ligação do LPS, a LBP, biomarcador da exposição a esta endotoxina. Além disso, os animais mostraram elevação do IKKβ / NF-κB assim como aumento das citocinas inflamatórias e do SOCS3

hipotalâmico. Em contrapartida, o IKKβ / NF- κB parece também regular a inibição de STAT3 em resposta ao incremento de SOCS3, embora o aumento de NF-κB seja observado apenas nos neurônios, indicando ativação, por vias diferentes das citocinas, para mediar a desregulação do equilíbrio energético nas situações de consumo excessivo de energia (Zhang et al., 2008).

Outra possibilidade capaz de justificar a elevação do IKKβ / NF-κB é o estresse do retículo endoplasmático. Ácidos graxos saturados podem ativar esse processo no hipotálamo (Milanski et al., 2009). A manutenção de exposição à dietas ricas em gordura acentua esse quadro de estresse, levando à ativação da sinalização inflamatória e apoptose (Hotamisligil, 2010; Cnop et al., 2012). Nas situações de consumo excessivo de energia, seja por dieta hiperlipídica ou no período de realimentação após jejum, o estresse do reticulo endoplasmático e a ativação do IKKβ / NF-κB têm efeito sinérgico no hipotálamo, o que conduz a mecanismos centrais de desequilíbrio energético (Zhang et al., 2008; Sasako et al., 2019). Dessa forma, o aumento na expressão de IKKβ / NF-κB indica que os grupos submetidos a RAI cursam com inflamação por via independente do TLR4.

A proteína JNK é ativada em resposta a uma variedade de sinais de estresse, entre eles a inflamação (Zhang et al., 2020). Nosso trabalho mostrou que ambos os grupos submetidos a RAI apresentaram maior expressão do gene JNK. Já foi descrito que a endotoxemia prolongada induziu maior expressão do gene JNK, que foi associada a inibição da sinalização da cascata de insulina no hipotálamo de ratos machos (Rorato et al., 2017). Por outro lado, a deleção do gene MyD88, no hipotálamo de camundongos obesos, não alterou a ativação da JNK (Kleinridders et al., 2009), indicando que a JNK pode ser ativada por outra via, provavelmente por indução de citocinas inflamatórias, aumentadas tanto em razão do consumo de dieta hiperlipídica (Dalby et al., 2018; Santos et al., 2019) como pela RAI (Han et al., 2014).

A quimiocina quimiotáctica CCL5 ou RANTES implicada no recrutamento de leucócitos, além de mediar a acumulação local de células T e macrófagos, está envolvida na gênese da inflamação crônica (Le Thuc et al., 2017). Nossos resultados apontaram aumento na expressão do gene CCL5 no grupo DIO-R, indicando que a alternância entre DIO e RAI se apresentou mais prejudicial do que os padrões alimentares isoladamente. Recentemente foi mostrado que o consumo de dieta hiperlipídica, em curto prazo, induz aumento da expressão de citocinas inflamatórias IL-1β, IL-6, TNF-α, CCL2, assim como do CCL5 no hipotálamo de camundongos machos, indicando inflamação subsequente ao

consumo de DIO (Cansell et al., 2021). Adicionalmente, o consumo deste tipo de dieta por tempo prolongado mantém elevada a expressão do CCL5 de forma crônica. Essa condição pode resultar em aumento da ingestão de alimentos e consequente ganho de massa corporal, dada a promoção de intensa ativação de neurônios hipotalâmicos e liberação do hormônio liberador de melanina (MCH) (Le Thuc et al., 2017).

Consumo excessivo de energia pode induzir à inflamação hipotalâmica por diferentes vias, entre elas, o estresse intracelular independente do receptor de membrana, causado pela entrada de ácidos graxos, glicose e aminoácidos; pela via do receptor TLR; e pela rota de receptores de citocinas / quimiocinas. Estas respostas primárias tem como objetivo ajustar o equilíbrio energético, entretanto, a exposição prolongada ao excesso de lipídios pode levar a desregulação e alterações hipotalâmicas que conduzem à obesidade (Cai & Liu, 2011). Neste trabalho, mostramos que a RAI, intercalada ou não com DIO, levou ao aumento da expressão de genes inflamatórios no hipotálamo, podendo existir prejuízo na resposta anorexígena, em razão da acentuada expressão de SOCS3 com possível prejuízo a resposta anorexígena da insulina e leptina. Assim, a crescente difusão desta prática alimentar associada a benefícios à saúde, permanece exigindo cautela e maior investigação, posto que sua prática em longo prazo pode levar à inflamação central e perda do balanço energético, conforme verificado no modelo animal do presente estudo.

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6. CONCLUSÕES

A nível periférico, a RAI associada com DIO resultou em efeitos sinérgicos favorecendo o ganho de massa corporal e incremento dos depósitos de gordura visceral total e do tecido adiposo marrom, além de causar hipertrofia do tecido adiposo branco e marrom. Também, a RAI levou a maior consumo alimentar e eficiência energética incrementada.

A nível central, a RAI associada ou não com DIO agravou o perfil inflamatório hipotalâmico, associado a prejuízo na resposta anorexígena causada por acentuada expressão de SOCS3 com possível prejuízo a resposta anorexígena da insulina e leptina.

Reunidos, estes achados são indicativos de que a prática da restrição alimentar intermitente, em longo prazo, não favorece a manutenção da perda de massa corporal, em razão dos prejuízos causados pela inflamação hipotalâmica e possível ausência na resposta alimentar, corroborado com o maior consumo alimentar, eficiência energética e aumento dos depósitos de gordura observados nos animais submetidos ao tratamento. Assim, a crescente difusão desta prática alimentar associada a benefícios a saúde, permanece exigindo cautela.

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ANEXOS

ANEXO A

Aprovação do projeto de pesquisa pela CEUA/UFRJ

UNIVERSIDADE FEDERAL DO RIO DE JANEIRO
CAMPUS UFRJ-MACAÉ
Professor Aloísio Teixeira

Macaé, 17 de Dezembro de 2015

Prezado

A Comissão de Ética em Experimentação Animal da Universidade Federal do Rio de Janeiro – Campus UFRJ – Macaé Prof. Aloísio Teixeira certifica que o projeto sob sua coordenação e intitulado **"Efeito de nutrientes, plantas, algas e atividade física sobre a lipogênese, regulação da ingestão alimentar, programação metabólica e marcadores de síndrome metabólica."**, onde é previsto protocolo com utilização de animais, foi aprovado por esta comissão, sob o número de referência MAC030 possuindo validade de 4 (quatro) anos.

Atenciosamente,

Prof. Andre Gustavo Calvano Bonavita
Presidente da Comissão de Ética no Uso de Animais
SIAPE: 2738212
Campus UFRJ – Macaé Prof. Aloísio Teixeira

Universidade Federal do Rio de Janeiro – Campus UFRJ – Macaé Prof. Aloísio Teixeira
Rua Aluízio da Silva Gomes, 50 - Granja dos Cavaleiros, Macaé - RJ - CEP: 27930-560
Tel. (22) ??? E-mail: ceua@macae.ufrj.br

ANEXO B

Artigo publicado na revista British Journal of Nutrition

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Fasting and refeeding cycles alter subcutaneous white depot growth dynamics and the morphology of brown adipose tissue in female rats

Mariana Rosas Fernández[1,2], Carlos Concha Vilca[2,3], Leandro O. Batista[2], Letícia S. Figueiredo[4], Rosane A. Ribeiro[4,5], Maria das Graças Tavares do Carmo[6] and Kelse T. Albuquerque[2,3]*

[1]*Graduate Program in Nutrition, Federal University of Rio de Janeiro, RJ, Brazil*
[2]*Laboratory of Experimental Nutrition, Federal University of Rio de Janeiro, RJ, Brazil*
[3]*Nutrition Department, Multicenter Graduate Program in Physiological Sciences, Federal University of Rio de Janeiro, RJ, Brazil*
[4]*Graduate Program in Bioactive Products and Biosciences, Federal University of Rio de Janeiro, RJ, Brazil*
[5]*Department of General Biology, Biological and Health Sciences Sector, State University of Ponta Grossa, PR, Brazil*
[6]*Department of Nutrition and Dietetics, Laboratory of Nutritional Biochemistry, Federal University of Rio de Janeiro, RJ, Brazil*

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Abstract

Intermittent food restriction (IFR) is used mainly for weight loss; however, its effects on adipose tissue are not known when alternating with an obesogenic diet. To demonstrate its effects on morphological dynamics of fat deposits, female Wistar rats were distributed into groups: standard control (ST-C), with commercial diet; DIO control (DIO-C), with a diet that induces obesity (DIO) during the first and last 15 d, replaced by a standard diet for thirty intermediate days; standard restricted (ST-R), with standard diet during the first and last 15 d, with six cycles of IFR at 50 % of ST-C; and DIO restricted (DIO-R), in DIO during the first and last 15 d, with six cycles of IFR at 50 % of DIO-C. At 105 d of life, white adipose tissue (WAT) and brown adipose tissue (BAT) deposits were collected, weighed and histology performed. The DIO-R group showed higher total food intake (DIO-R 10 768·0 (SEM 357·52) kJ/g *v.* DIO-C 8868·6 (SEM 249·25) kJ/g, $P < 0·0001$), energy efficiency during RAI (DIO-R 2·26 (SEM 0·05) g/kJ *v.* DIO-C 0·70 (SEM 0·03) g/kJ, $P < 0·0001$) and WAT (DIO-R 5·65 (SEM 0·30) g/100 g *v.* DIO-C 4·56 (SEM 0·30) g/100 g) than their respective control. Furthermore, IFR groups presented hypertrophy of WAT and BAT, as well as fibrosis in BAT. Thus, IFR can establish prospective resistance to weight loss by favouring changes in adipose tissue morphology, increased energy intake and efficiency. Finally, the DIO diet before and after IFR aggravates the damages caused by the restriction.

Key words: Intermittent fasting: Fat tissue: Brown adipose tissue fibrosis: Energy efficiency: Diet-induced obesity model

The adipose tissue derives from different cell lines and is divided into two compartments: subcutaneous (SUB) and visceral, which present functional and morphological differences[1]. It is essentially distinguished by white adipose tissue (WAT) and brown adipose tissue (BAT), exercising functions of energy storage and thermogenesis, respectively[2,3]. Additionally, both tissues exert an endocrine function, secreting a set of substances, such as hormones, adipokines and cytokines, which regulate energy homoeostasis, glucose, lipids and inflammation[4,5].

The principal difference between WAT and BAT compartments can be explained by different gene expressions and different progenitor cells[6]. BAT's colour stems from the density of mitochondria and rich vascularisation[7], its adipocytes in response to sympathetic nervous system action and other hormones oxidise their stored lipids, to produce heat, contributing to thermogenesis[8].

WAT has aroused clinical interest due to its expansion potential. The excessive accumulation of this tissue promotes the release of macrophages and the production of inflammatory cytokines, characterising the inflammatory process, observed in obesity, and determining other metabolic disorders[9,10]. Thus, the main objective of restrictive diets should be the loss of body weight accompanied by the reduction of WAT in order to promote a reduction in metabolic risks[11].

Abbreviations: BAT, brown adipose tissue; DIO, diet that induces obesity; DIO-C, DIO control; DIO-R, DIO restricted; EE, energy efficiency; IFR, intermittent food restriction; RET, retroperitoneal; RF, refeeding; ST-C, standard control; ST-R, standard restricted; SUB, subcutaneous; WAT, white adipose tissue.

* **Corresponding author:** Kelse Tibau de Albuquerque, email ktibau@yahoo.com.br

British Journal of Nutrition

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British Journal of Nutrition

Some dietary patterns may increase adiposity, including saturated fat and refined sugar consumption[12–15]. On the other hand, the high prevalence of obesity in the world has led to the practice of several dietary strategies suggested as inducing loss of body mass, which can result in physiological losses and establish a pro-obesogenic cycle in the body, with repercussions on adipogenesis[16]. From an observational point of view, in Brazil, these practices are often developed by individuals without compromise with healthier eating practices and deal with temporary behaviours and subsequent return to consumption of palatable diets, rich in energy, fat and sugar[17,18].

Rapid weight loss protocols are more practised by women than by men, due to the desire to lose weight resulting from greater body dissatisfaction[19–21]. Intermittent food restriction (IFR), also called intermittent fasting or fasting–refeeding (RF) cycles, is a strategy that became popular with the promise of loss of body mass. It consists of total or partial food restriction for hours in the day, on alternate days or on selected days during the week, followed by periods of RF, without limiting the amount of food eaten[22]. The proposal meets the expectations of some individuals who seek to lose weight, but with freedom in food consumption outside the short periods of food restriction, that is, after the restriction, they return to ingesting palatable foods, establishing eating behaviour that alternates between food restriction and hyperenergetic consumption.

In humans and experimental obese rodents, IFR seems to produce positive results in the loss of body mass, as well as in the improvement of markers related to cardiometabolic diseases[23,24]. These findings, however, are controversial, with evidence that IFR also has a negative effect on health, due to the increased activity of adipogenic enzymes and the consequent gain in fat mass[25–30].

Therefore, herein, we tried to represent human behaviour, in which women consume a hyperenergetic diet pattern during their youth; then in an attempt to lose weight quickly, they carry out restrictive diets, a period in which they strive to consume a healthy diet; however, they are unable to maintain this pattern of restriction and end up abandoning it, returning to the habitual consumption of a hyperenergetic diet. Considering the few investigations on the relationship of IFR on morphology and function of SUB and visceral adiposity, this study aimed to evaluate, in female rats, the effect of the alternation between periods of diet that induces obesity (DIO) and IFR in the morphological dynamics of white and brown fat deposits.

Materials and methods

Animal care and ethics

Wistar female rats were provided by our breeding facility and were housed in individual cages, under a 12 h light–12 h dark cycle with lights off at 20.00 hours, at 22 ± 1°C room temperature. The handling of rats and experimental procedures were performed according to ARRIVE guidelines, the rules of the National Council of Animal Experiments Control, and all experimentation received approval from the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro, *Campus* Macaé, under reference number MAC030. The same rats were used for all experiments, following the 3Rs principle reduction.

Experimental model

Forty-five-day-old *Wistar* females rats (*n* 40, 10 per group; 153·34 (SEM 1·57) g) were randomly distributed into four groups: standard control (ST-C; *n* 10), DIO control (DIO-C; *n* 10), standard restricted (ST-R; *n* 10) and DIO restricted (DIO-R; *n* 10), during 60 d of treatment. Groups ST-C and ST-R had access to filtered water and a standard diet (Nuvilab). The DIO-C and DIO-R groups received an obesity-induced diet (DIO) during the first 15 d, as well as, the last 15 d of treatment and consumed a standard diet and filtered water *ad libitum* between the 16th and 45th d of treatment. ST-R and DIO-R rats were submitted to IFR between the 16th and 45th d of treatment. IFR consisted of 2 d of food restriction in which the restricted groups received 50 % of the amount of chow consumed by the ST-C group, followed by 3 d of *ad libitum* feeding, completing six restriction cycles for 30 d (Fig. 1 shows the representation of diet treatments and IFR of experimental groups).

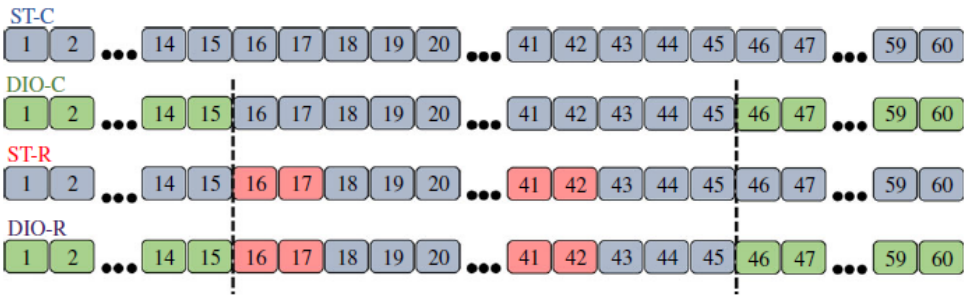

Fig. 1. Experimental design. Treatment timeline of standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R), DIO restricted (DIO-R) groups. ST-C rats consumed standard chow during all experimental period. DIO-C rats consumed DIO diet in the first and last 15 d of treatment, but between the 16th and 45th day of the experimental period fed on a standard diet and filtered water *ad libitum*. During the first and last 15 d, ST-R and DIO-R were fed with commercial standard chow or diet-induced obesity (DIO), respectively, and were submitted to intermittent food restriction (IFR) cycles between 16th and 45th day, receiving commercial chow and filtered water. ▭, Standard diet: *ad libitum* standard chow + *ad libitum* filtered water; ▭, IFR: 50 % of ST-C intake + *ad libitum* filtered water; ▭, DIO diet: 45 % hyperlipidic chow *ad libitum* + 20 % sucrose solution *ad libitum*.

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The standard diet consisted of commercial rodent chow (Nuvilab) and filtered water, both *ad libitum*. The chow composition was 4 % fat, 22 % protein, 42 % carbohydrates, 10 % minerals, 0·8 % P, 1 % vitamins; 8 % fibres and 12·5 % humidity, with an energy density of 12·2 kJ/g. The DIO diet consisted of high-fat chow (46 % fat of total energy) and 20 % sucrose in its drinking water, both *ad libitum*. The DIO diet was prepared in our laboratory and consists of 60 % of the standard chow, 2 % soya oil, 18 % lard, 13 % sugar and 7 % casein (Synth). The energy distribution was 46 % fat, 19 % protein and 35 % carbohydrates with an estimated supply of 18·2 kJ/g.

Assessment of food intake, body mass energy efficiency and visceral fat

Food and drinking water consumption and body mass were registered daily, between 08.00 and 09.00 hours, throughout the 60 d of treatment using an electronic balance (TOLEDO DO BRASIL). Body weight was assessed at the exact moment when the rat was resting on all four paws without moving for 3 s. Energy efficiency (EE) was obtained from the equation: EE = ((average body mass gain (g)/average energy consumption (kJ)) × (100)). At the end of the experimental period, after euthanasia of all rat groups, the visceral fat deposits (retroperitoneal (RET), mesenteric and gonadal) were removed and weighed and the visceral fat content (TOTAL) was estimated.

Serum biochemical analysis

After 60 d of DIO and/or IFR experimental period, all rat groups were food-deprived for 12 h and were euthanised by beheading with guillotine, between 08.00 and 10.00 hours. The blood was collected for analysis of TAG and total cholesterol using a colorimetric kit according to the manufacturer's instructions (TAG Liquiform/total cholesterol; Analisa) and was performed with a 505 nm wavelength spectrophotometer reading (Asys Expert 96; Biochrom).

Histological analysis

At the end of the experimental period, SUB and visceral WAT (from the thigh and RET areas, respectively) and the interscapular BAT were collected on histological cassettes, remaining in phosphate buffer (formaldehyde 10 %, methane 1·5 % and PBS 88·5 %) for 48 h. Afterwards, tissues were washed under running water and kept in 70 % alcohol until analysis. Before paraffin embedding, the tissue was dehydrated in increasing concentrations of alcohol and diaphanised in xylol. The samples were cut in sections of 5 µm in thickness, using a microtome (Lupetec MRP2015), and arranged in sheets, which were immersed in xylol, followed by decreasing concentrations of alcohol for hydration. Subsequently, the WAT and BAT sections were stained with haematoxylin and eosin, and BAT was also stained with picrosirius red (Sigma-Aldrich, Diadema), and the coverslips were quickly assembled. Images from WAT and BAT sections stained with haematoxylin and eosin or picrosirius red were captured using a digital camera (Olympus DP71; Olympus Optical) coupled with an optical microscope (Olympus BX51; Olympus Optical). For each female rat (*n* 10 per experimental group), eight histological random fields from each haematoxylin and eosin stained section obtained from the SUB or RET WAT, and BAT were registered. All morphometric measurements were performed with the aid of the Image J software (https://imagej.nih.gov/ij/download.html). All white or brown adipocyte per field registered were manually counting. The diameter of all SUB and RET adipocytes per field registered and the areas of at least fifty brown adipocytes per field registered were measured using the 'straight' and 'freehand' tools of Image J software, respectively. The pixel number obtained in each measure was automatically converted to micrometres through software calibration with a micrometre scale slide. In addition, all lipid vacuoles per brown adipocyte measured were manually counted[31,32]. For the quantification of BAT collagen deposition, the percentage area that was positive for picrosirius red staining was calculated in the BAT sections by determining the specific threshold for pink/red colour that represents stained collagen fibres, and a square was applied on this colour for recording, using Image J Software. Positive picrosirius red areas were automatically obtained through the conversion of total pixel numbers/intensities to micrometres. Data are expressed as the percentage of the positive-stained area/total tissue area examined[33].

Statistical analysis

The G*Power 3.1.9.2[34] software was used to design sample size in this study; the power analysis was carried out *a priori* during the design stage of the study. We calculated the sample size needed in each group to obtain a power of 0·80 and a significance level of 0·05, the effect size *f* was defined as 0·56 ($\eta^2_p = 0{\cdot}24$) based on our previous study[28] and in the literature[35–37]. The sample size obtained was of ten rats per group.

For multiple comparisons, one-way ANOVA was used, followed by the Bonferroni post-test, and a significance level of $P < 0{\cdot}05$ was adopted. The ST-C and DIO-C groups were considered controls, respectively, of the ST-R and DIO-R groups. The analyses were performed using the GraphPad Prism 6.0 Software, and the results are presented as mean values with their standard errors. The effect size was calculated using the statistical package Jamovi version 1.1.9.0 (https://www.jamovi.org/download.html). Eta-squared (η^2; small effect = 0·01, medium effect = 0·06, large effect = 0·14) and Cohen's *d* (small effect = 0·2, medium effect = 0·5, large effect = 0. 8) are reported when analyses were performed by ANOVA and in *post hoc* tests, respectively[38].

Results

Body mass behaviour

When observed per week, the difference in weight between the groups was not detected (Fig. 2(a)). No differences were observed before (PRE) and during the IFR; however, ANOVA revealed a main effect of diet in this period on the groups ($P < 0{\cdot}001$, $\eta^2 = 0{\cdot}505$). DIO-C and DIO-R groups showed significant body weight gain (1·16 (SEM 0·19) g, $P = 0{\cdot}0073$

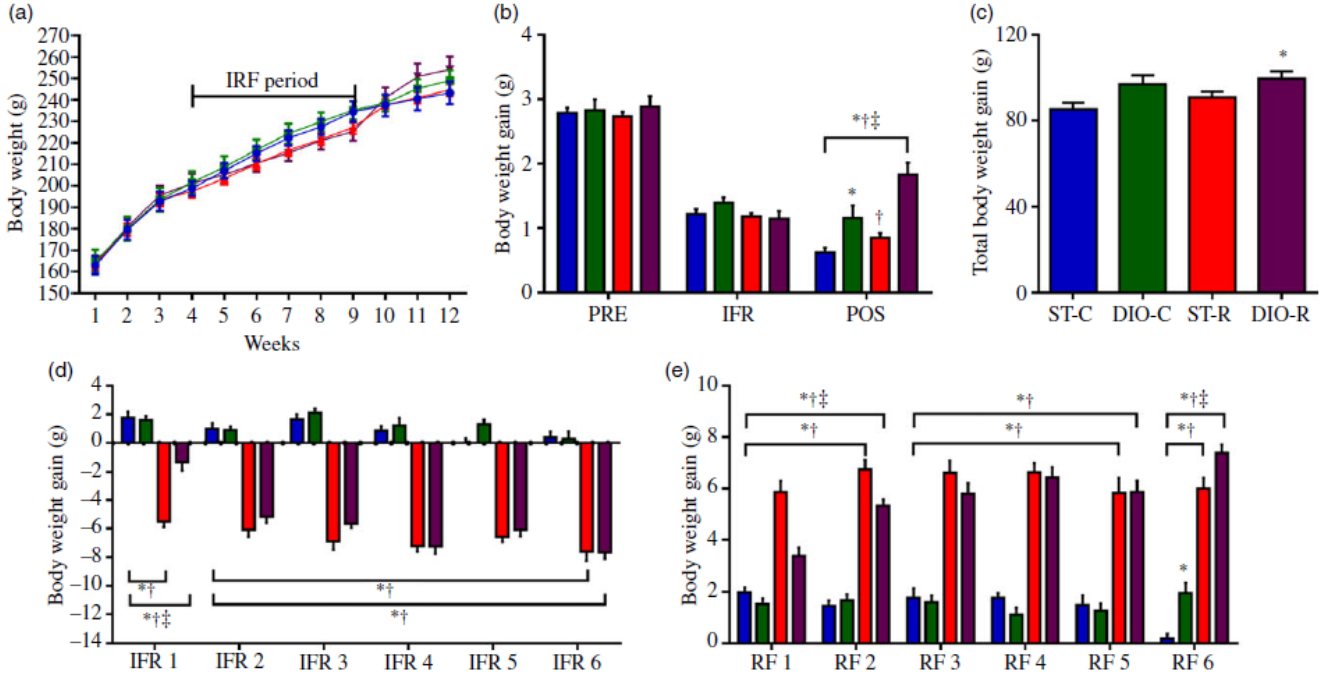

Fig. 2. Diet that induces obesity (DIO) consumption after intermittent food restriction (IFR) exacerbates body mass gain in female rats. (a) Timeline of body weight changes. —●—, ST-C; —■—, DIO-C; —▲—, ST-R; —▼—, DIO-R. (b) Body weight gain, before (PRE), during (IFR) and after (POS) IFR. (c) Total body weight gain at the end of the trial period. (d) Body weight gain on each effective restraint cycle during IFR. (e) Body weight gain in each refeeding (RF) cycle during IFR. ■, ST-C; ■, DIO-C; ■, ST-R; ■, DIO-R. Experimental groups: standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R), DIO restricted (DIO-R). Values are mean values with their standard errors (*n* 10). Data were analysed using one-way ANOVA followed by Bonferroni post-test. $P<0·05$ * *v.* ST-C, † *v.* DIO-C, ‡ *v.* ST-R.

and 1·83 (SEM 0·18) g, $P<0·0001$, respectively) than ST-C (0·63 (SEM 0·07) g). In addition, body weight gain, in the period following IFR (POS), was higher in DIO-R rats than that observed for DIO-C ($P=0·0008$). In this same period, the ST-R group showed significantly less body mass gain ($P<0·0001$), compared with the DIO-R group (Fig. 2(b)). The final body weight gain was significantly different among groups ($P=0·019$, $\eta^2=0·195$). The DIO-R group showed greater final body weight gain (102·69 (SEM 4·40) g, $P=0·0254$, Cohen's $d=0·134$) compared with the ST-C (87·64 (SEM 3·22) g) (Fig. 2(c)).

Throughout the six feed restriction cycles, the ST-R and DIO-R groups showed similar reductions of body mass, except in the first IFR cycle, in which DIO-R rats (−1·33 (SEM 0·59) g, $P<0·0001$) displayed a minor reduction when compared with that observed for ST-R (−5·55 (SEM 0·40) g) (Fig. 2(d)). Fig. 2(e) shows the six RF cycles of the IFR period; it is possible to observe that body mass gain was significantly higher in the ST-R and DIO-R groups, compared with the ST-C in all cycles. In the first two RF cycles (RF1 and RF2), the DIO-R rats showed a lower body mass gain (RF1 3·38 (SEM 0·34) g, $P<0·0001$ and RF2 5·33 (SEM 0·23) g, $P=0·0242$), than the ST-R group (RF1 5·87 (SEM 0·42) g and RF2 6·74 (SEM 0·38) g). But, at RF3, RF4 and RF5, DIO-R and ST-R groups maintained a similar body mass gain; however, in the last RF cycle (RF6), a significant difference was observed among these groups ($P=0·019$, $\eta^2=0·195$), in which the body mass gain of the DIO-R group was significantly higher (7·38 (SEM 0·33) g, $P=0·0283$, Cohen's $d=0·148$) than that observed for the ST-R group (6·00 (SEM 0·42) g) (Fig. 2(e)).

Intermittent food restriction increases intake and energy efficiency

DIO-C and DIO-R rats maintained a higher energy intake than the ST-C group, before (2820·96 (SEM 106·01) kJ/g, $P=0·0015$, Cohen's $d=1·021$ and 2872·81 (SEM 93·51) kJ/g, $P=0·0003$, Cohen's $d=1·074$ *v.* 1787·59 (SEM 43·9) kJ/g) (PRE), during (3978·65 (SEM 124·21) kJ/g, $P=0·036$, Cohen's $d=0·797$ and 4322·17 (SEM 260·37) kJ/g, $P<0·0001$, Cohen's $d=1·034$ *v.* 2784·17 (SEM 61·02) kJ/g) (IFR) and after (2537·50 (SEM 109·76) kJ/g, $P<0·0001$, Cohen's $d=1·726$ and 2806·70 (SEM 133·30) kJ/g, $P<0·0001$, Cohen's $d=1·978$ *v.* 1109·47 (SEM 63·55) kJ/g) (POS) IFR. ANOVA revealed a main effect of diet on the total amount of energy consumed throughout the whole treatment ($P<0·0001$, $\eta^2=0·829$); all groups were significantly different from ST-C, even DIO-R (10 768·00 (SEM 357·52) kJ/g, $P=0·0006$, Cohen's $d=0·722$) group differed significantly from its own control (DIO-C) (8868·60 (SEM 249·25) kJ/g) (Fig. 3(a)).

EE was significantly lower in the DIO-C and DIO-R groups (0·86 (SEM 0·04) g/kJ, $P<0·0001$, Cohen's $d=0·05$ and 0·87 (SEM 0·03) g/kJ, $P<0·0001$, Cohen's $d=0·05$ *v.* ST-C 1·28 (SEM 0·03) g/kJ) during the first 15 d of treatment, when these rats received a DIO. However, over the IFR period, ANOVA revealed a main effect of diet on EE ($P<0·0001$, $\eta^2=0·957$), in which EE was significantly higher in both ST-R and DIO-R groups, regardless of diet, indicating a greater anabolic capacity of these rodents in the feeding periods (2·35 (SEM 0·06) g/kJ, $P<0·0001$, Cohen's $d=0·139$ and 2·26 (SEM 0·05) g/kJ,

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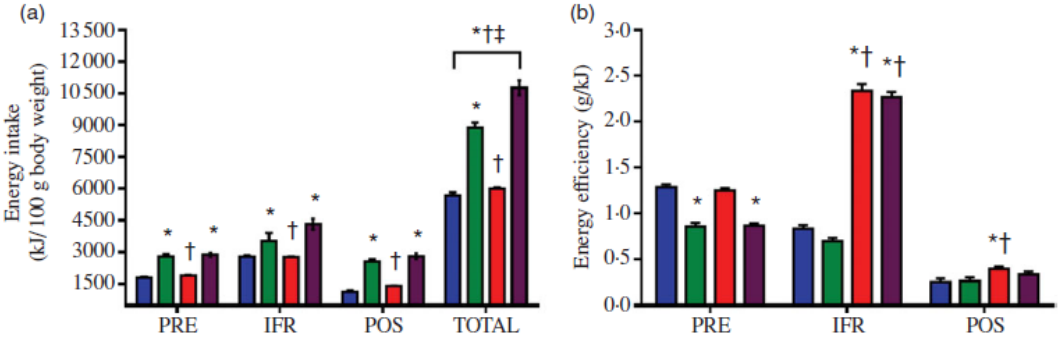

Fig. 3. Intermittent food restriction (IFR) increased energy intake and feed efficiency in female rats fed on a standard diet or diet that induces obesity (DIO). (a) Energy intake (in kJ) before (PRE), during (IFR) and after (POS) IFR and total (accumulated at the end of the experimental period). (b) Energy efficiency in the 15 d before IFR (PRE), in IFR and in the last 15 d of treatment (POS). Experimental groups: standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R). ■, ST-C; ■, DIO-C; ■, ST-R; ■, DIO-R. Values are mean values with their standard errors (*n* 10). Data were analysed by one-way ANOVA followed by Bonferroni post-test. $P < 0·05$ * *v.* ST-C, † *v.* DIO-C, ‡ *v.* ST-R.

Table 1. Serum content of TAG and total cholesterol (TC) at the end of treatment in standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R) female rats (*n* 8)‡
(Mean values with their standard errors)

	ST-C		DIO-C		ST-R		DIO-R	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
TAG (mg/dl)	137·47	2·84	152·52*	3·81	126·86†	1·65	142·68	2·53
TC (mg/dl)	142·52	6·26	154·57	4·71	161·44	6·47	159·23	3·35

‡ Data analysed using one-way ANOVA followed by Bonferroni *post-test.* $P < 0·05$ * *v.* ST-C, † *v.* DIO-C.

$P < 0·0001$, Cohen's $d = 0·132$ *v.* ST-C 0·83 (SEM 0·04) g/kJ). In the 15 d subsequent of IFR, only ST-R group (0·41 (SEM 0·02) g/kJ, $P = 0·044$, Cohen's $d = 0·014$) maintained a significantly higher EE than the ST-C (0·25 (SEM 0·03) g/kJ; Fig. 3(b)).

Analysis of serum lipids

Fasted DIO-C rats exhibited significantly higher serum TAG levels than ST-C and ST-R groups ($P < 0·0001$, $\eta^2 = 0·667$; Table 1). The total cholesterol levels were not different between the groups (Table 1).

Increase of adipose tissue after intermittent food restriction

At the end of the treatment, ANOVA revealed a significant effect of diet on WAT depots when evaluating RET ($P < 0·0001$, $\eta^2 = 0·477$), mesenteric ($P < 0·0001$, $\eta^2 = 0·408$), gonadal ($P < 0·0001$, $\eta^2 = 0·509$) and total white visceral fat ($P < 0·0001$, $\eta^2 = 0·529$). DIO-C (2·19 (SEM 0·21) g/100 g) and DIO-R rats (2·88 (SEM 0·12) g/100 g) had a significantly higher content of gonadal fat ($P < 0·0027$ and $P < 0·0001$, respectively), than the ST-C (1·26 (SEM 0·21) g/100 g). As for the total visceral white fat, DIO-C, DIO-R and ST-R groups (4·56 (SEM 0·30) g/100 g, $P < 0·0001$, Cohen's $d = 0·054$; 5·65 (SEM 0·30) g/100 g, $P < 0·0001$, Cohen's $d = 0·089$ and 3·64 (SEM 0·23) g/100 g, $P = 0·0110$, Cohen's $d = 0·025$) showed significantly higher deposits than ST-C (2·87 (SEM 0·31) g/100 g). Furthermore, DIO-R group displayed higher WAT deposits ($P = 0·0006$, Cohen's $d = 0·034$), when compared with its respective control (DIO-C) (Fig. 4(a)).

The DIO diet also had a significant effect on interscapular BAT ($P < 0·0001$, $\eta^2 = 0·735$), since DIO-C and DIO-R (0·13 (SEM 0·005) g/100 g, $P < 0·0001$ and 0·13 (SEM 0·004) g/100 g, $P < 0·0001$) groups exhibited significantly higher BAT amounts, than that observed for ST-C rats (0·08 (SEM 0·005) g/100 g). But, in the ST-R rats, BAT deposits were significantly lower (0·0958 (SEM 0·014) g/100 g, $P < 0·0001$), when compared with the DIO-R group (Fig. 4(b)).

Hyperplasia and hypertrophy of white subcutaneous adipose tissue after intermittent food restriction

The treatment showed significant differences on the size ($P < 0·0001$, $\eta^2 = 0·427$) and number ($P < 0·0001$, $\eta^2 = 0·854$) of the SUB white adipocytes among the groups. Adipocyte hypertrophy in SUB WAT was observed in all groups, when compared with ST-C rats (DIO-C 47·39 (SEM 0·90) μm, $P < 0·0001$; ST-R 48·88 (SEM 1·68) μm, $P < 0·0001$, and DIO-R 67·55 (SEM 0·70) μm, $P < 0·0001$ *v.* ST-C 36·82 (SEM 0·60) μm). But, DIO-R rats displayed higher hypertrophic adipocytes in SUB WAT ($P < 0·0001$), when compared with DIO-C (Fig. 5(b)). Also, adipocyte hyperplasia was observed in the SUB WAT of DIO-R (104·00 (SEM 3·75)) and ST-R groups (32·18 (SEM 3·44)), when compared with their controls ($P < 0·0001$, Cohen's $d = 0·544$).

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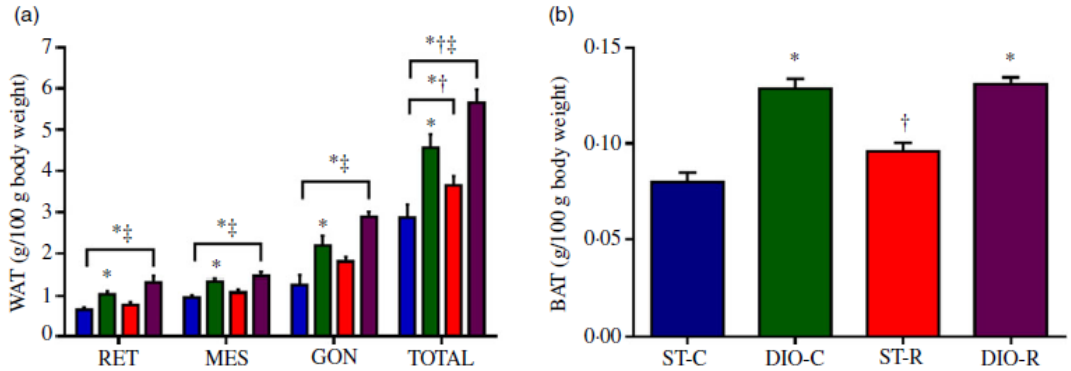

Fig. 4. Increase of adipose tissue after intermittent food restriction (IFR). (a) Mean values with their standard errors (*n* 10) of retroperitoneal (RET), mesenteric (MES), gonadal (GON) white fat pad weights, total white visceral fat stores (TOTAL), and in (b) interscapular brown adipose tissue weight in standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R) female rats at the end of the experimental period. ■, ST-C; ■, DIO-C; ■, ST-R; ■, DIO-R. Data were analysed by one-way ANOVA followed by Bonferroni post-test. $P < 0.05$ * *v.* ST-C, † *v.* DIO-C, ‡ *v.* ST-R.

Notably, adipocyte hyperplasia in this WAT store was 223% higher in DIO-R rats, when compared with the ST-R group ($P < 0.0001$, Cohen's $d = 0.629$; Fig. 5(c)).

Visceral adipose tissue hypoplasia and hypertrophy after intermittent food restriction

At the end of the treatment, significant differences on the size ($P < 0.0001$, $\eta^2 = 0.543$) and number ($P < 0.0001$, $\eta^2 = 0.121$) of adipocytes from the visceral WAT were observed between the groups. ST-R (254·12 (SEM 3·94) μm, $P < 0.0001$, Cohen's $d = 0.29$) and DIO-R groups (276·23 (SEM 9·83) μm, $P < 0.0001$, Cohen's $d = 0.36$) showed adipocyte hypertrophy ($P < 0.0001$, Cohen's $d = 0.30$), when compared with the ST-C (187·59 (SEM 4·14) μm) and DIO-C rats (202·17 (SEM 10·88) μm), respectively (Fig. 5(d)). Hypoplasia was observed only in DIO-R rats (51·71 (SEM 1·45), $P = 0.0133$), when compared with ST-C (67·22 (SEM 3·86)) (Fig. 5(e)).

Intermittent food restriction induces hypertrophy and fibrosis of brown adipose tissue

ANOVA revealed a significant effect of diet on the adipocytes size ($P < 0.0001$, $\eta^2 = 0.616$), lipid inclusions per field analysed ($P < 0.0001$, $\eta^2 = 0.346$) and adipocytes number ($P < 0.0001$, $\eta^2 = 0.671$) of BAT among the groups. BAT histology showed hypertrophy in all groups compared with ST-C (DIO-C 1244·82 (SEM 43·73) μm, $P < 0.0001$; ST-R 1198·29 (SEM 36·64) μm, $P < 0.0001$ and DIO-R 1259·65 (SEM 40·48) μm, $P < 0.0001$ *v.* ST-C 440·01 (SEM 17·69) μm; Fig. 5(f)). On the other hand, the number of lipid inclusions per brown adipocyte was significantly lower in ST-R (6·83 (SEM 0·20), $P < 0.0001$) and DIO-R rats (8·63 (SEM 0·29), $P = 0.0007$), when compared with the ST-C (10·06 (SEM 0·25)). But, DIO-C rats (13·16 (SEM 0·42), $P < 0.0001$) exhibited higher number of lipid inclusions per brown adipocyte (Fig. 5(g)). All groups showed BAT hypoplasia (DIO-C 61·36 (SEM 2·57), $P < 0.0001$; ST-R 59·86 (SEM 1·63), $P < 0.0001$ and DIO-R 59·56 (SEM 3·12), $P < 0.0001$; respectively), when compared with ST-C (172·79 (SEM 18·39); Fig. 5(h)).

The percentage of positive-picrosirius area that indicates collagen fibres deposition in BAT of all rat groups can be observed in Fig. 5(a) and (i). Both, DIO diet and IFR increased BAT fibrosis ($P < 0.0001$, $\eta^2 = 0.655$) in DIO-C (0·60 (SEM 0·02) %, $P < 0.0001$) or in ST-R (0·47 (SEM 0·03) %, $P < 0.0001$) and DIO-R rats (0·61 (SEM 0·03) %, $P < 0.0001$), when compared with ST-C (0·15 (SEM 0·02) %). Notably, collagen deposition in BAT of DIO-C and DIO-R groups was higher than that observed in ST-R (Fig. 5(i)).

Discussion

Here, for the first time, we demonstrated that obesity-inducing diet (DIO) consumption, rich in saturated fat and refined sugar, after an IFR period (IFR), characterising popular cyclical behaviour to get rapid loss of body mass, promoted hyperplasia and hypertrophy of SUB WAT in female rats, as well as brown adipocyte hyperplasia and fibrosis. These adiposity changes appear to be linked to a compensatory increase in energy intake and feed efficiency as a result of periods of IFR. Highlighting that at the contrary of which is expected with the reduction of food intake, herein we observed that IFR did not promote the effective reduction of body mass, and when DIO was provided in rats submitted to IFR, a higher body mass gain and adiposity were induced in DIO-R group, suggesting that IFR changes lipid metabolism at least in adipocytes, causing an economic phenotype which exacerbates body fat storage.

Rodents treated with DIO and submitted to IFR (DIO-R group) showed higher body mass gain at the end of the treatment, which may be due to the fact that in the last 15 d of treatment, a period in which the groups received DIO again (groups DIO-C and DIO-R), there was an increase in weight gain, compared with the corresponding controls (groups ST-C and ST-R). Despite this mass gain, the circulating levels of TAG and total cholesterol were not affected by IFR. These findings indicate that the performance of IFR resulted in damage to the DIO-R group, which was able to compensate for the loss of body mass, throughout the RF cycles, ending the treatment with body mass higher than the ST-C group. Additionally, food restriction, which occurred after the consumption of DIO, resulted in excessive

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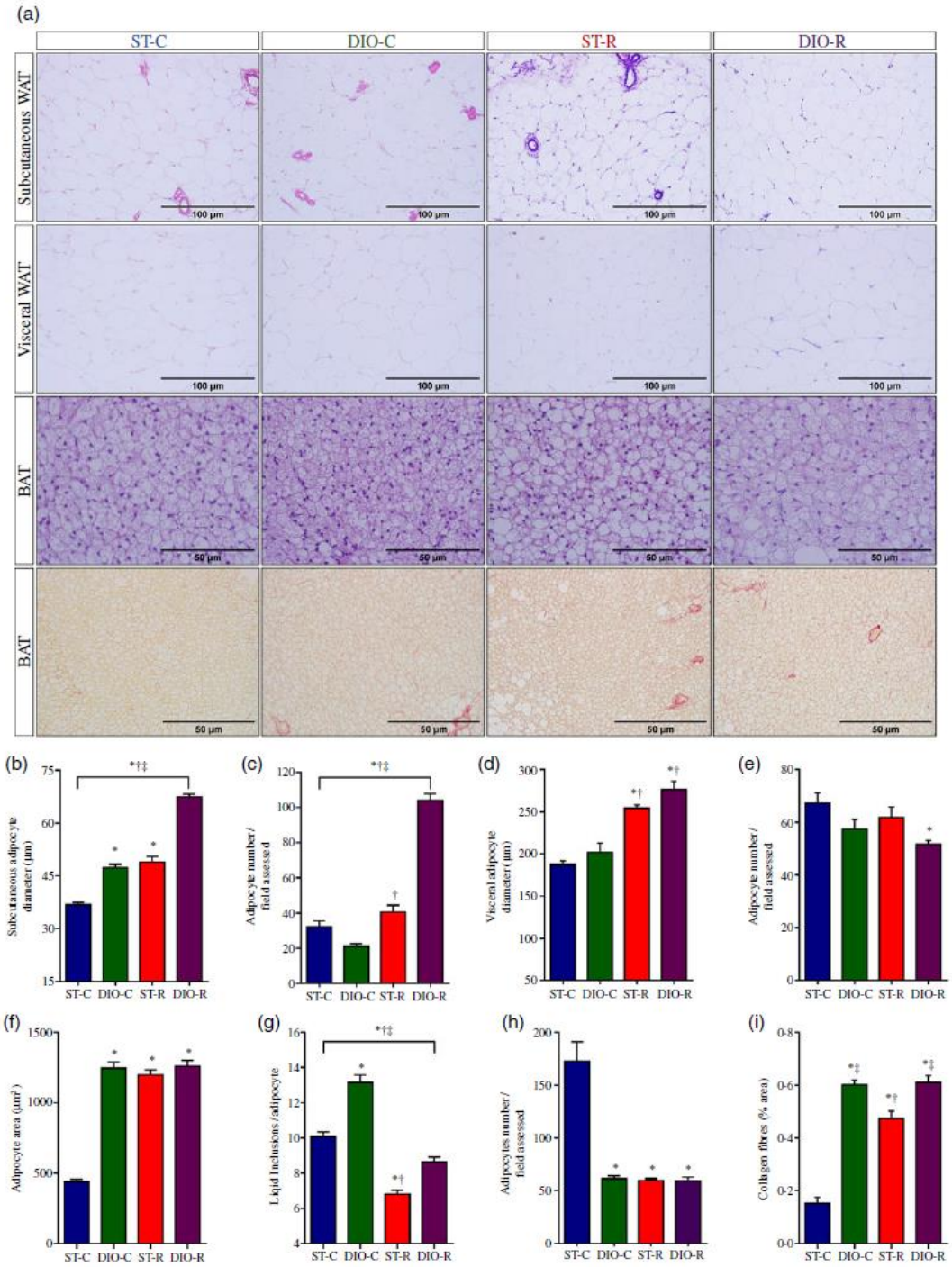

Fig. 5. Morphological alterations of white adipose tissue (WAT) and brown adipose tissue (BAT). (a) Representative images of histological sections (5 µm in thickness) of subcutaneous (thigh region) and visceral (retroperitoneal) WAT (scale bars 100 µm), and interscapular BAT (scale bars 50 µm) stained with haematoxylin and eosin. The lower panel shows representative histological sections of the intrascapular BAT stained with picrosirius red, for collagen deposition measurement in BAT of standard control (ST-C), DIO control (DIO-C), standard restricted (ST-R) and DIO restricted (DIO-R) female rats. Mean values with their standard errors (n 10) of diameter or number of white adipocytes from subcutaneous (b and c, respectively) or visceral fat pads (d and e, respectively), and the areas and number of brown adipocyte (f and g) and the number of lipid inclusions per brown adipocyte (h). Percentage of the picrosirius-positive area in BAT (i) of ST-C, DIO-C, ST-R and DIO-R female rats. Data were analysed by one-way ANOVA followed by Bonferroni post-test. $P < 0.05$ * *v.* ST-C, † *v.* DIO-C, ‡ *v.* ST-R.

energy consumption. Studies carried out in both experimental rodents and humans consider IFR to be an efficient strategy for a loss of body mass and improvement in the markers of metabolic diseases[39,40]. In the present study, the absence of differences in body mass between the ST-R and DIO-R groups, at the end of the treatment, can be partly explained by the energy compensation in the RF periods, which may lead to greater mass regain in the long term.

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The consumption of DIO during the first 15 d of treatment reduced EE. In this sense, it was shown that rats fed exclusively with foods rich in saturated fat showed less EE, increasing the oxidation of fatty acids, in order to disadvantage the accumulation of lipids[41]. Here, during the IFR period, ST-R and DIO-R rats tripled the EE compared with their respective controls, demonstrating the body's effectiveness in recovering the loss of body mass during the IFR periods, a fact that justifies the absence of difference in the final body mass between the groups. However, in the last 15 d of treatment, the ST-R group was the most affected by IFR, since even returning to *ad libitum* consumption of a standard diet, it maintained high EE. As for DIO-R rats, the difference was not significant, but it seems to remain greater during this period. Similarly, but in male mice, Dankel *et al.*[32] also observed an increase in body mass gain and EE in animals that intercalated IFR with a hyperenergetic diet, resulting in greater recruitment of adipocytes in epididymal WAT.

The improvement in EE may also explain the increase in the total white visceral fat deposits of the ST-R and DIO-R rats in relation to their respective controls, allowing greater energy storage in the WAT. In addition, increased deposits of interscapular BAT were identified in the DIO-C and DIO-R groups, indicating that the obesogenic diet negatively affects BAT. The high energy consumption results in higher TAG storage in the visceral and SUB WAT; however, with the maintenance of this dietary pattern, there is a storage of fatty acids in other tissues, characterising ectopic fat[10]. It is recognised that through excessive consumption of fatty acids, WAT adipocytes suffer from hyperplasia and hypertrophy; however, this growth is limited[9]. Thus, despite is not addressed in our study, it is possible that the IFR protocol performed here could also increase fat storage ectopically, which will be considered in future investigations.

Changes in the morphology of SUB WAT of DIO-R rats demonstrated that IFR aggravated the increase in adiposity induced by DIO. The growth dynamics of the tissue was impaired by both hypertrophy and hyperplasia of the cells. These findings diverge from other studies carried out with male mice, which indicated that IFR improves the damage caused by the consumption of an obesogenic diet. A decrease in inguinal and epididymal tissue has been demonstrated due to the transdifferentiation process of white adipocytes, known as browning. This process leads to increased expression of uncoupling protein 1[31,35], which increases thermogenesis in the tissues that store energy and reduces the overload in BAT[42]. However, in our study, ST-R and DIO-R rats showed hypertrophy in visceral WAT, whereas hypoplasia was observed only in DIO-R, which could be the cause of lipolysis of this tissue due to IFR treatment. Studies using experimental rodents and in humans have shown that sex can determine differences in the energy storage, so that in females there is greater hypertrophy and hyperplasia of SUB WAT compared with visceral, as a way of protecting against insulin resistance[36,43]. The difference in fat deposition is mainly due to hormonal differences, such as the oestrogen that favours lipolysis; hormone receptors of the β type present in greater quantity in the abdominal area or of the α type in greater proportion in the gluteus femoral area; enzyme concentrations and increased insulin sensitivity in females[44].

Obesity establishes a profile of adipose tissue hyperplasia, mainly to protect the body from damage caused by the circulation of high amounts of NEFA. When the adipose tissue loses its expansion capacity, becoming more lipolytic, thus, the lipotoxicity process begins, with consequent ectopic adipogenesis[45,46]. Okuno *et al.*[47] showed that adipose tissue presents healthy expansion, featuring protective properties, and that oxidative stress inhibits this expansion, modifying the tissue architecture and exacerbating inflammation. Studies showed that prolonged IFR caused increased stress and elevated serum corticosterone concentration in rats[48], as well as increased net ROS production, leading to oxidative imbalance[49]. In our study, although the content of reactive oxygen and corticosterone species was not assessed, hypertrophy was identified in BAT and WAT, as well as fibrosis in BAT, which could be resulted from the increase in the number of reactive oxygen species.

We also verified hypertrophy of BAT in DIO rats, which may constitute a physiological response aimed at minimising the damage caused by the diet. It was described that palatable diets cause an increase in thermogenesis in BAT, as a mechanism to dissipate excess energy in the form of heat[50]. Kuipers *et al.*[42] showed that 1 d of exposure to a high-fat diet may be enough to promote the first signs of BAT bleaching. In fact, we verified that the BAT of the DIO-C and DIO-R groups presented hypoplasia, while the restricted groups (ST-R and DIO-R) lost the multilocularity characteristic of this tissue. The presence of small lipid vacuoles in BAT increases the area of contact with the lipase, promoting effective lipolysis and consequent thermogenesis[51]. Therefore, the decrease in multilocularity, which was an effect evidenced in both ST-R and DIO-R rats, is an important indicative of loss of the main function of BAT in these rodents, leading the BAT to storage than dissipate energy as heat.

In addition, intense thermogenesis in BAT also causes tissue injury through the depletion of the mitochondrial complex, leading to loss of main function and tissue atrophy[52]. In our study, DIO-C as well as restricted groups (ST-R and DIO-R) exhibited BAT fibrosis, indicative of BAT impaired function. The expansion of WAT causes hypoxia, leading to increased expression of hypoxia-inducible factor 1-alpha, stimulating changes in the extracellular structure, and fibrillar collagen deposition in the tissue[53].

Both hypertrophy in SUB and visceral WAT as well as hypertrophy, hypoplasia and decreased lipid inclusions in BAT observed in the ST-R and DIO-R groups can result from the frequency of restriction cycles. It has been shown that in humans, the 24-h fast increases lipolysis and fatty acid re-esterification by 2·8 and 2·5 times, respectively[54,55]. Repeated restraint and RF cycles lead to an increase in lipolysis during the fasting period, an increase in circulating fatty acids and re-esterification, generating an increase in abdominal and hepatic fat deposits[25,56]. Thus, the increase in EE and compensatory energy consumption in rats may have concomitantly promoted the increase in WAT and BAT.

In summary, IFR, when associated with DIO, results in synergistic effects, favouring long-term gain in body mass, with increased deposits of total visceral fat and BAT content, as well as promoting hypertrophy in WAT and BAT. These results indicate

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that IFR, in female rats, in this experimental design that tried to mimic the human eating behaviour, in the pursuit of weight loss, does not favour effective prolonged body mass loss, since it induces greater energy consumption, increased EE and adiposity effects aggravated by alternating periods of consumption of DIO and IFR.

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The authors declare that there are no conflicts of interest.

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