UNIVERSIDADE FEDERAL DE JUIZ DE FORA
INSTITUTO DE CIÊNCIAS BIOLÓGICAS

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AVALIAÇÃO DO EFEITO DE ÁCIDO LINOLÉICO CONJUGADO PROVENIENTE DE FONTE NATURAL E SINTÉTICA SOBRE A COMPOSIÇÃO CORPORAL, SENSIBILIDADE À INSULINA E PERFIL DE LIPÍDEOS SÉRICOS EM RATOS WISTAR

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Dissertação de Mestrado do Curso de Pós-Graduação em Ciências Biológicas: Área Genética e Biotecnologia, para obtenção do título de Mestre em Ciências Biológicas: Área: Genética e Biotecnologia.

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Juiz de Fora
2015

Ficha catalográfica elaborada através do Programa de geração
automática da Biblioteca Universitária da UFJF,
com os dados fornecidos pelo(a) autor(a)

Almeida, Mariana Macedo de.
AVALIAÇÃO DO EFEITO DE ÁCIDO LINOLÉICO CONJUGADO PROVENIENTE DE FONTE NATURAL E SINTÉTICA SOBRE A COMPOSIÇÃO CORPORAL, SENSIBILIDADE À INSULINA E PERFIL DE LIPÍDEOS SÉRICOS EM RATOS WISTAR / Mariana Macedo de Almeida. -- 2015.
74 f. : il.

Orientador: Raúl Marcel González Garcia
Coorientador: Fernando César Ferraz Lopes
Dissertação (mestrado acadêmico) - Universidade Federal de Juiz de Fora, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Ciências Biológicas: Imunologia e Genética, 2015.

1. Ácido Linoléico Conjugado . 2. CLA cis-9, trans-11. 3. CLA trans-10, cis-12. 4. Diabetes tipo 2. 5. Obesidade. I. Garcia, Raúl Marcel González, orient. II. Lopes, Fernando César Ferraz , coorient. III. Título.

MARIANA MACEDO DE ALMEIDA

Avaliação do efeito de ácido linoleico conjugado proveniente de fonte natural e sintética sobre a composição corporal, sensibilidade à insulina e perfil de lipídeos séricos em ratos Wistar

Dissertação de Mestrado submetida à banca examinadora do Curso de Pós-Graduação em Ciências Biológicas – Ênfase em Genética e Biotecnologia, da Universidade Federal de Juiz de Fora, como parte dos requisitos necessários para obtenção do Grau de Mestre em Ciências Biológicas.

Aprovada em: 04 / 03 / 15.

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Aos meus pais

AGRADECIMENTOS

Agradeço à Embrapa Gado de Leite pelo apoio e incentivo à pesquisa. Ao CNPq, FAPEMIG e UFJF pelo auxílio financeiro que tornou possível a realização deste trabalho. À CAPES pela concessão de bolsa de pós-graduação. Aos meu pais, Suzana e Douglas, pelo amor, eterna dedicação e confiança. A minha irmã, Carolina, pela inspiração que me motiva. Ao meu namorado, Lucas, pelo carinho e apoio incondicional. A toda a minha família por sempre estar ao meu lado e ser meu porto seguro. Ao meu orientador Raúl Marcel Garcia, pela oportunidade, confiança e orientação. Ao meu co-orientador, Fernando César Lopes, e ao pesquisador da Embrapa Gado de Leite, Marco Antônio da Gama, pelas oportunidades proporcionadas, por tornar viável a realização deste trabalho e pela orientação. Aos colegas do Laboratório de Biologia Celular e Molecular, por toda ajuda prestada, aprendizado e momentos de alegria. Aos meus amigos, com quem sempre pude contar e dividir as dificuldades e felicidades vivenciadas durante este trabalho.

RESUMO

A prevalência de sobrepeso e obesidade está crescendo em taxa alarmante no mundo. Essas condições fornecem maior risco para doenças crônicas, incluindo diabetes tipo 2. Nesse contexto, o ácido linoléico conjugado (CLA) tem atraído considerável atenção, principalmente devido às suas propriedades antiobesidade e antidiabética. O presente estudo objetivou avaliar de forma independente os efeitos do CLA proveniente de fontes natural e sintética sobre o risco de obesidade, sensibilidade à insulina e perfil de lipídeos séricos. Cinquenta ratos Wistar machos foram atribuídos aos seguintes tratamentos dietéticos (n=10/ grupo), por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga padrão e 2,3% de OS; Manteiga enriquecida com CLA (M-CLA): dieta contendo 21,7% de manteiga enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS; Controle Hiperlipídico (CH): dieta contendo 24,0% de OS e CLA Sintético (S-CLA): dieta contendo 1,5% de mistura de isômeros de CLA (Luta-CLA 60) e 22,5% de OS. Os animais alimentados com M-CLA tiveram menores níveis de insulina em jejum do que aqueles alimentados com dieta MC, enquando os níveis de PPARγ no tecido adiposo foram aumentados no grupo M-CLA comparado com o grupo MC. Além disso, R-QUICK foi menor nos animais alimentados com MC do que naqueles alimentados com CN, enquanto nenhuma diferença no R-QUICK foi observada entre os grupos CN e M-CLA. Os níveis de colesterol HDL e triglicérides foram maiores nos ratos alimentados com CLA-M. Portanto, a manteiga alto CLA preveniu a hiperinsulinemia, aumentou o colesterol HDL e causou hipertrigliceridemia. A dieta S-CLA não teve efeito sobre a ingestão alimentar e composição alimentar. Os ratos alimentados com S-CLA apresentaram menores níveis da proteína PPARγ no tecido adiposo, hiperinsulinemia, hiperglicemia, índice de HOMA aumentado e maiores níveis de colesterol HDL. Portanto, cautela deve ser tomada antes que suplemetos sintéticos contendo CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sejam recomendados.

Palavras-chave: Ácido Linoléico Conjugado (CLA); CLA *cis*-9, *trans*-11; CLA *trans*-10, *cis*-12; CLA *trans*-10, *cis*-12; Dislipidemia; Manteiga; Obesidade; Rato; Sensibilidade à insulina.

ABSTRACT

The prevalence of overweight and obesity is increasing at an alarming rate. These conditions are a major risk for chronic diseases, including type 2 diabetes. In this context, conjugated linoleic acid (CLA) has attracted considerable attention, mainly due to its anti-obesity and anti-diabetic properties. The present study aimed to independently evaluate the effects of CLA from natural and synthetic sources on obesity risk, insulin sensibility and profile of serum lipids. Fifty male Wistar rats were assigned to the following dietary treatments (n=10/group), for 60 days: Normolipidic Control (CN): diet containing 4.0% soybean oil (SO); Control Butter (MC): diet containing 21.7% standard butter and 2.3% SO; and CLA enriched Butter (M-CLA): diet containing 21.7% *cis*-9, *trans*-11 CLA-enriched butter and 2.3% SO; Hyperlipidic Control (CH), diet containing 24.0% SO; Synthetic CLA (S-CLA), diet containing 1.5% of an isomeric CLA mixture (Luta-CLA 60) and 22.5% SO. M-CLA-fed rats had lower serum insulin levels at fasting than those fed with the MC diet, while the PPARγ protein levels in adipose tissue was increased in M-CLA-fed rats compared to MC-fed rats. Furthermore, R-QUICK was lower in MC than in CN group, while no differences in R-QUICK were observed among CN and M-CLA. Serum HDL cholesterol and triacylglycerol levels were higher in M-CLA-fed rat. Thus, feeding rats on a high-fat diet containing butter naturally enriched in cis-9, trans-11 CLA prevented hyperinsulinemia, increased HDL cholesterol levels and caused hypertriglyceridemia. The S-CLA diet had no effect on dietary intake and body composition. S-CLA-fed rats had lower levels of PPARγ protein in adipose tissue, hyperinsulinemia, hyperglycemia, increased HOMA index and higher levels of serum HDL cholesterol. Therefore, caution should be taken before synthetic supplements containing *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are recommended.

Keywords: Butter; Conjugated linoleic acid (CLA); *Cis*-9, *trans*-11 CLA; Diabetes; Dyslipidemia; Insulin sensitivity; Obesity; Rat; *Trans*-10, *cis*-12 CLA.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

CLA	Ácido Linoléico Conjugado
WHO	*World Health Organization*
EPM	Erro Padrão da Média
ICB	Instituto de Ciências Biológicas
CBR	Centro da Biologia da Reprodução
PPARγ	Receptores Ativados por Proliferadores de Peroxissoma gama
HDL	*High density lipoprotein*
LDL	*Low density protein*
NEFA	Ácido Graxos não Esterificados
HOMA	*Homeostatic Model Assessment*
R-QUICKI	*Revised Quantitative Insulin Sensitivity Check Index*
TOTG	Teste Oral de Tolerência à Glicose
AGS	Ácidos Graxos Saturados
VLDL	*Very low density lipoprotein*
CN	Controle Normolipídico
MC	Manteiga Controle
M-CLA	Manteiga Alto CLA
CH	Controle Hiperlipídico
S-CLA	CLA Sintético

SUMÁRIO

1 INTRODUÇÃO

1.1 Panorama Geral

A prevalência do sobrepeso e obesidade está aumentando em taxas alarmantes pelo mundo (KELLY et al., 2008). De acordo com a *World Health Organization* (WHO, 2014a), historicamente considerados problemas apenas em países de alta renda, sobrepeso e obesidade agora estão dramaticamente em crescimento também em países de baixa e média renda. Em 2008 mais de meio bilhão de adultos estavam obesos no mundo (WHO, 2014b) e é projetado que este número aumente para 1,12 bilhão em 2030 (KELLY et al., 2008). Além disso, sobrepeso e obesidade são os maiores fatores de risco para várias doenças crônicas, como o diabetes mellitus tipo 2, cuja epidemia global está estreitamente relacionada com o aumento das taxas de sobrepeso e obesidade (CHEN et al., 2012). Isso ocorre porque o acúmulo de tecido adiposo visceral, característico da condição de sobrepeso e/ou de obesidade, é um fator de risco independente para resistência à insulina (CHEN et al., 2012), inflamação subclínica, dislipidemia e aterosclerose (ALEXOPOULOS et al., 2014).

Diabetes mellitus é uma importante causa de mortalidade e morbidade pelo mundo, com efeitos prejudiciais sobre a expectativa de vida e os custos do sistema de saúde (DANAEI et al., 2011). O diabetes mellitus tipo 2 compreende 90% do total das pessoas com diabetes ao redor do mundo, e é caracterizada pela uso ineficaz da insulina pelo organismo (WHO, 2014c). O número de pessoas com diabetes mellitus mais do que dobrou nas últimas três décadas (DANAEI et al., 2011), e foi previsto que o número aumentará para 439 milhões em 2030 (SHAW et al., 2010). Além disso, a condição de prediabetes, que envolve indivíduos com prejuízo na tolerância à glicose e/ou glicemia em jejum aumentada, também tem aumentado rapidamente pelo mundo (CHEN et al., 2012).

1.2 Biologia do Tecido Adiposo

A obesidade é caracterizada pelo excesso de massa adiposa, que se acumula em consequência do desequilíbrio entre a ingestão e o gasto energético, além da contribuição de outros fatores nutricionais, ambientais e genéticos (ROSS e DESAI, 2014). O acúmulo de tecido adiposo visceral é o principal fator de risco para o desenvolvimento de componentes da síndrome metabólica como dislipidemia com perfil aterogênico (hipertrigliceridemia e redução de colesterol HDL), intolerância à glicose, resistência à insulina, hipertensão e doenças cardiovasculares. Obesos frequentemente exibem estados pró-trombótico e pró-inflamatório exacerbados, o que contribui muito para a piora nos quadros de resistância à insulina e desenvolvimento de diabetes tipo 2, bem como para doenças cardiovasculares (AHIMA, 2011).

O desenvolvimento destas co-morbidades associadas à obesidade se deve ao fato de que o tecido adiposo branco é um importante órgão endócrino e alterações em hormônios derivados de adipócitos estão associadas a diversas patologias. Entre as principais substâncias secretadas por adipócitos (adipocinas), encontram-se a leptina e a adiponectina, as quais exercem os mais variados efeitos biológicos (AHIMA e LAZAR, 2008).

A leptina é um hormônio peptídico e sua concentração sérica está diretamente relacionada ao índice de massa corporal (IMC), mais precisamente ao conteúdo total de gordura de um indivíduo (LUSTIG, 2004). Ela atua principalmente sobre os sistemas de neuropeptídios hipotalâmicos responsáveis pelo controle do apetite e do gasto energético. A leptina inibe a produção de peptídeos orexigênicos (NPY, AgRP, orexinas) e estimula a de peptídeos anorexigênicos (alfa-MSH, CART), resultando em efeito anorexigênico, além de aumentar a taxa metabólica basal por diferentes mecanismos. As ações da leptina são mediadas pela ligação ao seu receptor de forma longa, OBRb, com subsequente ativação da via JAK2-STAT3 (AHIMA E OSEI, 2004; SAHU, 2003).

A adiponectina é uma proteína exclusivamente expressa pelo tecido adiposo e apresenta efeitos antidiabéticos. A redução da concentração dessa proteína está relacionada com resistência insulínica. Níveis plasmáticos de adiponectina têm sido reportados como diminuídos em humanos obesos, particularmente naqueles com

obesidade visceral, e correlacionam inversamente com a resistância à insulina (KADOWA et al., 2006).

O aumento da massa adiposo ocorrido na obesidade é determinado pelo aumento do tamanho do adipócito (hipertrofia) e/ou do seu número (hiperplasia). Modificações no tamanho (diâmetro e volume) de adipócitos maduros ocorrem via processos de lipogênese e lipólise. Já as modificações no número de adipócitos dependem da diferenciação dos pré-adipócitos em adipócitos, processo denomidado adipogênese (QUEIROZ et al., 2009).

O receptor gama ativado por proliferadores de peroxissomas (PPARγ) é um dos reguladores centrais da adipogênese. Entretanto, interconectado ao seu papel na diferenciação de adipócitos, o PPARγ regula a sensibilidade à insulina ativando transcricionalmente genes envolvidos na sinalização da insulina, absorção de glicose, e absorção e estoque de ácidos graxos (BROWN e MCINTOSH, 2003). Tiazolidinedionas (TZDs) são agentes terapêuticos largamente empregados para o tratamento da diabetes tipo 2. TZDs têm sido propostos na melhora da resistência à insulina, através da ligação e ativação do PPARγ no tecido adiposo, promovendo, assim, diferenciação de adipócitos e aumento do número de adipócitos pequenos que são mais sensíveis à insulina (KADOWA et al., 2006). Os agonistas de PPARγ da classe das tiazolidinedionas, também podem melhorar a sensibilidade à insulina aumentando os níveis de adiponectina (KADOWA et al., 2006).

1.3 Ácido Linoléico Conjugado

Existem evidências convincentes de que a dieta desenvolve importante papel na prevenção de várias doenças não transmissíveis, incluindo a obesidade e a diabetes mellitus tipo 2 (KAUR, 2014). Nesse contexto, o ácido linoléico conjugado (*Conjugated Linoleic Acid* – CLA) tem atraído considerável atenção na comunidade científica devido às suas propriedades promotoras da saúde que foram relatadas em numerosos estudos *in vitro* e com animais (BHATTACHARYA et al., 2006). O termo CLA refere-se a um grupo de isômeros posicionais e geométricos do ácido linoléico (C18:2 *cis*-9, *cis*-12) (WANG e JONES, 2004), cujas duas duplas ligações estão conjugadas (separadas por uma ligação simples). Isômeros de CLA são

predominantemente encontrados na fração lipídica de carne, leite e outros produtos derivados de ruminantes (LAWSON et al., 2001). Embora aproximadamente vinte isômeros já tenham sido identificados em produtos de ruminantes (ROACH et al., 2002), o CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 são os mais biologicamente ativos (KENNEDY et al., 2010) (Figura 1) e os principais isômeros de CLA encontrados em preparações comerciais (HALADE et al., 2010).

A atividade biológica do CLA foi descoberta acidentalmente quando investigava-se as propriedades carcinogênicas da carne bovina (PARIZA et al., 1979). Porém, ao contrário do esperado, este estudo demonstrou que os ácidos graxos presentes na carne apresentavam atividade anticarcinogênica, efeito posteriormente atribuído ao CLA componente da fração lipídica (HA et al., 1987). Desde então, diversos estudos com modelos animais e culturas de células derivadas de humanos e animais têm relatado uma série de efeitos benéficos do ácido linoléico conjugado, tais como: redução da deposição de gordura, aumento do ganho de massa magra, redução dos efeitos negativos de dietas que induzem perda de peso, redução da hipertensão, propriedade antidiabetogênica, efeito anticarcinogênico e antiaterogênico, melhora da função imune, redução da inflamação, redução de efeitos catabólicos da resposta imune e redução de asma em modelos animais, aumento do crescimento de roedores jovens (COOK et al., 1993; MILLER et al., 1994; PARIZA et al., 2001; WHIGHAM et al., 2001; BASSAGANYA-RIERA et al., 2002; NOONE et al., 2002; WHIGHAM et al., 2002; ALBERS et al., 2003; BASSAGANYA-RIERA et al., 2003; BELURY, 2003; HENRIKSEN et al., 2003; KRITCHEVSKY, 2003; NAGAO et al., 2003).

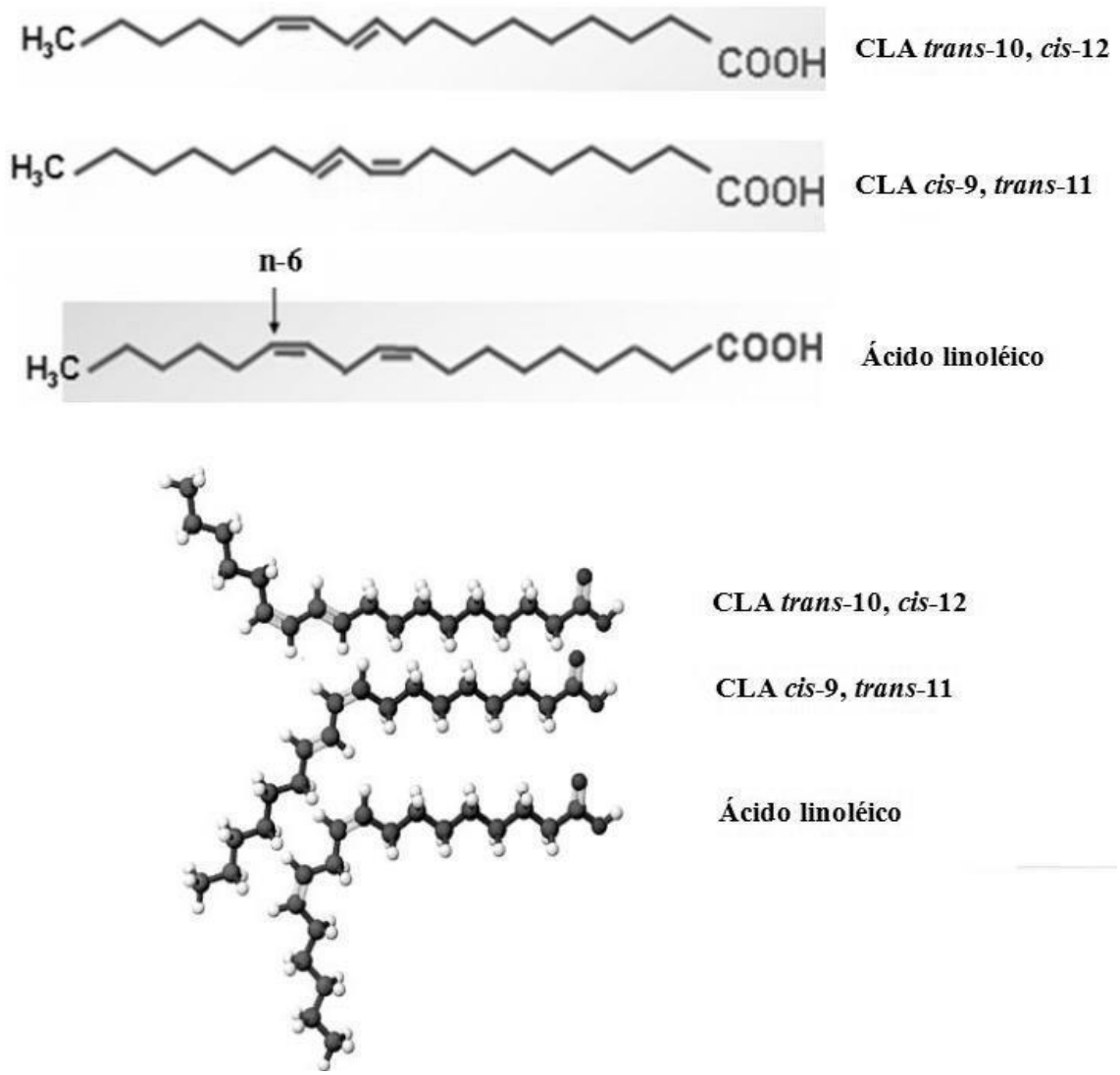

Figura 1. Representações das estruturas químicas do ácido linoléico e de isômeros de ácido linoléico conjugado (CLA) ativos, o CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12.
Fontes: BENJAMIN e SPENER (2009); Adaptado de PARIZA (2004).

1.4 Produção natural e sintética de CLA

O ácido linoléico conjugado é naturalmente produzido no trato gastrointestinal de ruminantes através do metabolismo e biohidrogenação de ácidos graxos poli-insaturados de 18 carbonos, monoinsaturados e poli-insaturados de cadeia longa, o que resulta não apenas na produção de ácido esteárico (C18:0), mas também de uma vasta gama de intermediários de biohidrogenação ruminal, especialmente ácidos graxos *trans* e conjugados (CHILLIARD et al., 2007). Além disso, alguns

intermediários da biohidrogenação ruminal são transformados pelos tecidos corporais do ruminante, especialmente pela glândula mamária, onde a enzima delta-9 dessaturase atua através da adição de dupla ligação *cis*-9 em diferentes ácidos graxos (CHILLIARD et al., 2007). Esta ação da delta-9 dessaturase sobre o ácido vacênico (C18:1 *trans*-11) é responsável pela maior parte da síntese de CLA *cis*-9, *trans*-11 (ácido rumênico) (KAY et al., 2004) (Figura 2). Devido à relação precursor:produto entre o C18:1 *trans*-11 e CLA *cis*-9, *trans*-11, alimentos ricos em ácido rumênico são também ricas fontes de ácido vacênico (LOCK et al., 2005). A síntese endógena de CLA *cis*-9, *trans*-11 a partir de C18:1 *trans*-11 via delta-9 dessaturase também tem sido relatada em humanos (TURPEINEN et al., 2002) e em outras espécies (SANTORA et al., 2000; CORL et al., 2003).

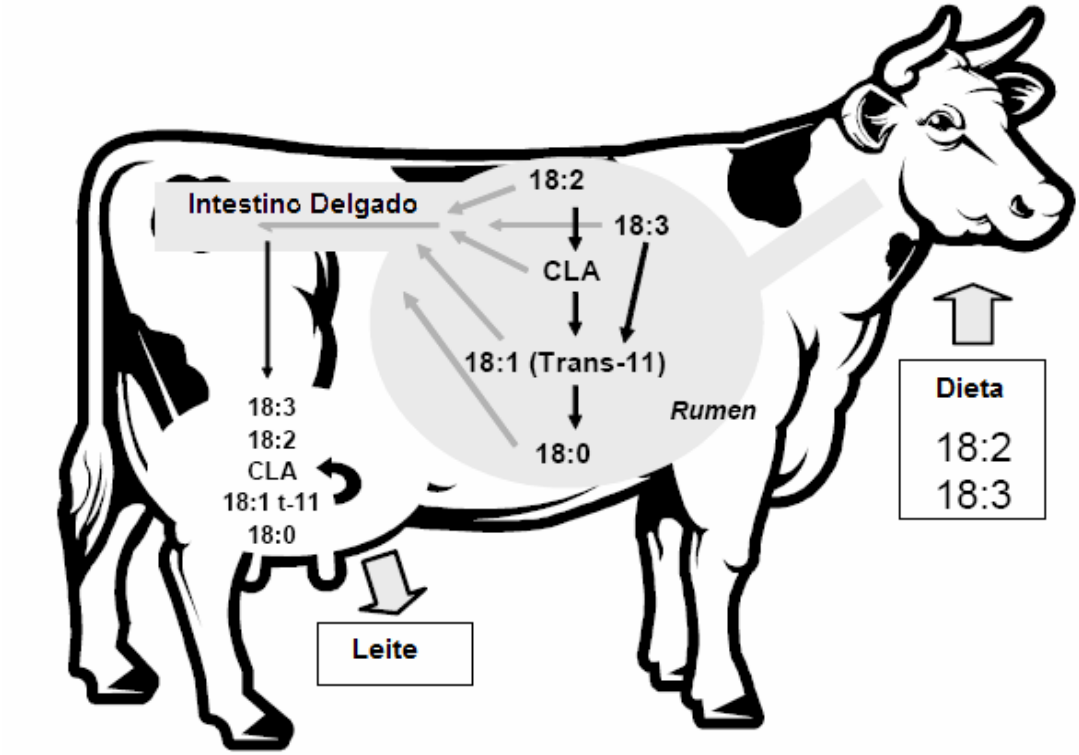

Figura 2. Produção de Ácido Linoléico Conjugado (CLA) no leite de ruminantes. Esta síntese ocorre principalmente a partir de ácido graxos poli-insaturados presentes na dieta do animal
Fonte: Adaptado de KENNELLY e BELL (2007).

Dessa forma, o CLA *cis*-9, *trans*-11 é o isômero de ácido linoléico conjugado mais abundante encontrado na gordura de produtos derivados de ruminantes (75% a 90% do CLA total) (BAUMAN et al., 2003) (Tabela 1), enquanto o isômero CLA *trans*-10, *cis*-12 é encontrado em pequenas concentrações em fontes naturais (BAUMAN et al., 2000; EULITZ et al., 1999). Entretanto, o incremento do teor desse último

isômero pode ser obtido quando dietas com baixo teor de fibra e elevado teor de lipídeos poli-insaturados são fornecidas aos animais (PIPEROVA et al., 2000). Da mesma forma, aumentos significativos nas concentrações de CLA *cis*-9, *trans*-11 e do seu precursor, ácido vacênico (C18:1 *trans*-11), podem ser obtidos pela manipulação da dieta de ruminantes (BAUMAN et al., 2000). Assim, a composição de ácidos graxos e, consequentemente, de CLA varia amplamente em função da dieta fornecida ao ruminante (JURJANZ et al., 2004; LOOR et al., 2005; ROY et al., 2006). Incrementos significativos na concentração de CLA do leite podem ser obtidos pelo fornecimento de forragens frescas e/ou suplementação da dieta com óleos vegetais (COLLOMB et al., 2006; DEWHURST et al., 2006; CHILLIARD et al., 2007) (Tabela 2).

Tabela 1. Percentual de CLA e de CLA *cis*-9, *trans*-11 em carne, leite e derivados.

Alimentos	**Total de CLA (% de gordura)**	**CLA *cis*-9, *trans*-11 (% do total de CLA)**
Leite UHT	0,80	-
Leite homogeneizado	0,55	92
Produtos Lácteos		
Leite Condensado	0,63-0,70	82
Queijo Cheddar	0,40-0,53	78-82
Queijo Cottage	0,45-0,59	83
Queijo Mussarela	0,34-0,50	78-95
Manteiga	0,47-0,94	78-88
Iogurte integral	0,38-0,88	83-84
Iogurte *light*	0,44	86
Sorvete	0,36-0,50	76-86
Carnes		
Carne bovina moída	0,16-0,43	72-85
Carne de vitelo	0,27	84
Carne suína	0,06-0,13	25-82
Carne de frango	0,09-0,15	67-84
Carne de peru	0,20-0,25	40-76

Fonte: Adaptado de DHIMAN et al. (2005)

As preparações comerciais de ácido linoléico conjugado são constituídas, principalmente, de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 (HALADE et al., 2010). Elas são produzidas a partir do ácido linoléico proveniente de óleo de açafrão ou de girassol em condição alcalina (KENNEDY et al., 2010). O uso do CLA sintético permitiu a avaliação de efeitos isômero específicos (HALADE et al., 2010), uma vez que evidências indicam que vários efeitos biológicos do CLA são devido à ação isolada do CLA *cis*-9, *trans*-11 e do CLA *trans*-10, *cis*-12, enquanto outros podem ser induzidos e/ou melhorados pela atuação sinérgica desses dois isômeros (PARIZA et al., 2001). A mistura sintética de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 tem sido mostrada como tendo propriedades anti obesidade (WANG e JONES, 2004; WHIGHAM et al., 2007), como diminuição de massa gorda, aumento de massa magra (HALADE et al., 2010) e redução do consumo energético (KENNEDY et al., 2010). Suplementos sintéticos de CLA contendo mistura de CLA *cis*-9, *trans*-11 e *trans*-10, *cis*-12 na razão de 50:50 (v:v) já têm sido comercializados para redução de massa gorda (FDA, 2008). Entretanto a segurança dessa mistura de isômeros de CLA para auxiliar na perda de peso não é unanimidade na comunidade científica e seu uso não é autorizado em alguns países, como, por exemplo, Brasil e Austrália (ANVISA, 2007; FSANZ, 2008).

Tabela 2. Fatores da dieta que afetam a concentração de CLA na gordura do leite de ruminates.

Fatores da dieta	**Total de CLA (% de gordura)**
Modo de apresentação da forragem	
Pasto	0,59-2,21
Silagem	0,34-0,86
Feno	0,79
Inclusão de óleo vegetal na dieta[1]	
Óleo de soja (3-4%)	0,71-2,13
Óleo de linhaça (4,4-5,3%)	1,67-1,70
Óleo de girassol (5,3%)	2,44
Óleo de canola (3-3,3%)	0,51-1,10

[1]Valores entre parênteses referem-se à porcentagem de inclusão do óleo vegetal na dieta (% da matéria seca total). Adaptado de DHIMAN et al. (2005).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar em ratos Wistar machos adultos os efeitos da suplementação com ácido linoléico conjugado de origem natural e sintética sobre fatores de risco associados à obesidade e diabetes mellitus tipo 2.

2.2 OBJETIVOS ESPECÍFICOS

Avaliar em ratos Wistar machos adultos o efeito do CLA proveniente de fontes natural e sintética sobre:

- Consumo alimentar e composição corporal;
- Níveis de Receptores Ativados por Proliferadores de Peroxissoma (PPAR)γ no tecido adiposo retroperitoneal;
- Concentração sérica de insulina, glicose, ácidos graxos não esterificados (NEFA), leptina e adiponectina;
- Índices de HOMA e R-QUICKI, que denotam sensibilidade à insulina;
- Teste Oral de Tolerância à Glicose (TOTG);
- Concentração sérica de colesterol total, triglicérides, colesterol LDL e colesterol HDL.

3 MATERIAL E MÉTODOS

3.1 DECLARAÇÃO DE ÉTICA

Os protocolos para uso de animais neste estudo foram aprovados pelo Comitê de Ética em Experimentação Animal da Universidade Federal de Juiz de Fora (UFJF), Minas Gerais, Brasil. Os números das permissões são 054/2012 e 053/2012 para os estudos do ácido linoléico conjugado de fonte natural e sintética, respectivamente. Todos os procedimentos foram realizados de acordo com as recomendações do *Guide for the Care and Use of Laboratory Animals* (NATIONAL RESEARCH COUNCIL, 2011).

3.2 ANIMAIS

Cinquenta ratos Wistar machos (*Rattus norvegicus* BERKENHOUT, 1769), com 60 dias de idade e 250 - 300 g de massa corporal, provenientes do Centro da Biologia da Reprodução (CBR) da UFJF foram usados no estudo. Todos os animais foram acomodados em grupos, em gaiolas no biotério do Laboratório de Biologia Celular e Molecular do Instituto de Ciências Biológicas (ICB). As gaiolas dos animais foram mantidas em ambiente com temperatura controlada (23 ± 2ºC) e fotoperíodo de 12 horas (07h00min às 19h00min - claro e 19h00min às 07h00min - escuro) (Figura 3). Água e ração foram ofertadas *ad libitum* aos animais durante todo o período experimental.

3.3 DELINEAMENTO EXPERIMENTAL

A avaliação do ácido linoléico conjugado de origem natural e sintética foi

realizada em dois experimentos independentes, porém conduzidos simultaneamente. Para avaliação do CLA de origem natural, um grupo de ratos recebeu dieta hiperlipídica com 24% de gordura, sendo 21,7% na forma de manteiga naturalmente enriquecida com CLA (n=10), e outro grupo recebeu dieta hiperlipídica com 24% de gordura, sendo 21,7% na forma de manteiga sem enriquecimento (n=10). Para avaliação do ácido linoléico conjugado de origem sintética, um grupo recebeu dieta hiperlipídica com 24% de gordura, sendo 1,5% na forma de isômeros sintéticos de CLA e 22,5% na forma de óleo de soja (n=10), e outro grupo recebeu dieta hiperlipídica com 24% de gordura na forma de óleo de soja (n=10).

Figura 3. Câmara com condições controladas onde as gaiolas dos animais de experimentação foram mantidas durante os tratamentos dietéticos. Fonte: Arquivo pessoal.

De forma simultânea à condução dos grupos dietéticos anteriormente citados, um grupo experimental recebeu dieta normolipídica com 4% de gordura na forma de óleo de soja (n=10). Esse último grupo experimental foi utilizado tanto na avaliação do CLA de origem natural como sintética. Dessa forma, para a investigação do ácido linoléico conjugado oriundo de fonte natural, foram utilizadas comparações entre os grupos de animais que receberam a dieta normolipídica, aquela com manteiga

naturalmente enriquecida com CLA ou aquela contendo manteiga controle. Para a investigação do ácido linoléico conjugado de origem sintética, foram utilizadas comparações entre os grupos que receberam a dieta normolipídica, aquela com CLA sintético ou aquela hiperlipídica com óleo de soja.

3.4 ÁCIDO LINOLÉICO CONJUGADO DE FONTE NATURAL

3.4.1 FABRICAÇÃO DAS MANTEIGAS EXPERIMENTAIS

As manteigas experimentais utilizadas no estudo foram produzidas na Embrapa Gado de Leite (Juiz de Fora, Minas Gerais, Brasil). Manteiga controle e manteiga enriquecida com CLA *cis*-9, *trans*-11 foram produzidas a partir do leite de vacas (Holandês x Gir) alimentadas com dietas compostas de silagem de milho e concentrado sem óleo de girassol, ou capim elefante picado e concentrado contendo óleo de girassol na concentração de 4,5% da matéria seca da dieta, respectivamente. As manteigas foram produzidas como descrito por ORTIZ-GONZALEZ et al. (2007). As principais etapas de produção das manteigas estão representadas em forma de esquema na figura 4.

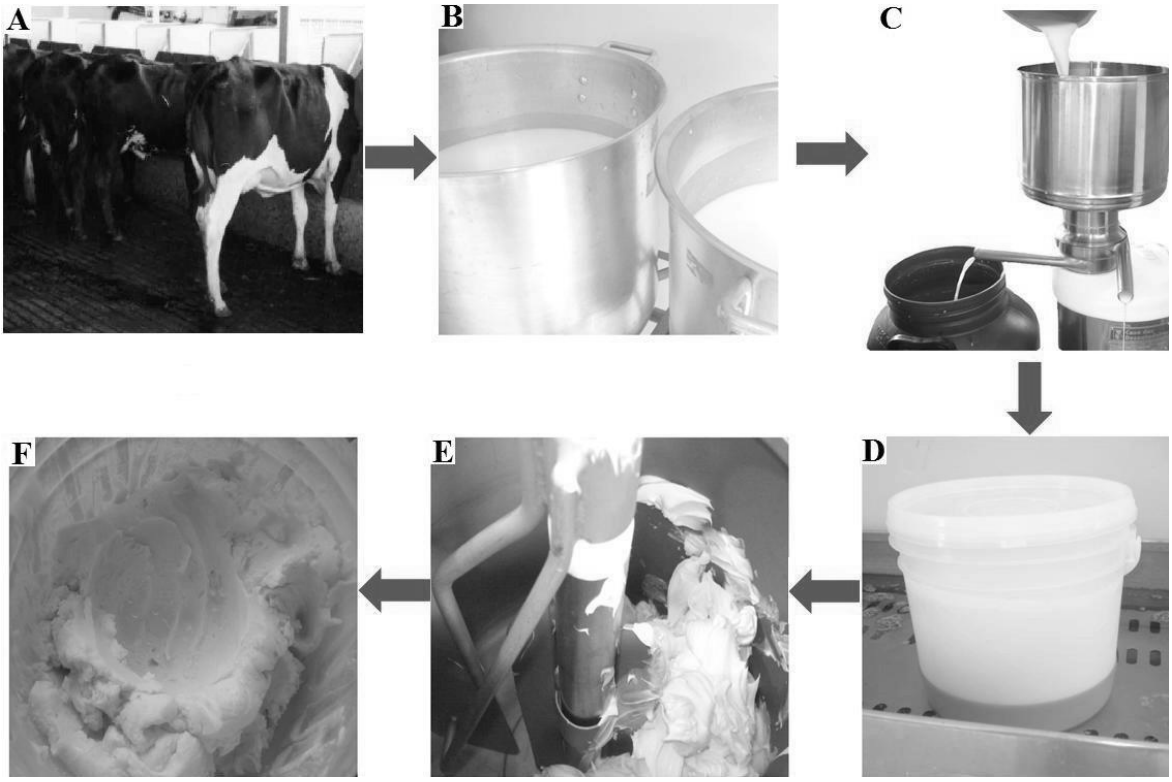

Figura 4. Principais etapas da produção das manteigas experimentais. Vacas mestiças recebendo as dietas para produção de leite enriquecido ou não com CLA (**A**). Imediatamente após a ordenha, o leite foi aquecido a 45 a 50°C (**B**) e, em seguida, processado em desnatadeira elétrica (modelo 18GR, Casa das Desnatadeiras, Goiânia, GO) para separação do creme (**C**) que foi posteriormente submetido a processo de pasteurização lenta, a 65 a 68°C, durante 30 minutos (**D**). Homogeneização do creme (**E**), e posterior processo de malaxagem, no qual as manteigas foram lavadas com água fria e, depois de retirado o excesso de água (**F**), acondicionadas em potes plásticos previamente identificados, que foram adequadamente vedados para prevenir entrada de ar e armazenados sob temperatura de -20°C. Fonte: Arquivo Pessoal

3.4.2 TRATAMENTOS DIETÉTICOS

Depois de sete dias de período de aclimatação, no qual os animais receberam ração comercial (Nuvital, Colombo, PR, Brasil), os ratos foram aleatoriamente distribuídos às dietas experimentais (n=10/grupo), por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e Manteiga alto CLA *cis*-9, *trans*-11 (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS (Tabela 3).

Tabela 3. Composição lipídica das dietas experimentais

	Grupos Experimentais (n=10/grupo)		
	CN (**C**ontrole **N**ormolipídico)	**MC** (**M**anteiga **C**ontrole)	**M-CLA** (**M**anteiga Alto **CLA**)
Óleo de Soja	4%	2,3%	2,3%
Manteiga Controle	------	21,7%	------
Manteiga Alto CLA *cis*-9 *trans*-11	------	------	21.7%

OS foi incluído em ambas dietas MC e M-CLA para atingir as necessidades dos ácidos linoléico e linolênico para ratos adultos (REEVES et al., 1993). Todas as dietas foram produzidas de acordo com o *American Institute of Nutrition* (AIN-93M) (REEVES et al., 1993). Os ingredientes foram cuidadosamente misturados para obtenção de uma massa homogênea que foi usada para produção de péletes produzidos manualmente. Os péletes foram preparados semanalmente, evaporados com nitrogênio e estocados a -20ºC em porções diárias em potes de polietileno, visando minimizar a oxidação de ácidos graxos. Os péletes e a forma de armazenamento estão demonstrados na figura 5. A composição das dietas purificadas está apresentada na tabela 4.

Figura 5. Representação dos péletes experimentais produzidos manualmente e seu acondicionamento em potes de polietileno. Fonte: Arquivo pessoal.

Tabela 4. Composição centesimal das dietas experimentais

Ingrediente	% da dieta
Amido de milho[1]	46,6 ou 29,1[a,b,c]
Amido de milho dextrinizado[1]	15,5
Caseína[2]	14,0 ou 17,3[a,b,c]
Sacarose[2]	10,0
Celulose[1]	5,0
AIN-93 mineral mix[2]	3,5
AIN-93 vitamina mix[2]	1,0
L-Cistina[1]	0,18
Bitartarato de colina[1]	0,25
Terc-Butilhidroquinona[2]	0,01
OS[3] ou Manteiga[4]+OS[a,b,c]	4,0 ou 24,0[a,b,c]

[1,2]Ingredientes das dietas experimentais foram adquiridos da Rhoster (Araçoiaba da Serra, SP, Brasil) e Farmos (Rio de Janeiro, RJ, Brazil); [3]Óleo de soja (OS); [4]Manteiga Controle ou Manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11. [a]Dieta Controle Normolipídico consistiu de 46,6% de amido de milho, 14,0% de caseína e 4,0% de OS; [b]Dieta Manteiga Controle consistiu de 29,1% de amido de milho, 17,3% de caseína e 21,7% de Manteiga Controle + 2,3% OS; [c]Dieta Manteiga Alto CLA *cis*-9, *trans*-11 consistiu de 29,1% de amido de milho, 17,3% de caseína e 21,7% Manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 + 2,3% OS.

Amostras de péletes (50 g) de cada dieta foram aleatoriamente coletadas e analisadas no Laboratório de Análise de Alimentos da Embrapa Gado de Leite (Juiz de Fora) para composição química, de acordo com métodos de referência (GOERING e VAN SOEST, 1970; AOAC, 2000). A determinação do perfil de ácidos graxos das dietas experimentais foi realizada no Laboratório de Cromatografia da Embrapa Gado de Leite (Juiz de Fora). Os lipídeos totais foram extraídos como descrito por HARA e RADIN (1978) usando mistura 3:2 (vol:vol) de hexano e isopropanol (4,5 mL/g de pélete), seguido por 67 g/L de solução de sulfato de sódio (3 mL/g de pélete). Ésteres metílicos de ácidos graxos (FAME) foram obtidos por transmetilação por catálise básica, usando reagente de metilação (1 g de ácido oxálico em 30 mL de éter dietil), recém preparado e cloreto de cálcio foi adicionado para remoção de resíduos de etanol. Os FAME foram determinados por cromatografia gasosa (cromatógrafo modelo 6890 N; Agilent Technologies Brasil Ltda., Barueri, Brasil) equipado com detector de ionização de chama e com coluna capilar de sílica fundida CP-Sil 88 (100 m × 0,25 mm × 0,2 μm de espessura do filme; Varian Inc., Mississauga, ON). Condições de operação incluíram temperaturas

do injetor e detector ambos a 250°C, H_2 como gás de arraste (1 mL/min), fluxo do detector de ionização de chama a 35 mL/min, N_2, como gás *makeup*, a 30 mL/min, e purificação do ar a 286 mL/min. A temperatura inicial foi de 45°C por 4 min, a taxa de aumento de temperatura foi de 13°C/min até atingir 175°C, que manteve-se por 27 min. A partir de então, o aumento de temperatura foi de 4°C/min até atingir 215°C, que manteve-se por 35 min (CRUZ-HERNANDEZ et al., 2007). Os FAME foram identificados por comparação com quatro padrões de referência de FAME (Supelco37 mix #47885-U, linoleic acid isomers mix #47791, CLA isomers mix #05632; Sigma-Aldrich, St. Louis, MO, e Nu-Chek GLC-463); isômeros *trans*-18:1 secundários foram identificados de acordo com a ordem de eluição relatada sob as mesmas condições cromatográficas (KRAMER et al., 2001; CRUZ-HERNANDEZ et al., 2007). A composição de ácidos graxos dos péletes das dietas CN, MC e M-CLA foi expressa como porcentagem de peso do total de ácidos graxos usando fatores de respostas relativos teóricos descritos por WOLFF et al. (1995) (Tabela 5). A representação dos cromatogramas sobrepostos na região de eluição do CLA dos péletes das dietas MC e M-CLA está demonstrada na figura 6.

Tabela 5. Composição química e perfil de ácidos graxos das dietas experimentais.

	Tratamentos Dietéticos		
	CN[2]	**MC[3]**	**M-CLA[4]**
	Composição química, % de matéria seca da dieta		
Conteúdo de matéria seca (%)	79,1	86,8	85,4
Gordura	3,11	17,6	17,4
Proteína Bruta	13,1	16,0	16,2
Cinza	2,76	2,98	3,09
Fibra em detergente neutro	2,76	3,55	3,26
Carboidrato	55,4	44,8	43,4
	Composição Energética		
Energia de Carboidrato (%)	73,4	44,6	43,9
Energia de Proteína (%)	17,4	15,9	16,4
Energia de Gordura (%)	9,35	39,5	39,6
Kcal/g	2,39	3,49	3,38
	Ácidos Graxos (g/100 g do total de ácidos graxos)		
C4:0	n.d.[1]	3,16	2,95
C5:0	n.d.	0,03	0,01
C6:0	n.d.	1,69	1,37
C7:0	n.d.	0,02	0,01
C8:0	n.d.	1,00	0,64

Tabela 5. Composição química e perfil de ácidos graxos das dietas experimentais. (*Continuação*)

	Tratamentos Dietéticos		
	CN[2]	**MC[3]**	**M-CLA[4]**
	Ácidos Graxos (g/100 g do total de ácidos graxos)		
C9:0	n.d.	0,03	0,01
C10:0	n.d.	2,07	1,14
C10:1 *cis*-9	n.d.	0,26	0,12
C11:0	n.d.	0,02	0,01
C12:0	n.d.	2,37	1,25
C12:1 *cis*-9/C13:0	n.d.	0,16	0,08
C14:0	0,52	8,71	5,74
C15:0 *iso*	n.d.	0,20	0,25
C15:0 *anteiso*	n.d.	0,41	0,47
C14:1 *cis*-9	n.d.	0,83	0,46
C15:0	n.d.	0,95	0,90
C16:0	11,7	29,3	19,7
C16:1 *trans*-9	n.d.	0,03	0,03
C17:0 *isso*	n.d.	0,32	0,51
C16:1 *cis*-9+C17:0 *anteiso*	n.d.	1,51	1,16
C17:0	n.d.	0,49	0,51
C17:1 *cis*-9	n.d.	0,18	0,19
C18:0	4,25	9,02	13,9
C18:1 *trans*-4	n.d.	0,02	0,07
C18:1 *trans*-5	n.d.	0,02	0,06
C18:1 *trans*-6/7/8	n.d.	0,31	0,80
C18:1 *trans*-9	n.d.	0,23	0,57
C18:1 *trans*-10	n.d.	0,30	0,81
C18:1 *trans*-11	n.d.	1.09	4.03
C18:1 *trans*-12	n.d.	0.29	0.65
C18:1 *trans*-13/14	n.d.	0.24	0.49
C18:1 *cis*-9/*trans*-15	23,8	20,3	25,9
Minor *cis*-C18:1 isomers (c11+c12+c13)	1,43	0,83	1,03
C18:1 *trans*-16	n.d.	0,23	0,36
C18:1 *cis*-14	n.d.	0,05	0,10
C19:0/C18:1 *cis*-15	n.d.	0,11	0,11
C18:2 *trans*-9 *trans*-12	n.d.	0,01	0,01
C18:2 *cis*-9 *trans*-12	0,09	0,04	0,06
C18:2 *trans*-9 *cis*-12	n.d.	0,03	0,04
C18:2 *cis*-9 *cis*-12	49,5	8,04	7,15
C20:0	0,36	0,18	0,20
C18:3 *cis*-6, *cis*-9 *cis*-12	n.d.	0,02	0,01
C20:1 *cis*-11	n.d.	0,06	0,12
C18:3 *cis*-9 *cis*-12 *cis*-15	6,16	0,96	0,89
CLA *cis*-9 *trans*-11	n.d.	0,53	1,66
CLA *trans*-10 *cis*-12	n.d.	0,01	0,01

Tabela 5. Composição química e perfil de ácidos graxos das dietas experimentais. (*Continuação*)

	Tratamentos Dietéticos		
	CN[2]	**MC**[3]	**M-CLA**[4]
	Ácidos Graxos (g/100 g do total de ácidos graxos)		
CLA *trans*-11 *cis*-13	n.d.	0,01	0,02
C21:0	n.d.	0,03	0,03
C20:2 *cis*-11, *cis*-14	n.d.	0,02	0,02
C22:0	0,41	0,11	0,13
C20:3 n-6	n.d.	0,05	0,04
C20:4 n-6	n.d.	0,10	0,08
C23:0	n.d.	0,03	0,01
C20:5 n-3 (EPA)	n.d.	0,02	0,01
C24:0	0,15	0,06	0,06
C22:5 n-3 (DPA)	n.d.	0,06	0,06
C22:6 n-3 (DHA)	n.d.	n.d.	n.d.

[1]n.d.: não detectado
[2]Controle Normolipídico, dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC), dieta contendo 21,7% de manteiga controle e 2,3% de OS; [4]Manteiga alto CLA *cis*-9, *trans*-11 (M-CLA), dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS.

O conteúdo de CLA *cis*-9, *trans*-11 nas dietas MC e M-CLA foi calculado como segue: (Conteúdo de matéria seca da dieta) x (Conteúdo de gordura x 0,95) x (Concentração de CLA *cis*-9, *trans*-11 em g/100g do total de ácidos graxos). Os 5% descontados do conteúdo de gordura deve-se à correção devido à concentração de glicerol das moléculas de triglicerídeos (GLASSER et al., 2010). Baseado nos cálculos acima, os conteúdos de CLA *cis*-9, *trans*-11 nas dietas MC e M-CLA foram 0,075% e 0,235%, respectivamente. Entretanto, considerando que, aproximadamente, 11% de ácido vacênico (C18:1 *trans*-11) é convertido de forma endógena em ácido rumênico em roedores (SANTORA et al., 2000), o aumento esperado de CLA *cis*-9, *trans*-11 nos níveis teciduais dos ratos alimentados com CLA-M é aproximadamente de 15% maior do que os níveis teciduais deste isômero em ratos alimentados com MC.

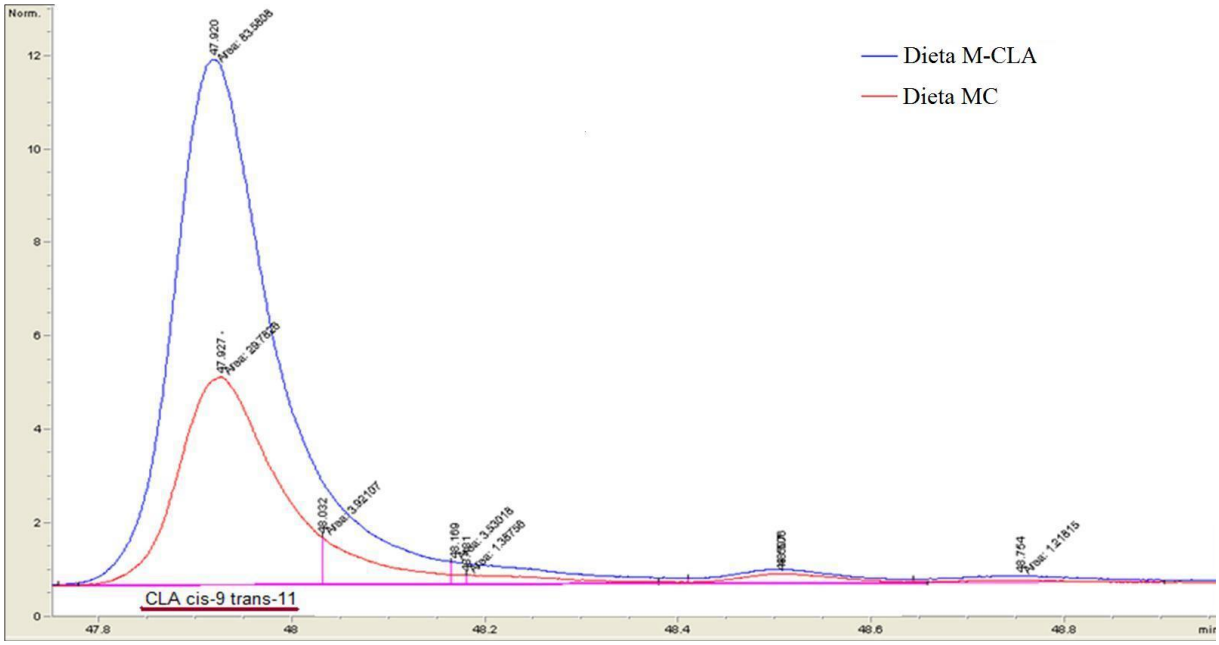

Figura 6. Cromatogramas sobrepostos dos péletes das dietas experimentais MC, contendo 21,7% de manteiga controle e 2,3% de óleo de soja (OS), e M-CLA, contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. É mostrada a região de eluição do Ácido Linoléico Conjugado (CLA), com destaque para o isômero CLA *cis*-9 *trans*-11. Foi utilizado cromatógrafo Agilent Technologies (6890N) equipado com coluna CPSil88 (100m x 0,25mm x 0,2µm) e detector de ionização de chama com condições de operação descritas por CRUZ-HERNANDEZ et al. (2007). Fonte: Arquivo pessoal.

3.5 ÁCIDO LINOLÉICO CONJUGADO DE FONTE SINTÉTICA

3.5.1 TRATAMENTOS DIETÉTICOS

Depois de sete dias de período de aclimatação, no qual os animais receberam ração comercial (Nuvital, Colombo, PR, BR), os ratos foram aleatoriamente distribuídos às dietas experimentais (n=10/grupo), por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Controle Hiperlipídico (CH): dieta contendo 24,0% de OS; CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 e 22,5% de OS. Luta-CLA 60 (BASF AG, São Paulo, Brasil) é composto de 60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 (Tabela 6).

Tabela 6. Composição lipídica das dietas experimentais.

	Grupos Experimentais (n=10/grupo)		
	CN (**C**ontrole **N**ormolipídico)	**CH** (**C**ontrole **H**iperlipídico)	**S-CLA** (**CLA S**intético)
Óleo de Soja	4%	24%	22,5%
Luta-CLA 60	------	------	1,5%

As dietas experimentais foram produzidas e armazenadas conforme descrito no item *3.4.2*. A composição das dietas purificadas está apresentada na tabela 7.

Tabela 7. Composição centesimal das dietas experimentais

Ingrediente	**% da dieta**
Amido de milho[2]	46,6 ou 29,1[a,b,c]
Amido de milho dextrinizado[2]	15,5
Caseína[1]	14,0 ou 17,3[a,b,c]
Sacarose[1]	10,0
Celulose[2]	5,0
AIN-93 mineral mix[1]	3,5
AIN-93 vitamina mix[1]	1,0
L-Cistina[2]	0,18
Bitartarato de colina[2]	0,25
Terc-Butilhidroquinona[1]	0,01
OS[3] ou CLA sintético[4]+OS[a,b,c]	4,0 ou 24,0[a,b,c]

[1,2]Ingredientes das dietas experimentais foram adquiridos da Rhoster (Araçoiaba da Serra, SP, Brasil) e Farmos (Rio de Janeiro, RJ, Brasil); [3]Óleo de soja (OS); [4]Luta-CLA 60 (BASF AG, São Paulo, Brasil) composto de 60% de CLA na proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12; [a]Dieta Controle Normolipidico consistiu de 46.6% de amido de milho, 14,0% de caseína e 4,0% OS; [b]Dieta Controle Hiperlipídico consistiu de 29,1% de amido de milho, 17,3% de caseína e 24% de OS; [c]Dieta CLA sintético consistiu de 29,1% de amido de milho, 17,3% de caseína e 22,5% de OS + 1,5% de CLA sintético.

Amostras de péletes (50 g) de cada dieta foram aleatoriamente coletadas e analisadas para composição química e análise do perfil de ácidos graxos, conforme descrito no item *3.4.2* (Tabela 8). A representação dos cromatogramas sobrepostos na região de eluição do CLA dos péletes das dietas CN, CH e S-CLA está demonstrada na figura 7.

Tabela 8. Composição química e perfil de ácidos graxos das dietas experimentais.

	CN[2]	**CH**[3]	**S-CLA**[4]
	Composição química, % de matéria seca		
Conteúdo de matéria seca (%)	79,1	88,4	88,3
Gordura	3,11	21,1	20,9
Proteína Bruta	13,1	14,8	15,2
Cinza	2,76	2,95	2,95
Fibra em detergente neutro	2,76	3,89	3,00
Carboidrato	55,4	42,7	44,5
	Composição Energética		
Energia de Carboidrato (%)	73,4	40,7	41,7
Energia de Proteína (%)	17,3	14,1	14,3
Energia de Gordura (%)	9,25	45,2	44,0
Kcal/g	2,39	3,71	3,77
	Ácidos Graxos (g/100 g do total de ácidos graxos)		
C14:0	0,52	0,13	0,12
C16:0	11,7	10,8	10,6
C18:0	4,25	4,06	4,06
C18:1 *cis*-9/*trans*-15	23,8	22,4	23,5
C18:2 *cis*-9 *cis*-12	49,5	52,4	49,8
C20:0	0,36	0,35	0,30
C20:1 *cis*-11	n.d. [1]	0,03	0,03
C18:3 *cis*-9 *cis*-12 *cis*-15	6,16	6,58	5,81
CLA *cis*-9 *trans*-11	n.d.	n.d.	1,70
CLA *trans*-10 *cis*-12	n.d.	n.d.	1,77
C22:0	0,41	0,30	0,21
C24:0	0,15	0,05	0,07

[2]Controle Normolipídico, dieta contendo 4% de óleo de soja (OS); [3]Controle Hiperlipídico, dieta contendo 24% de OS; [4]CLA Sintético, dieta contendo 1.5% de Luta-CLA 60 (BASF AG, São Paulo, Brasil) e 22,5% de OS. Luta-CLA 60 é composto de 60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12.
[1]n.d.: não detectado.

O conteúdo de isômeros de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 na dieta S-CLA foi calculado conforme anteriormente citado no item *3.4.2*. Os conteúdos de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 na dieta S-CLA foram ambos de 0,3%.

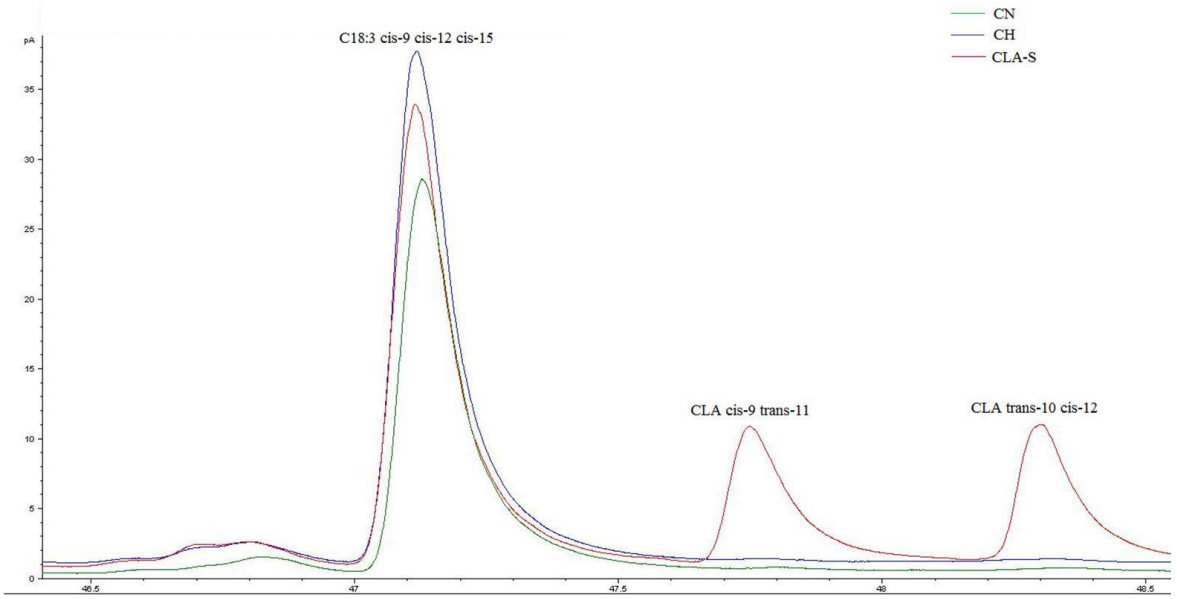

Figura 7. Cromatogramas sobrepostos dos péletes das dietas experimentais MC, contendo 21,7% de manteiga controle e 2,3% de óleo de soja (OS), e M-CLA, contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. É mostrada a região de eluição do Ácido Linoléico Conjugado (CLA), com destaque para o isômero CLA *cis*-9 *trans*-11. Foi utilizado cromatógrafo Agilent Technologies (6890N) equipado com coluna CPSil88 (100m x 0,25mm x 0,2µm) e detector de ionização de chama com condições de operação descritas por CRUZ-HERNANDEZ et al. (2007). Fonte: Arquivo pessoal.

3.6 DELINEAMENTO DURANTE OS TRATAMENTOS DIETÉTICOS

Os animais receberam ração (F_i) *ad libitum* diariamente (entre 11h00min e 12h00min) e as sobras foram pesadas no dia seguinte (F_f), imediatamente antes do fornecimento de outra F_i. A média da ingestão alimentar (gramas/animal/dia) foi estimada como segue: (F_i - F_f)/5 (número de animais por gaiola). A massa corporal individual foi medida a cada cinco dias durante todo o período de experimentação. Depois do período de tratamento dietético, os ratos foram submetidos a jejum por 12 horas (7h00min até 19h00min) e amostras de sangue foram coletadas da veia caudal para determinações da glicemia, usando o método da glicose oxidase (BERGMEYER e BERNT, 1974). Imediatamente após as determinações glicêmicas, os animais foram anestesiados com injeção intraperitoneal de solução de xilazina (10 mg/kg)/quetamina (90 mg/kg), e eutanaziados por exsanguinação total. Determinações glicêmicas foram realizadas anteriormente à anestesia porque foi mostrado que ela causa hiperglicemia (AYALA et al., 2010). Depois da eutanásia, amostras de sangue, tecido adiposo e carcaças foram analisadas para parâmetros

relacionados à sensibilidade à insulina, perfil de lipídeos séricos e composição corporal.

3.7 ANÁLISE DA COMPOSIÇÃO QUÍMICA DA CARCAÇA

As carcaças foram evisceradas, fatiadas, estocadas a -80°C, liofilizadas (modelo Liotop L120; Liobras, São Carlos, Brasil) e moídas em moinho de faca. As carcaças foram pesadas antes e depois da liofilização para determinação do conteúdo de matéria seca. O conteúdo de umidade, cinzas, proteína e lipídeos foram determinados em triplicata no Laboratório de Análise de Alimentos da Embrapa Gado de Leite (Juiz de Fora), de acordo com métodos de referência (AOAC, 2000). O conteúdo de proteína foi quantificado, usando o método de Kjeldahl (AOAC, 2000) com o equipamento Foss (modelo Kjeltec 8400, Foss, Hillerød, Dinamarca) e o conteúdo de lipídeo foi determinado usando o procedimento Ankom com extrator Ankom (modelo XT10, Ankom Technology, Nova York, USA). As principais etapas do processamento da carcaça são mostradas na figura 8.

3.8 ANÁLISE DO NÍVEL DE PROTEÍNA PPARγ POR *WESTERN BLOT*

Amostras de tecido adiposo retroperitoneal foram homogeneizadas em tampão de lise [Tris-HCl: 50 mM, pH 7,4, $Na_4P_2O_7$: 30 mM, NP-40: 1%, Triton (1%), SDS: 0,1%, NaCl: 150 mM, EDTA: 5 mM, NaF: 50 mM, plus Na_3VO_4: 1 mM e coquetel inibidor de protease (Roche Diagnostics, Mannheim, Alemanha)] usando um homogeneizador Ultra-Turrax (IKA Werke, Staufen, Suíça). Depois da centrifugação (7500×g for 5 min), os homogenatos foram estocados a −20°C até o ensaio de SDS-PAGE. O conteúdo total de proteínas no homogenato foi determinado por ensaio com *kit* BCA (Pierce, Illinois, EUA). Os conteúdos das proteínas Receptores Ativados por Proliferadores de Peroxissoma (PPAR)γ e β-tubulina (controle de carga) em amostras de tecido adiposo retroperitoneal foram avaliados por incubação com anticorpos primários monoclonais (anti-PPARγ e anti-

β-tubulin; 1:1000; da Abcam, Cambridge, Reino Unido) durante a noite a 4°C, seguida da utilização de anticorpos secundários apropriados (1 hora; 1:7000 anticorpo da Sigma-Aldrich Co., Missouri, USA) e estreptavidina (1 hora; 1:7000; Zymed, California, USA). As bandas das proteínas foram visualizadas por quimioluminescência com *kit* ECL Plus (GE Healthcare Life Sciences, Buckinghamshire, UK) seguida da exposição ao ImageQuant™ LAS 500 (GE Healthcare Life Sciences). Área e densidade das bandas foram quantificadas pelo software Image J (Media Cybernetics, Maryland, EUA). Os resultados foram normalizados pelo conteúdo de β-tubulina e expressos como relativo (%) ao grupo normolipídico.

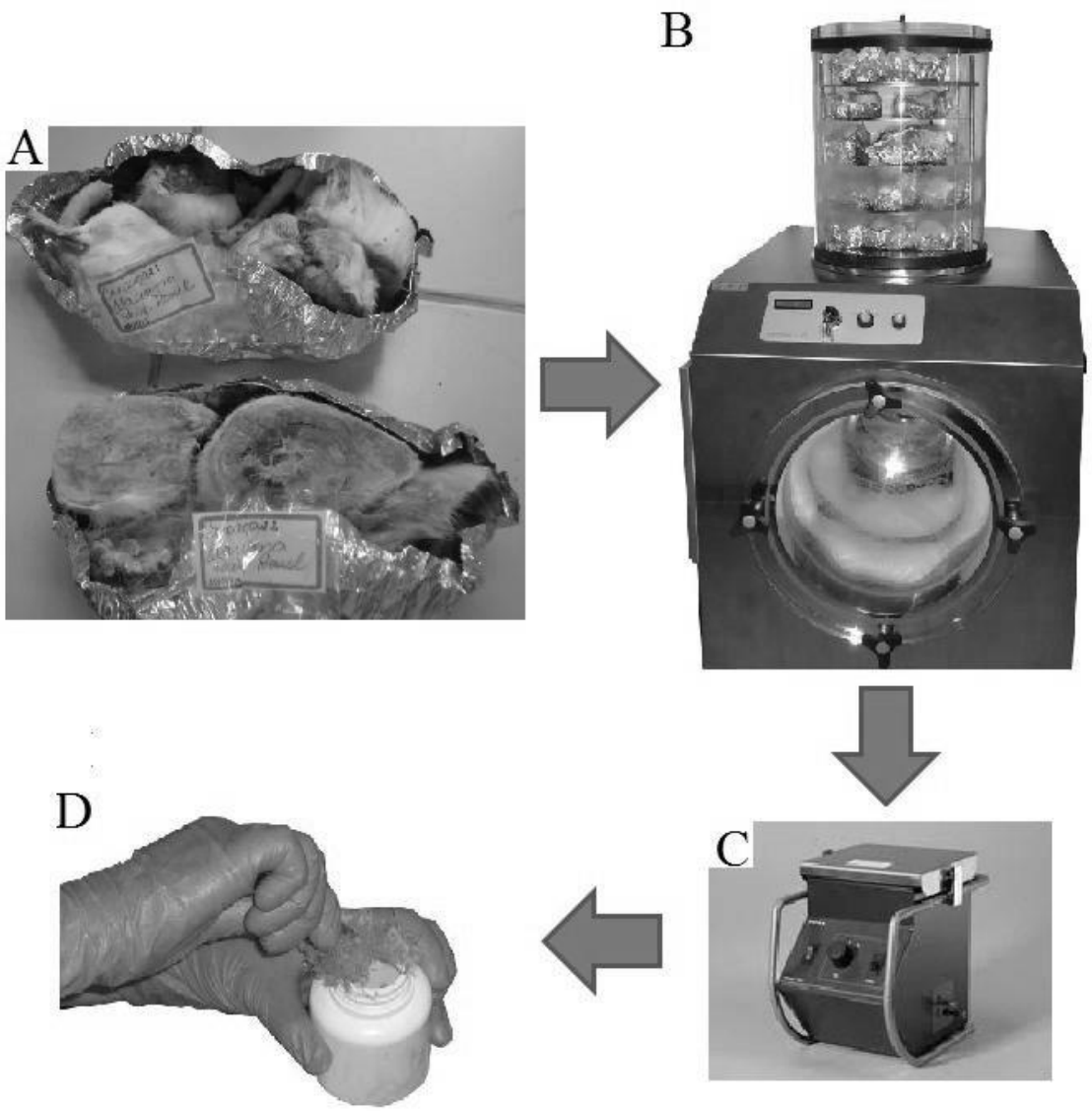

Figura 8. Principais etapas de processamento das carcaças dos animais para avaliação da composição corporal. As carcaças foram evisceradas, fatiadas (**A**), estocadas a -80°C, liofilizadas (**B**), moídas em moinho de faca (**C**) e acondicionadas em potes (**D**), os quais foram vedados e armazenados a -20°C até o momento das análises de umidade, cinzas, proteína e lipídeo.

3.9 PARÂMETROS HORMONAIS E BIOQUÍMICOS

Amostras de sangue foram coletadas dos animais eutanasiados por punção cardíaca e centrifugadas (5714×*g* por 5 min) para a separação do soro. Níveis de insulina sérica foram determinados usando *kit* ELISA de insulina para rato (Mercodia, Uppsala, Suécia). Níveis séricos de ácidos graxos não esterificados (NEFA) foram analizados usando *kit* colorimétrico (Randox Laboratories, Antrim, Reino Unido), enquanto leptina e adiponectina foram analisadas usando os *kits* ELISA de Leptina (R&D Systems, Minneapolis, EUA) e Adiponectina (EMD Millipore, Missouri, EUA), respectivamente. Níveis séricos de colesterol (ALLAIN et al., 1974), triglicérides (FOSSATI e PRENCIPE, 1982), colesterol HDL (proteína de alta densidade) (KOSTNER et al., 1979) e colesterol LDL (proteína de baixa densidade) (BACHORIK e ROSS, 1995) foram determinados por colorimetria usando equipamento BT 3000 do Wiener laboratories.

3.10 HOMA E R-QUICKI

Índice de HOMA (*Homeostatic Model Assessment*) foi calculado como segue: [insulina de jejum (ng/ml)×glicose de jejum (mM)]/22.5. Um índice de HOMA alto denota baixa sensibilidade à insulina (WALLACE et al., 2004a), embora deva ser reconhecido que o modelo de HOMA não tenha sido validado para o uso com modelos de animais (WALLACE et al. 2004b).

O índice de R-QUICKI (*Revised Quantitative Insulin Sensitivity Check Index*) é também usado para avaliar sensibilidade à insulina (PERSEGHIN et al. 2001). Este índice foi calculado como segue: [1/log insulina de jejum (mU/mL)+log glicose de jejum (mg/dL)+log NEFA (mmol/L)] (PERSEGHIN et al., 2001).

3.11 TESTE ORAL DE TOLERÂNCIA À GLICOSE (TOTG)

Depois de 55 dias sob as dietas experimentais, os ratos foram submetidos a jejum por 12 horas (7h00min até 19h00) e receberam solução de glicose 50% (2g/kg de massa corporal) através de gavagem oral (ANDREAZZI et al. 2009; KISS et al., 2012; ELAHI-MOGHADDAM et al., 2013). Amostras de sangue foram coletadas da veia caudal para as determinações glicêmicas usando o método de glicose oxidase (BERGMEYER e BERNT, 1974) nos tempos 0, 30, 60, 90, 120 e 240 minutos após a gavagem. Devido à razões previamente descritas, anestesia não foi usada no TOTG. Mudanças na concentração da glicemia durante o teste oral de tolerância à glicose foram avaliadas através da estimativa da área total sob a curva que foi calculada usando o método do trapezóide (TAI, 1994).

3.12 ANÁLISES ESTATÍSTICAS

As análises estatísticas foram realizadas usando o Prism 5.0 (GraphPad Software, Inc). Para avaliação dos resultados do ensaio do ácido linoléico conjugado de origem natural foram estabelecidas as comparações entre os grupos dietéticos CN, MC e M-CLA, e para investigação dos resultados do ensaio do ácido linoléico conjugado de origem sintética foram comparados os grupos experimentais CN, CH e S-CLA. Dessa forma, dois conjuntos de dados independentes foram obtidos, cada um deles analisados por one-way ANOVA seguido de Newman-Keuls. Os resultados foram expressos com média ± E.P.M. (erro padrão da média). Efeitos de tratamento e diferenças entre médias foram considerados significativos quando $p < 0,05$.

4 RESULTADOS

4.1 ÁCIDO LINOLÉICO CONJUGADO DE FONTE NATURAL

A média do consumo alimentar dos ratos alimentados com as dietas CN, MC e M-CLA foi de 26,45 ± 1,06, 20,96 ± 0,37 e 21,33 ± 0,49 g/dia/rato, respectivamente (Figura 9A). O consumo alimentar das dietas MC e M-CLA foi 20,76% e 19,54% menor que o consumo alimentar de CN, respectivamente, enquanto nenhuma diferença foi observada entre MC e M-CLA (Figura 9A). A média da ingestão energética dos ratos alimentados com as dietas CN, MC, M-CLA foi de 63,19 ± 2,52, 73,21 ± 1,31 e 72,01 ± 1,67 Kcal/dia/rato, respectivamente (Figura 9B). O consumo de energia observado nos ratos alimentados com as dietas MC e M-CLA foi 15,85% e 13,95% maior que o consumo dos ratos alimentados com a dieta CN, respectivamente, mas não houve diferença estatística entre MC e M-CLA (Figura 9B). A média da massa corporal inicial dos animais alimentados com as dietas CN, MC e M-CLA foi de 274,50 ± 5,18, 261,60 ± 3,09 e 276,00 ± 5,99 g, respectivamente, com nenhuma diferença estatística entre os grupos experimentais (Figura 9C). A média da massa corporal final dos animais alimentados com as dietas CN, MC e M-CLA foi de 433,90 ± 10,11 g, 443,80 ± 4,69 g e 454,00 ± 9,65 g, respectivamente (Figura 9C), com nenhuma diferença entre os tratamentos (Figura 9C). Não foi observada diferença no ganho de massa corporal (expresso como porcentagem da massa corporal inicial) entre os grupos experimentais (Figura 9D).

No que diz respeito à composição química da carcaça, não houve diferença nos conteúdos de umidade, lipídeos, proteína e cinzas entre os tratamentos dietéticos (Figura 10).

Os níveis da proteína PPARγ no tecido adiposo foram diminuídos em 58,70% e 62,35% nos ratos alimentados com a dieta MC em comparação com aqueles que receberam as dietas CN e M-CLA, respectivamente (Figura 11).

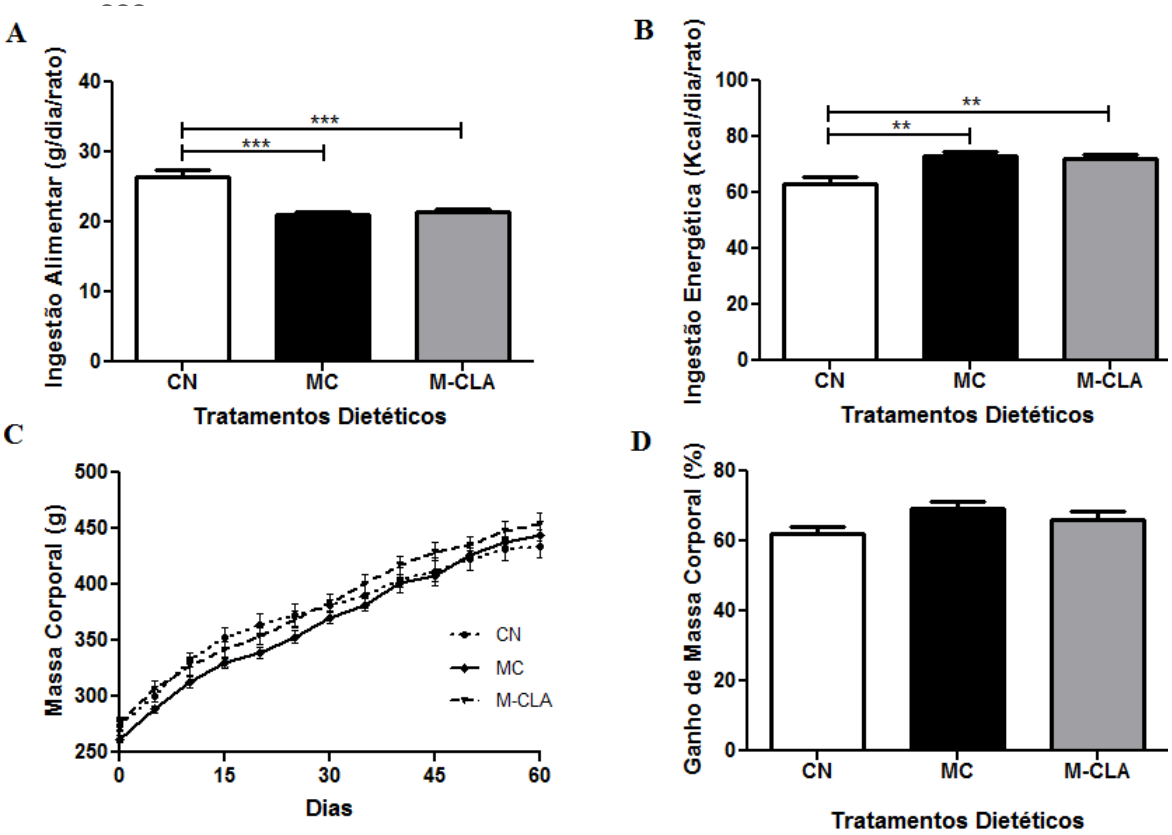

Figura 9. Efeito de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 sobre ingestão dietética e massa corporal. Consumo alimentar (**A**), consumo energético (**B**), massa corporal (**C**) e ganho de massa (**D**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e Manteiga Alto CLA (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. ***p*<0,01, ****p*<0,001.

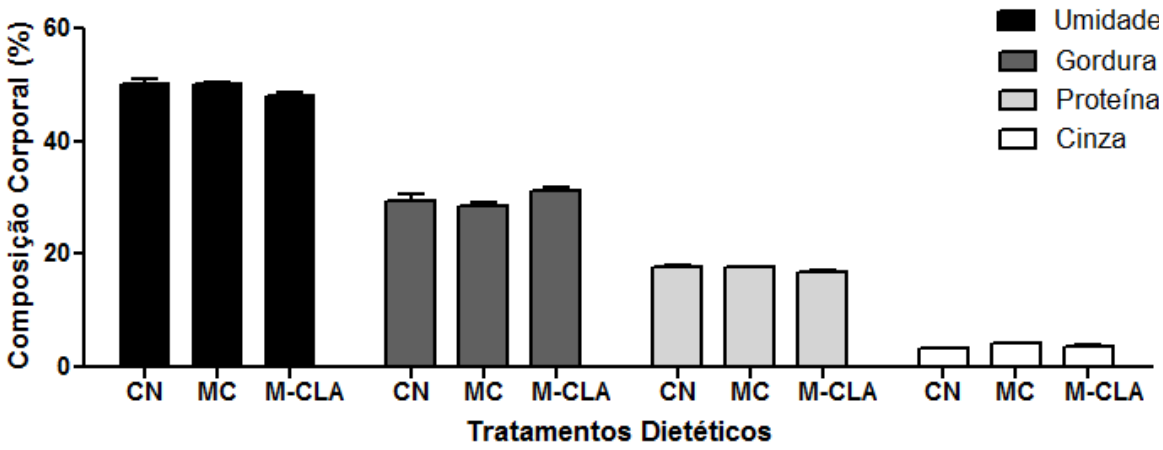

Figura 10. Efeito de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 sobre composição química da carcaça de ratos Wistar machos. Os animais foram atribuídos aos seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e Manteiga Alto CLA (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10

ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls.

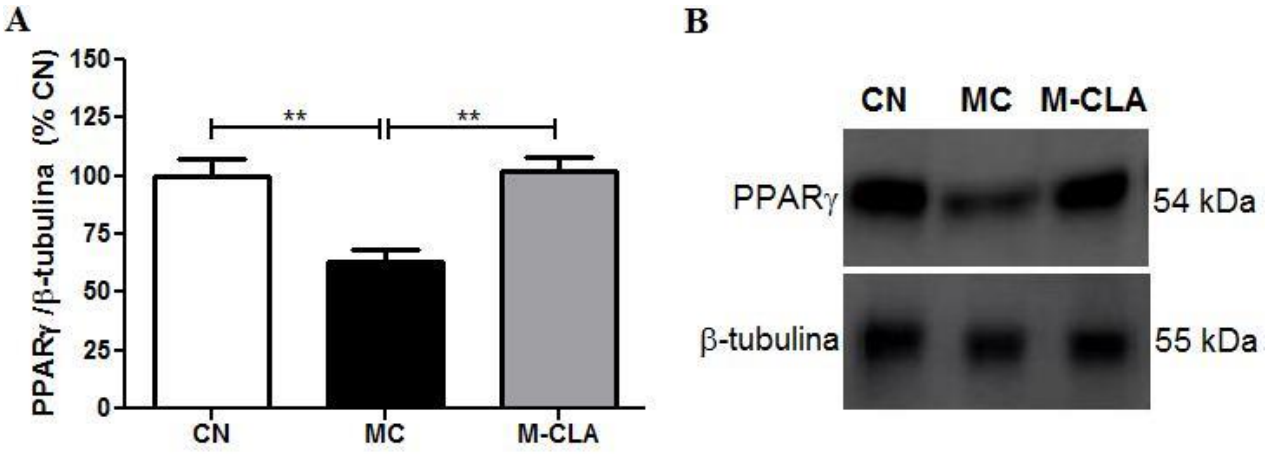

Figura 11. Efeito de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 sobre níveis da proteína PPARγ no tecido adiposo retroperitoneal. Níveis de PPARγ (**A**) e blots representativos para PPARγ e β-tubulina (controle de carga) (**B**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e Manteiga Alto CLA (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. ** $p<0,01$.

Os níveis de insulina sérica nos ratos alimentados com a dieta M-CLA foram menores (11,60%) que naqueles alimentados com a dieta MC. A concentração sérica de insulina no grupo MC foi maior (27,76%) do que no grupo CN, enquanto que não houve diferença entre os valores dos grupos CN e M-CLA (Figura 12A). A glicemia (Figura 12B) e as concentrações séricas de NEFA, leptina e adiponectina (Tabela 9) não diferiram entre os tratamentos dietéticos.

O índice de HOMA não foi modificado entre os tratamentos dietéticos (Figura 13A). Entretanto, o grupo MC apresentou menor índice R-QUICKI (13,63%) que o grupo CN, enquanto nenhuma diferença foi observada entre os valores dos grupos M-CLA e CN (Figura 13B).

Não houve diferença na área sob a curva do TOTG entre os grupos experimentais (Tabela 9).

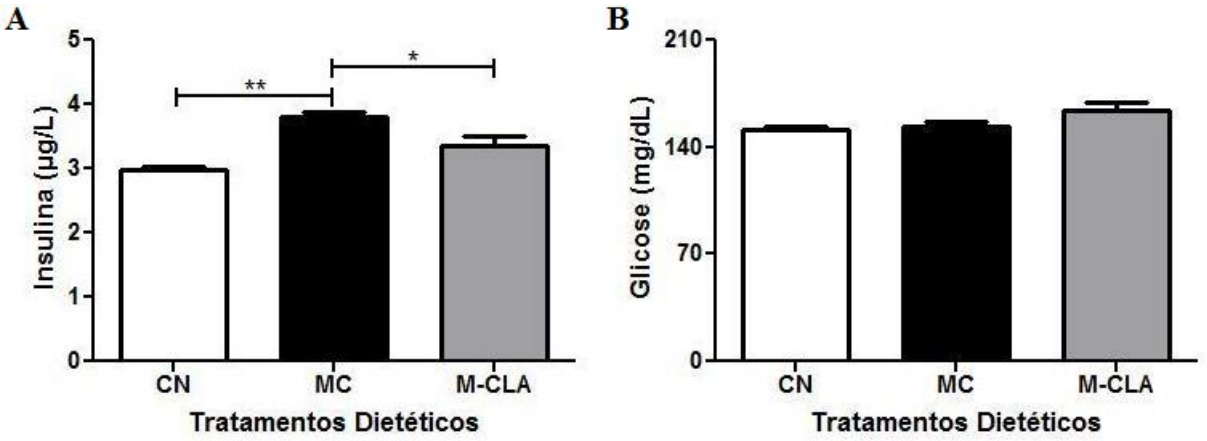

Figura 12. Efeito de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 sobre metabólitos séricos. Insulina (**A**), glicose (**B**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e Manteiga Alto CLA (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. **p*<0,05, ***p*<0,01.

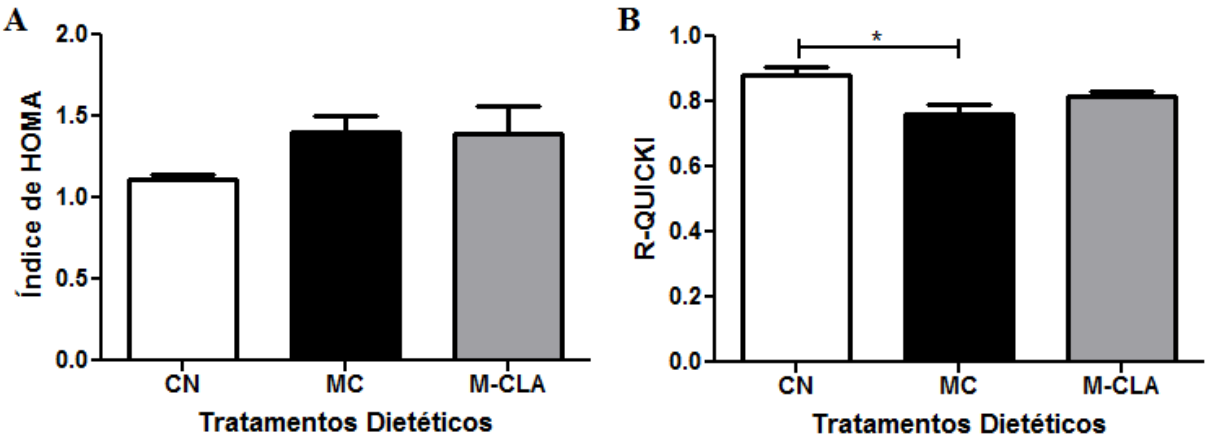

Figura 13. Efeito de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 sobre índices de sensibilidade à insulina. Índice de HOMA (**A**) e R-QUICKI (**B**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e Manteiga Alto CLA (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. **p*<0,05.

Tabela 9. Metabólitos séricos de ratos Wistar alimentados com manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 por 60 dias.

	Tratamentos Dietéticos		
	CN[1]	**MC[2]**	**CLA-M[3]**
	Metabólitos Séricos e Área sob a Curva		
NEFA (mmol/L)	0.375±0.023	0.325±0.017	0.354±0.022
Leptina (ng/mL)	2.21±0.21	2.59±0.26	2.72±0.35
Adiponectina (ng/mL)	15310±1408	16610±1407	14260±1117
Área sob a Curva[4]	13180±1505	12330±1158	14390±1398

Dados são apresentados como média ± E.P.M (n=10 ratos/grupo). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. Asterisco denota diferença estatisticamente significativas comparado com CN ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). [1]Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); [2]Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e [3]Manteiga Alto CLA (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. [4]Área sob a curva calculada a partir do Teste Oral de Tolerância à Glicose.

Os níveis séricos de colesterol não diferiram entre os grupos M-CLA e CN, enquanto os valores observados em MC foram 25,78% e 17,01% menores do que em CN e M-CLA, respectivamente (Figura 14A). Os níveis séricos de triglicérides em M-CLA foram aumentados em 58,81% e 49,54% quando comparados aos observados nos grupos CN e MC, respectivamente (Figura 14B). Os níveis de colesterol HDL foram aumentados em 10,08% e 25,76% nos ratos alimentados com M-CLA quando comparados com aqueles de ratos alimentados com as dietas CN e MC, respectivamente (Figura 14C). Não houve diferença estatística nos níveis de colesterol LDL entre os ratos alimentados com as dietas M-CLA e MC, mas os valores observados nesses grupos foram 39,68% e 36,88% menores do que o do grupo CN, respectivamente (Figura 14D).

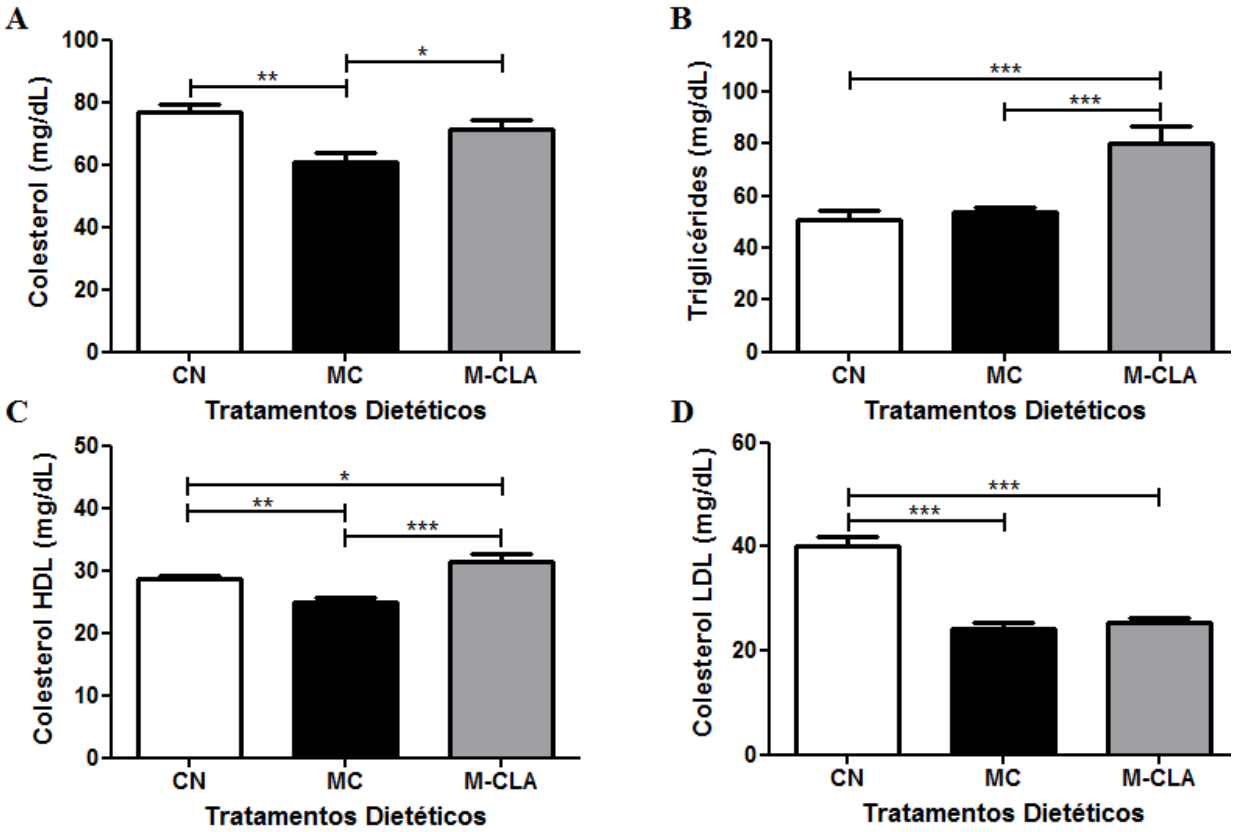

Figura 14. Efeito de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 sobre lipídeos séricos. Colesterol (A), triglicérides (B), colesterol HDL (C) e colesterol LDL (D) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Manteiga Controle (MC): dieta contendo 21,7% de manteiga controle e 2,3% de OS; e Manteiga Alto CLA (M-CLA): dieta contendo 21,7% de manteiga naturalmente enriquecida com CLA *cis*-9, *trans*-11 e 2,3% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. *p<0,05, **p<0,01, ***p<0,001.

4.2 ÁCIDO LINOLÉICO CONJUGADO DE FONTE SINTÉTICA

A média do consumo alimentar dos ratos alimentados com as dietas CN, CH e S-CLA foi de 26,45 ± 1,06, 18,90 ± 0,51 e 19,39 ± 0,36 g/dia/rato, respectivamente (Figura 15A). O consumo alimentar das dietas CH e S-CLA foi 28,55% e 26,69% menor do que aquele observado na dieta CN, respectivamente, enquanto nenhuma diferença foi observada entre CH e S-CLA (Figura 15A). A média da ingestão energética dos ratos alimentados com as dietas CN, CH e S-CLA foi de 63,19 ± 2,52, 70,17 ± 1,89 e 73,10 ± 1,34 Kcal/dia/rato, respectivamente (Figura 15B). O consumo de energia observado nos ratos alimentados com as dietas CH e S-CLA foi 11,05% e 15,68% maior do que o consumo dos ratos alimentados com a dieta CN,

respectivamente, mas não houve diferença estatística entre CH e S-CLA (Figura 15B). A média da massa corporal inicial dos animais alimentados com as dietas CN, CH e S-CLA foi de 274,50 ± 5,18, 264,30 ± 4,75 e 259,20 ± 5,67 g, respectivamente, com nenhuma diferença estatística entre os grupos experimentais (Figura 15C). No décimo dia, a massa corporal dos ratos alimentados com as dietas CH e S-CLA foram diminuídas em comparação com a dos animais alimentados com a dieta CN (Figura 15C). A média da massa corporal final dos animais alimentados com as dietas CN, CH e S-CLA foi de 433,90 ± 10,11 g, 421,00 ± 12,17 g e 425,10 ± 9,61 g, respectivamente (Figura 15C), com nenhuma diferença entre os tratamentos (Figura 15C). Não foi observada diferença no ganho de massa corporal (expresso como porcentagem da massa corporal inicial) entre os grupos experimentais (Figura 15D).

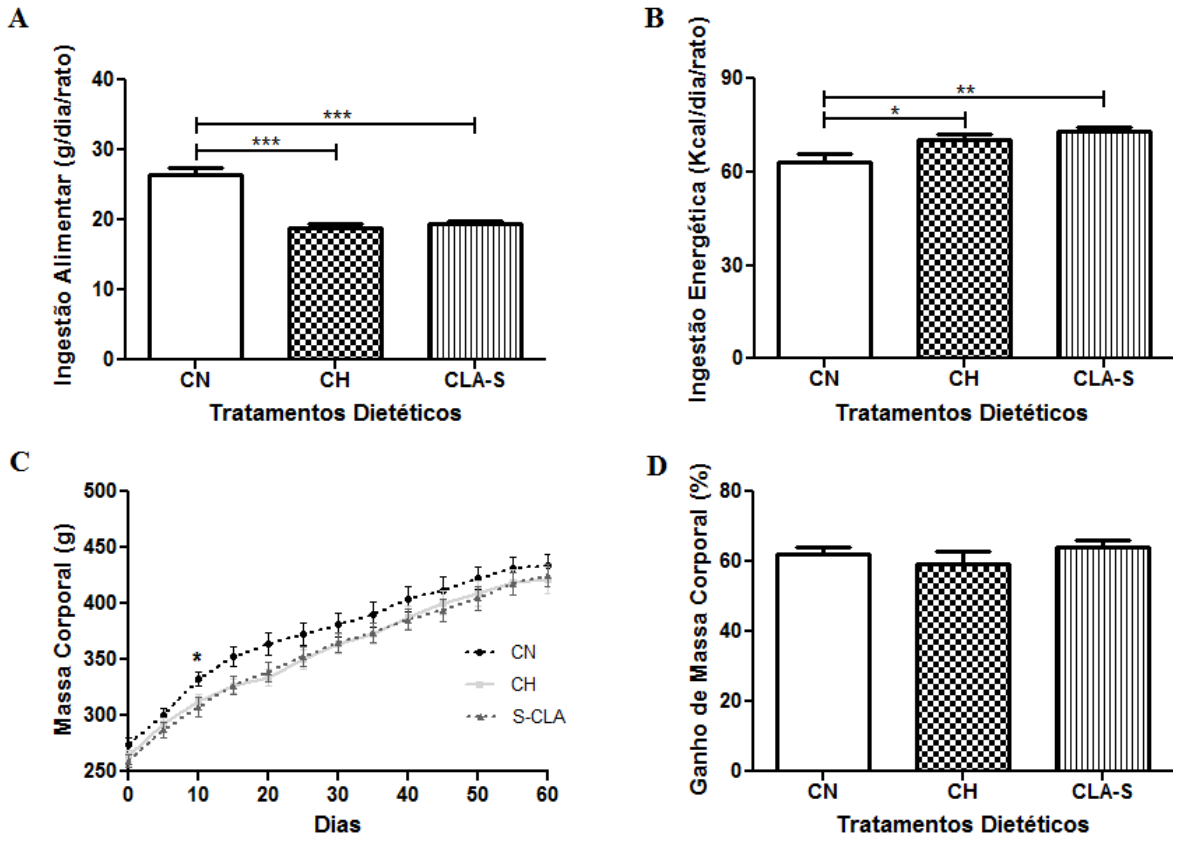

Figura 15. Efeito da mistura sintética CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sobre ingestão dietética e massa corporal. Consumo alimentar (**A**), consumo energético (**B**), massa corporal (**C**) e ganho de massa (**D**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Controle Hiperlipídico (CH): dieta contendo 24% OS; e CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 (60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 e 22,5% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. *p<0,05, **p<0,01, ***p<0,001.

No que diz respeito à composição química da carcaça, não houve diferenças nos conteúdos de umidade, lipídeos, proteína e cinzas entre os tratamentos dietéticos (Figura 16).

Os níveis da proteína PPARγ no tecido adiposo foram diminuídos em 38,06 % e 29,80 % nos ratos alimentados com a dieta S-CLA em comparação com os daqueles alimentados com as dietas CN e CH, respectivamente (Figura 17).

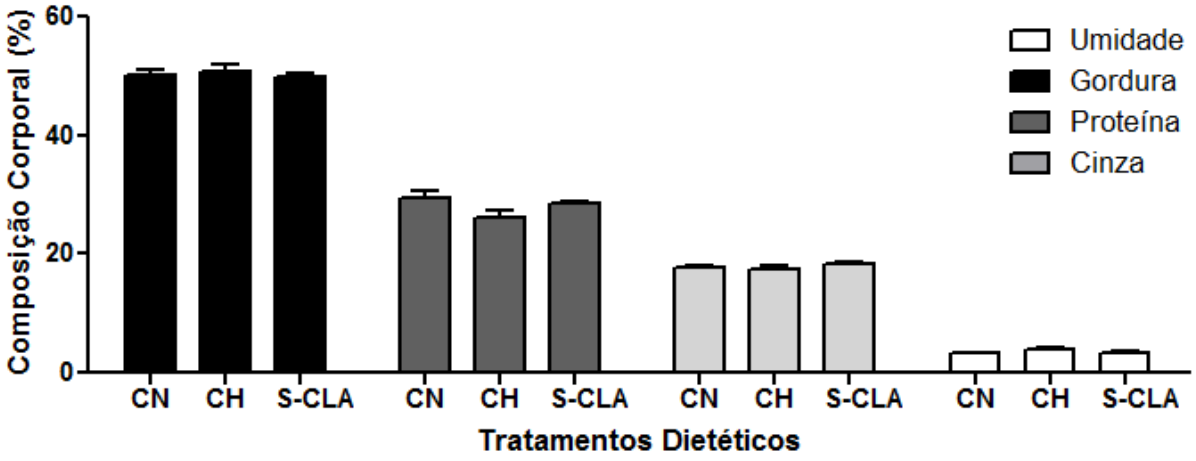

Figura 16. Efeito da mistura sintética CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sobre composição química da carcaça de ratos Wistar machos. Os animais foram atribuídos aos seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Controle Hiperlipídico (CH): dieta contendo 24% OS; e CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 (60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 e 22,5% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls.

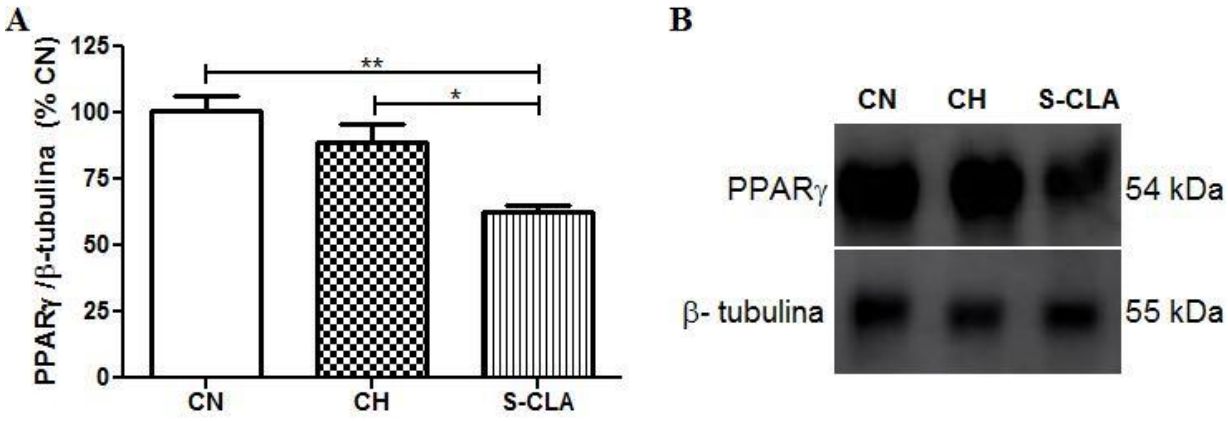

Figura 17. Efeito da mistura sintética CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sobre níveis da proteína PPARγ no tecido adiposo retroperitoneal. Níveis de PPARγ (**A**) e blots representativos para PPARγ e β-tubulina (controle de carga) (**B**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Controle Hiperlipídico (CH): dieta contendo 24% OS; e CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 (60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 e 22,5% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. *p<0,05, **p<0,01.

Os níveis de insulina sérica foram aumentados em 17,17% e 25,63% nos ratos alimentados com S-CLA em comparação com aqueles alimentados com as dietas CN e CH, respectivamente (Figure 18A), bem como a concentração da glicose sanguínea foi aumentada em 8,90% e 5,42% nos ratos alimentados com S-CLA em comparação com os animais alimentados com as dietas CN e CH, respectivamente (Figura 18B). Não houve diferenças nos níveis séricos de NEFA entre os ratos alimentados com as dietas S-CLA e CH, mas os valores observados nesses grupos foram 12,53% e 21,60% menores do que aqueles do grupo CN, respectivamente (Tabela 10). As concentrações séricas de leptina e adiponectina não diferiram entre os tratamentos dietéticos (Tabela 10).

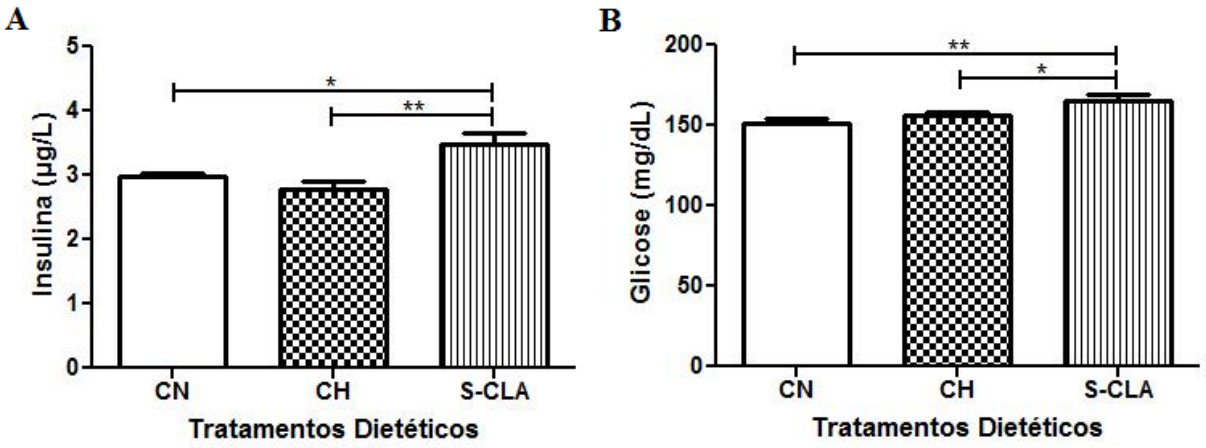

Figura 18. Efeito da mistura sintética CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sobre metabólitos séricos. Insulina (A), glicose (B), ácido graxos não esterificados (NEFA) (C), e leptina (D) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Controle Hiperlipídico (CH): dieta contendo 24% OS; e CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 (60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 e 22,5% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. **p*<0,05, ***p*<0,01.

O índice de HOMA foi aumentado em 29,85% e 32,74% nos ratos alimentados com S-CLA em comparação com aqueles alimentados com as dietas CN e CH, respectivamente (Figura 19A). O índice de R-QUICKI não foi modificado pelos tratamentos dietéticos (Figura 19B).

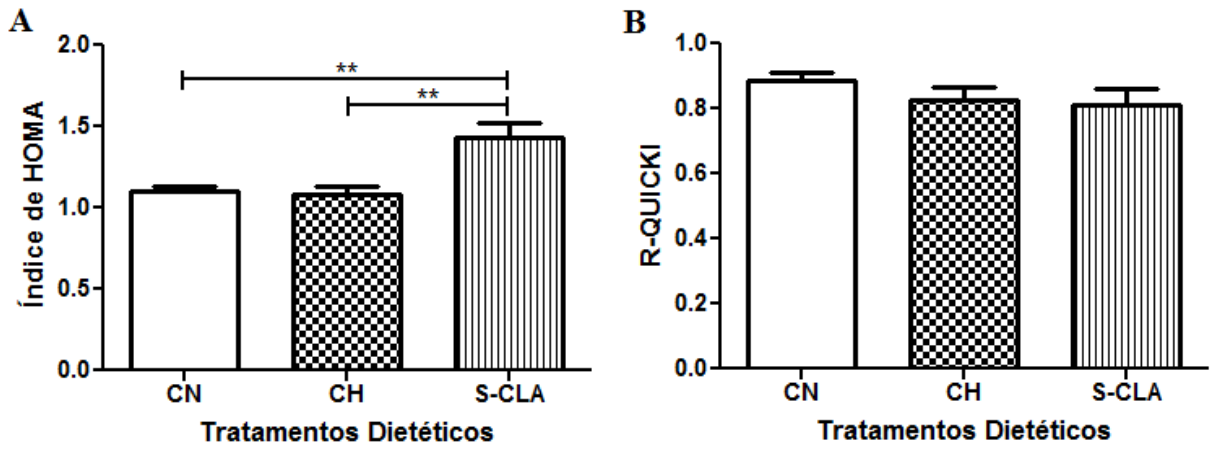

Figura 19. Efeito da mistura sintética CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sobre índices de sensibilidade à insulina. Índice de HOMA (**A**) e R-QUICKI (**B**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Controle Hiperlipídico (CH): dieta contendo 24% OS; e CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 (60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 e 22,5% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. ***p*<0,01.

A área sob a curva do TOTG não foi modificada pelos tratamentos dietéticos (Tabela 8).

Tabela 10. Metabólitos séricos de ratos Wistar alimentados com mistura de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 por 60 dias.

	Tratamentos Dietéticos		
	CN[1]	**CH[2]**	**CLA-S[3]**
	Metabólitos Séricos e Área sob a Curva		
NEFA (mmol/L)	0,375±0,023	0,294±0,025*	0,328±0,021
Leptina (ng/mL)	2,21±0,21	1,98±0,19	1,80±0,16
Adiponectina (ng/mL)	15310±1408	14870±914.5	13260±1129
Área sob a Curva[4]	13180±1505	14610±1021	17400±2007

Dados são apresentados como média ± E.P.M (n=10 ratos/grupo). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. Asterisco denota diferença estatisticamente significativas comparado com CN (*p<0.05, **p<0.01, ***p<0.001). [1]Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); [2]Controle Hiperlipídico (CH): dieta contendo 24% OS; e [3]CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 (60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 e 22,5% de OS. [4]Área sob a curva calculada a partir do Teste Oral de Tolerância à Glicose.

Os níveis séricos de colesterol dos ratos alimentados com as dietas CH e S-CLA foram diminuídos em 18,27% e 11,96% comparados aos dos animais alimentados com a dieta CN, respectivamente (Figura 20A). Da mesma forma, os níveis de triglicérides dos ratos alimentados com as dietas CH e S-CLA foram,

respectivamente, reduzidos em 31,29% e 26,73% comparados aos dos animais alimentados com a dieta CN (Figura 20B). Os níveis de colesterol HDL foram aumentados em 10,08% e 23,29% nos animais alimentados com S-CLA comparados com os dos ratos alimentados com as dietas CN e CH, respectivamente (Figura 20C). Não houve diferença nos níveis de colesterol LDL entre os grupos S-CLA e CH, mas esses valores foram 28,32% e 23,60% menores que aqueles observados nos animais alimentados com CN, respectivamente (Figura 20D).

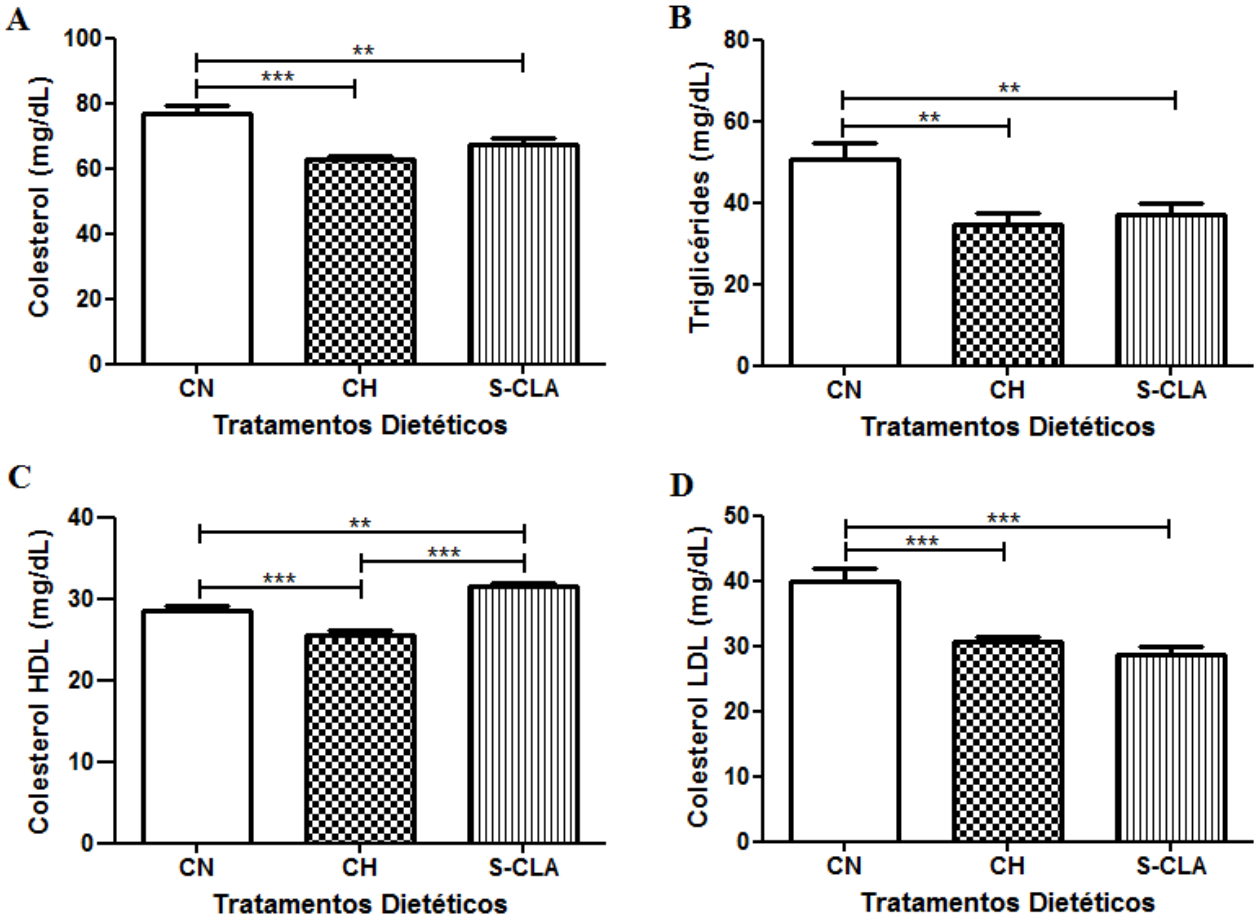

Figura 20. Efeito da mistura sintética CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sobre lipídeos séricos. Colesterol (**A**), triglicérides (**B**), colesterol HDL (**C**) e colesterol LDL (**D**) de ratos Wistar machos alimentados com os seguintes tratamentos dietéticos por 60 dias: Controle Normolipídico (CN): dieta contendo 4,0% de óleo de soja (OS); Controle Hiperlipídico (CH): dieta contendo 24% OS; e CLA Sintético (S-CLA): dieta contendo 1,5% de Luta-CLA 60 (60% de CLA com proporção de 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 e 22,5% de OS. Todos os dados estão apresentados como valores de média ± E.P.M. (n=10 ratos/grupos). Diferenças estatisticamente significativas foram determinadas por Anova seguido por Newman-Keuls. ***p*<0,01, ****p*<0,001.

5 DISCUSSÃO

5.1 ÁCIDO LINOLÉICO CONJUGADO DE FONTE NATURAL

Nos últimos anos, o CLA tem recebido mais atenção como suplemento dietético (HALADE et al., 2010), entretanto poucos estudos avaliaram os efeitos do ácido linoléico conjugado proveniente de fonte natural sobre o metabolismo da insulina, glicose e lipídeos. Nesse trabalho, foi demonstrado os efeitos dietéticos de manteiga enriquecida com CLA *cis*-9, *trans*-11 em ratos Wistar com 60 dias de idade, sobre a ingestão alimentar e composição corporal, bem como sobre o metabolismo da insulina, glicose, triglicérides, colesterol, NEFA, leptina e adiponectina.

Neste estudo, não houve diferenças no consumo alimentar, ganho de peso e composição corporal entre os ratos alimentados com manteiga enriquecida com CLA *cis*-9, *trans*-11 ou manteiga controle, porque o CLA *cis*-9, *trans*-11 por si só não está envolvido em mecanismos antiobesidade. Estas propriedades são especificamente atribuídas ao CLA *trans*-10, *cis*-12 (KENNEDY et al., 2010). A ingestão alimentar e energética dos grupos MC e M-CLA diferiram dos valores observados no grupo CN devido ao alto conteúdo de gordura das dietas enriquecidas com manteigas. Ratos alimentados com MC e M-CLA adaptaram à maior densidade energética das respectivas dietas por meio da redução do consumo alimentar diário comparado com o grupo CN, como relataram TARLING et al. (2009). O consumo energético diário maior nos ratos alimentados com MC e M-CLA do que naqueles do grupo CN, pode ser atribuído à maior palatabilidade das dietas hiperlipídicas, o que está diretamente relacionado ao maior consumo energético (STUBBS e WHYBROW, 2004). Dietas hiperlipídicas são mais palatáveis devido ao alto conteúdo de gordura (STUBBS e WHYBROW, 2004).

Experimentos têm mostrado que o PPARγ é um importante regulador adipogênico (KENNEDY et al., 2010) e, interconectado ao seu papel na diferenciação de adipócitos, o PPARγ regula a sensibilidade à insulina, ativando transcricionalmente genes envolvidos na sinalização da insulina, absorção de

glicose, e absorção e estoque de ácidos graxos (BROWN e MCINTOSH, 2003). Ratos alimentados com CLA-M apresentaram conteúdo aumentado de PPARγ no tecido adiposo quando comparados com ratos alimentados com MC. Isto pode ser atribuído ao maior (213,20%) fornecimento de CLA *cis*-9, *trans*-11 proveniente da dieta contendo manteiga enriquecida com CLA em comparação com a dieta contendo manteiga controle. Estudos têm demonstrado que o CLA *cis*-9, *trans*-11 aumenta a expressão de PPARγ, cuja regulação negativa pode levar à resistência à insulina (BROWN et al., 2003). Foi demonstrado que a mistura de CLA com 0,286% de CLA *cis*-9, *trans*-11 aumentou a expressão de RNAm de PPARγ no tecido adiposo de ratos Wistar, o que foi relacionado à melhor sensiblidade à insulina (ZHOU et al., 2008). Além disso, foi mostrado que a depleção de PPARγ no tecido adiposo causa resistência à insulina, já que a ação diminuída de PPARγ em adipócitos maduros, leva à redução da expressão de genes chave requeridos para a sinalização de insulina em adipócitos (FLOYD e STEPHENS, 2012). A ativação constitutiva adipócito específica de PPARγ em adipócitos maduros pode regular a sensibilidade à insulina corporal (SUGII et al., 2009). Dessa forma, a manteiga enriquecida em CLA *cis*-9, *trans*-11 foi observada como tendo mecanismo de ação dependente de PPARγ, promovendo uma regulação positiva da sua expressão protéica no tecido adiposo, e, consequentemente, prevenindo a redução de PPARγ, como foi observado pela dieta contendo manteiga controle.

Ratos alimentados com manteiga enriquecida com CLA *cis*-9, *trans*-11 tiveram menores níveis de insulina sérica de jejum do que os ratos alimentados com manteiga controle. Dessa forma, a dieta CLA-M preveniu a hiperinsulinemia em jejum, resultado potencialmente benéfico. De acordo com o *European Group for the Study of Insulin Resistance*, a insulina em jejum é o melhor e mais simples índice disponível para resistência à insulina, que é definida pela presença de hiperinsulinemia em jejum (BALKAU e CHARLES, 1999). Além disso, foi anteriormente demonstrado que o aumento gradual da insulina sérica no estado de jejum reflete em sensibilidade à insulina diminuída (LINDEBERG et al., 1999). O índice de HOMA não diferiu entre os grupos experimentais, entretanto o índice de R-QUICKI, que também denota sensibilidade à insulina (PERSEGHIN et al., 2001), foi menor no grupo MC comparado com o grupo CN, enquanto não houve diferença entre os grupos CN e M-CLA. Portanto, o índice de R-QUICKI mostra que a dieta contendo manteiga controle induz resistência à insulina comparada à dieta controle

normolipídico, uma condição que não foi observada no grupo CLA-M e pode estar associada ao conteúdo reduzido de PPARγ no tecido adiposo de ratos alimentados com MC (FLOYD e STEPHENS, 2012).

O efeito benéfico da manteiga enriquecida com CLA *cis*-9, *trans*-11 sobre o nível de insulina em jejum pode ser devido ao maior fornecimento de CLA *cis*-9, *trans*-11 proveniente da dieta contendo manteiga enriquecida com CLA em comparação à dieta contendo manteiga controle. Foi anteriormente mostrado que animais alimentados com 0,25% de CLA *cis*-9, *trans*-11 na dieta tiveram a concentração de insulina sérica diminuída no jejum (HALADE et al., 2010). Como observado na Tabela 4, as concentrações de vários ácidos graxos foram também alteradas na dieta M-CLA quando comparado com a dieta MC. Por exemplo, houve maior (269,72%) fornecimento de ácido vacênico proveniente da dieta CLA-M comparado à dieta MC, o que contribuiu para o aumento do nível tecidual de CLA *cis*-9, *trans*-11 nos ratos alimentados com CLA-M (SANTORA et al., 2000). Ademais existiu menor (32,06%) fornecimento de ácidos graxos saturados (AGS) de cadeia curta e média na dieta M-CLA comparado à dieta MC, o que pode também contribuir para a diminuição dos níveis séricos de insulina em jejum no grupo M-CLA, já que tem sido sugerido que dietas com alto teor de AGS apresentaram efeitos de hiperinsulinemia (SOLFRIZZI et al., 2009; NARDI et al., 2014). Apesar dos parâmetros alterados dos ratos alimentados com MC, as áreas sob a curva dos teste de tolerância oral à glicose não diferiram entre CN, MC e CLA-M, portanto as dietas experimentais não foram responsáveis por intolerância à glicose.

A concentração sérica de NEFA é um fator de risco para diabetes tipo 2 porque a combinação de níveis elevados de ácidos graxos não esterificados e de glicose levam à secreção diminuída de insulina, prejuízos na expressão do gene insulina e morte de células β por apoptose (POITOUT et al., 2010). Estudo anteriores mostraram que o CLA *cis*-9, *trans*-11 reduziu os níveis de NEFA (HALADE et al., 2010). Entretanto, na presente investigação, não houve diferenças entre os grupos. A ausência de efeito da manteiga enriquecida com CLA *cis*-9, *trans*-11 sobre a concentração sérica de NEFA pode ser atribuída à alterada biodisponibilidade e bioatividade do CLA *cis*-9, *trans*-11 presente na gordura da manteiga. Uma hipótese similar foi desenvolvida quando menores efeitos de carne com alto teor de CLA comparado com o CLA sintético foram observados sobre a proteômica de tecidos sensíveis à insulina (RUNGAPAMESTRY et al., 2012). Leptina é uma adipocina que

desenvolve papel no metabolismo da glicose e sensibilidade à insulina (BLÜHER, 2014). No presente estudo não houve diferença estatística no nível de leptina sérica entre os grupos experimentais. Provavelmente, porque o teor de massa gorda não foi modificado entre os tratamentos dietéticos e a leptina circulante é positivamente correlacionada com a adiposidade (HALADE et al., 2010). Similarmente, foi demonstrado que o CLA *cis*-9, *trans*-11 não alterou os níveis séricos de leptina (BROWN et al., 2003; MARTINS et al., 2009; HALADE et al., 2010). A adiponectina é uma adipocina exclusivamente produzida por adipócitos e está relacionada com aumento de sensibilidade à insulina no fígado (YAMAUCHI et al., 2001). Entretanto, não foram encontradas diferenças nas concentrações séricas de adiponectina nos animais alimentados com a dieta CLA-M em comparação com aqueles alimentados com as dietas CN e MC. Similarmente, foi demonstrado que o CLA *cis*-9, *trans*-11 não alterou os níveis séricos de adiponectina em roedores (HALADE et al., 2010).

Nesse estudo, as concentrações de colesterol e colesterol LDL não foram modificadas pela dieta M-CLA comparadas às dietas CN e MC. Similarmente, nenhum efeito do CLA *cis*-9, *trans*-11 sobre os níveis de colesterol e colesterol LDL foram também mostrados anteriormente (MOLONEY et al., 2007; JOSEPH et al., 2011). A maior concentração de colesterol LDL nos ratos alimentados com dieta CN pode ser devido aos altos níveis de carboidrato (73,39% de energia) desta dieta, já que foi demonstrado que quando o carboidrato da dieta foi aumentado de 50% para 67% da energia da dieta, o nível de triglicérides em jejum aumentou (PARKS, 2001), o que é comumente relacionado ao aumento de precursores de colesterol LDL no sangue, de lipoproteínas de densidade muito baixa (VLDL), e consequentemente, aumento dos níveis de colesterol LDL (COX e GARCÍA-PALMIERI, 1990). A diminuição do colesterol total nos ratos alimentados com a dieta MC foi relacionada ao baixo nível de colesterol HDL nesse grupo, o que é fator de risco para diabetes mellitus tipo 2 (CHEN et al., 2012).

Níveis séricos aumentados de triglicérides em ratos alimentados com M-CLA podem ser decorrentes dos maiores (160,37%) conteúdos dos isômeros C18:1 *trans*-9 e C18:1 *trans*-10 na dieta CLA-M comparado com a dieta MC. Foi demonstrado que o alto nível de C18:1 *trans*-9 tem correlação com o aumento da concentração plasmática de triglicérides (CASSAGNO et al., 2005), bem como o alto conteúdo de C18:1 *trans*-10 (ANADÓN et al., 2010). No que diz respeito aos efeitos do CLA *cis*-9, *trans*-11 sobre o nível sérico de triglicérides, estudos em animais

alimentados com este isômero de CLA não mostraram modificação na concentração de triglicérides (BISSONAUTH et al., 2006; LIU et al., 2012). Por outro lado, ratos alimentados com a dieta M-CLA apresentaram o nível sérico de colesterol HDL aumentado, o que é um resultado potencialmente benéfico, porque este reduz o risco de evento carviovascular (BARTER, 2013) e também tem efeito positivo sobre o controle glicêmico (DREW et al., 2009; FRYIRS et al., 2010; BARTER, 2013). O alto nível de colesterol HDL em ratos alimentados com M-CLA pode ser atribuído à maior concentração de CLA *cis*-9, *trans*-11, como também foi relatado por NESTEL et al. (2006). Similarmente, foi demonstrado que manteiga clarificada enriquecida com CLA aumentou a concentração plasmática de colesterol HDL em ratos Wistar (CHINNADURAI et al., 2013). Entretanto, é possível que o maior fornecimento de ácido oléico proveniente da dieta M-CLA comparado à dieta MC pode também ter contribuído para níveis de colesterol HDL aumentados, já que foi demonstrado que o ácido oléico resulta em acréscimo de HDL colesterol (BERMUDEZ et al., 2011). Além disso, houve menor (36,91%) fornecimento de ácidos láurico (C12:0) e mirístico (C14:0) na dieta M-CLA comparado à dieta MC, o que pode também ter contribuído para o aumento dos níveis de HDL colesterol no grupo M-CLA, já que foi demonstrado que dietas enriquecidas com ácidos láurico e mirístico diminuem a concentração de HDL colesterol (KHOSLA et al., 1997). Por outro lado, a dieta M-CLA apresentou maior (147,82%) teor do isômero C18:1 *trans*-9, o qual tem sido associado com diminuição de níveis de colesterol HDL (FILIP et al., 2010). Dessa forma, foi hipotetizado que os ácidos graxos relacionados ao aumento de colesterol HDL sérico foram capazes de atuar sinergicamente, prevalecendo sobre efeitos negativos do isômero C18:1 *trans*-9 sobre os níveis de colesterol HDL, resultando em maior concentração desta lipoproteína nos ratos alimentados com M-CLA. Entretando, no que diz respeito aos níveis de triglicérides, já tem sido demonstrado com animais alimentados com manteiga naturalmente enriquecida com CLA *cis*-9 *trans*-11 que esta dieta não promoveu efeitos sobre a concentração plasmática de triglicérides (LOCK et al., 2005). Portanto, é possível hipotetizar que os altos conteúdos de isômeros C18:1 *trans*-9 e C18:1 *trans*-10 na dieta M-CLA prevaleceram sobre a ausência de efeitos do CLA *cis*-9 *trans*-11 sobre os níveis de triglicérides, resultando em maior concentração de triglicérides no ratos alimentados com M-CLA.

5.2 ÁCIDO LINOLÉICO CONJUGADO DE FONTE SINTÉTICA

Suplementos sintéticos de CLA contendo 50:50 (v:v) da mistura de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 têm sido recomendados para o auxílio na perda de peso (FDA 2008). Entretanto, a autorização do uso da mistura de isômeros de CLA como suplemento alimentar não é consenso e mais estudos são necessários para investigar o seu papel na prevenção da obesidade e efeitos adversos relacionados ao consumo de CLA, principalmente aqueles associados com resistência à insulina e dislipidemia (FSANZ, 2011; ANVISA 2007). Portanto, nesse trabalho, foram avaliados os efeitos dietéticos de uma dieta contendo mistura de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 em ratos Wistar com 60 dias de idade sobre a prevenção do risco de obesidade, bem como sobre a sensibilidade à insulina e perfil de lipídeos séricos.

Nesse estudo, não houve diferença no consumo alimentar entre os ratos alimentados com dieta S-CLA e aqueles que receberam a dieta CH. O efeito do CLA sobre o consumo alimentar permanece controverso (HOUSE et al., 2005). Vários estudos relataram que o CLA tem pouco ou nenhum efeito sobre o consumo alimentar (DELANY et al., 1999; SISK et al., 2001; YAMASAKI et al., 2003), enquanto outros trabalhos relataram redução na ingestão de alimentos (SZYMCZYK et al., 2000; OSTROWSKA et al., 2003). Os consumos alimentar e energético observados nos grupos CH e S-CLA diferiram dos valores do grupo CN devido ao alto conteúdo de gordura das dietas CH e S-CLA. Os ratos alimentados com as dietas CH e S-CLA adaptaram à maior densidade energética destas dietas por meio da redução do consumo alimentar diário, quando comparado ao grupo CN, como foi relatado por TARLING et al. (2009). A ingestão energética foi maior nos ratos alimentados com CH e S-CLA do que no grupo CN, o que provavelmente pode ser atribuído à palatabilidade aumentada das dietas hiperlipídicas, o que é diretamente relacionado ao maior consumo energético (STUBBS e WHYBROW, 2004). Dietas hiperlipídicas são mais palatáveis, porque o conteúdo de gordura é um dos fatores que contribuem para a palatabilidade dos alimentos (STUBBS e WHYBROW, 2004).

Não houve diferença no ganho de massa corporal entre os grupos dos tratamentos dietéticos. Em outro estudo, uma mistura equimolar de CLA *cis*-9, *trans*-

11 e CLA *trans*-10, *cis*-12 foi fornecida para ratos Wistar na concentração de 1% da dieta (0,5% do isômero CLA *trans*-10 *cis*-12), e nenhuma diferença na massa corporal final depois de seis semanas foi encontrada entre os ratos tratados com CLA e controles (ARIAS et al., 2011). Similarmente, não foi demonstrado efeito no ganho de massa corporal entre os animais controle e aqueles alimentados com 0,25% de CLA *cis*-9, *trans*-11 e 0,25% de CLA *trans*-10, *cis*-12 (HALADE et al., 2010). Na presente investigação, não houve efeito de tratamentos dietéticos sobre a composição corporal. Resultados similares foram observados em estudo anterior no qual ratos Wistar foram alimentados com dieta contendo 1% de cada um dos isômeros de CLA (MIRAND et al., 2004).

Apesar da ausência de efeitos do CLA sobre a composição corporal, os níveis no tecido adiposo de PPARγ, um regulador adipogênico mestre (KENNEDY et al., 2010), foi menor no grupo S-CLA do que nos grupos CN e CH, o que a princípio pode parecer contraditório já que a composição corporal não foi alterada. Entretanto, diferenças no conteúdo de PPARγ no tecido adiposo não são necessariamente associadas com mudanças na composição corporal, já que o PPARγ não é a única proteína envolvida na adipogênese e, interconectado com o papel do PPARγ na diferenciação de adipócitos, esta proteína também regula a sensibilidade à insulina, como foi descrito no item *5.1* (BROWN e MCINTOSH 2003). Foi demonstrado que a depleção de PPARγ no tecido adiposo causa resistência à insulina, já que a ação de PPARγ diminuída em adipócitos maduros, leva à redução da expressão de genes requeridos para a sinalizaçãdo da insulina nos adipócitos (FLOYD e STEPHENS, 2012). Efeitos do ácido linoléico conjugado sobre PPARγ foram relatados como sendo isômero-específico, com o CLA *trans*-10, *cis*-12 regulando negativamente, e o CLA *cis*-9, *trans*-11 regulando positivamente sua expressão no tecido adiposo (BROWN et al., 2003). Portanto, o presente estudo sugere que o efeito do CLA *trans*-10, *cis*-12 sobre PPARγ prevaleceu sobre o efeito do CLA *cis*-9, *trans*-11 sobre a mesma proteína, consequentemente os ratos alimentados com dieta S-CLA apresentaram níveis reduzidos de PPARγ no tecido adiposo.

Ratos alimentados com dieta contendo a mistura de isômeros de CLA apresentaram maiores níveis de insulina sérica em jejum do que os ratos alimentados com as dietas controles normolipídica e hiperlipídica. A hiperinsulinemia em jejum é um importante parâmetro uma vez que já foi demonstrado que o aumento gradual da insulina sérica no estado de jejum reflete em diminuição da

sensibilidade à insulina (LINDEBERG et al., 1999). Os ratos alimentados com S-CLA apresentaram maior glicemia e índice de HOMA aumentado comparado com os animais alimentados com as dietas CN e CH, o que denota baixa sensibilidade à insulina no grupo S-CLA (WALLACE et al., 2004a). Estes resultados estão de acordo com estudos anteriores que mostraram que animais alimentados com dieta contendo a mistura de 0,25% de CLA *cis*-9, *trans*-11 e 0,25% de CLA *trans*-10, *cis*-12 apresentaram hiperinsulinemia e hiperglicemia associadas com resistência à insulina, demonstratada pelo aumento do índice de HOMA (HALADE et al., 2010). Por outro lado, as áreas sob as curvas do teste oral de tolerância à glicose não diferiram entre os tratamentos dietéticos. Portanto, é possível hipotetizar que apesar da mesma quantidade de glicose sérica observada nos grupos CN, CH e S-CLA durante o TOTG, maiores níveis de insulina podem estar sendo requeridos pelos ratos alimentados com S-CLA (HALADE et al., 2010; NORRIS e RICH, 2012).

De acordo com o índice R-QUICK, que denota sensibilidade à insulina (PERSEGHIN et al., 2001), não houve diferença entre os grupos de tratamentos dietéticos, apesar da dieta S-CLA ter apresentado efeitos negativos sobre a homeostase da glicose e insulina. Possivelmente, isto ocorreu porque este índice considera os níveis de NEFA, que apresentaram-se inalterados pela dieta S-CLA comparado com os das dietas CN e CH. Similarmente, foi demonstrado por HALADE et al. (2010) que a mistura de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 não modificou os níveis séricos de NEFA. Maiores níveis de NEFA nos ratos alimentados com CN podem ser relacionados com os altos níveis séricos de colesterol e de triglicérides desse grupo, como demonstrado por WARNEKE et al. (2014). No que diz respeito aos níveis séricos de leptina, não houve diferença entre os grupos experimentais, possivelmente porque o teor de massa gorda não foi modificado entre os tratamentos dietéticos e a leptina circulante é positivamente correlacionada com a adiposidade. Similarmente, foi anteriormente relatado que a mistura de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 não modificou níveis plasmáticos de leptina (BOUTHEGOURD et al., 2002). A dieta CLA-S não modificou a concentração sérica de adiponectina em comparação com as dietas CN e CH, resultado similar ao encontrado por HALADE et al. (2010) em roedores.

Os ratos alimentados com as dietas CH e S-CLA apresentaram menores níveis séricos de colesterol, triglicérides e colesterol LDL, comparado com os dos animais alimentados com a dieta CN. Possivelmente, isto foi devido ao elevado teor

de carboidrato (73,39% de energia) na dieta CN, já que foi demonstrado que quando o teor de carboidrato da dieta foi aumentado de 50% para 67% de energia, há aumento dos triglicérides de jejum (PARKS, 2001), o que é comumente relacionado ao aumento de precursores de colesterol LDL no sangue, as VLDL, e, consequentemente, aumento dos níveis de colesterol LDL (COX e GARCÍA-PALMIERI, 1990). Todas essas mudanças nos ratos alimentados com CN contribuíram para as maiores concentrações de colesterol, triglicérides e colesterol LDL observadas neste grupo. Os ratos alimentados com a dieta contendo a mistura de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 apresentaram favoráveis mudanças no perfil de lipoproteínas séricas comparados aos animais alimentados do grupo CH, já que a dieta contendo isômeros de CLA foi responsável por altos níveis de colesterol HDL enquanto as concentrações séricas de colesterol total, triglicérides e colesterol LDL não foram modificadas comparadas à dieta controle hiperlipídico. Similarmente, foi mostrado que animais suplementados com dieta contendo 1% da mistura 50:50 (v:v) de CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 apresentaram colesterol total plasmático e colesterol LDL inalterados, comparados aos valores do grupo controle (LEDOUX et al., 2007). No que diz respeito ao efeito do CLA sobre o nível sérico de triglicérides, outros estudos em animais mostraram que a concentração de triglicérides não foi modificada pela suplementação com CLA *cis*-9, *trans*-11 (MITCHELL et al., 2005; BISSONAUTH et al., 2006; LIU et al., 2012) ou com CLA *trans*-10, *cis*-12 (MITCHELL et al., 2005). O alto nível de colesterol HDL nos ratos alimentados com S-CLA provavelmente é atribuído ao CLA *cis*-9, *trans*-11, como também foi reportado por NESTEL et al. (2006). Os altos níveis de colesterol HDL nos animais alimentados com S-CLA é resultado potencialmente benéfico devido ao seu potencial em diminuir o risco de eventos cardiovasculares (BARTER, 2013) e ao efeito positivo sobre o controle glicêmico (DREW et al., 2009; FRYIRS et al., 2010; BARTER, 2013). Entretando, apesar dessas propriedades promotoras da saúde associadas ao colesterol HDL, o aumento de sua concentração nos animais alimentados com S-CLA não foi capaz de prevenir a hiperinsulinemia, hiperglicemia ou a resistência à insulina deste grupo. Portanto, é possível hipotetizar que os efeitos negativos sobre o metabolismo da insulina proveniente da diminuição do nível de PPARγ no tecido adiposo, prevaleceu sobre os potenciais efeitos positivos relacionados ao aumento sérico de colesterol HDL sobre o controle glicêmico e insulinêmico.

6 CONCLUSÃO

A manteiga enriquecida com CLA *cis*-9, *trans*-11 aumentou significativamente o colesterol HDL sérico e preveniu a hiperinsulinemia em jejum, o que pode ser atribuído aos maiores níveis de CLA *cis*-9, *trans*-11, ácido vacênico, ácido oléico e menores níveis de AGS de cadeia média e curta proveniente da manteiga enriquecida em CLA comparado com a manteiga controle. Entretanto, a manteiga enriquecida em CLA também causou hipertrigliceridemia, o que pode ser associado com o concomitante aumento nos conteúdo de isômeros C18:1 *trans*-9 e C18:1 *trans*-10 na manteiga enriquecida com CLA. Estudos adicionais são ainda necessários antes que o ácido linoléico conjugado de fonte natural possa ser utilizado para diminuição de fatores de risco relacionados à diabetes mellitus tipo 2.

Embora a dieta contendo CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 aumente significativamente o colesterol HDL sérico, essa mistura de isômeros de CLA não modificou a composição corporal, o que demonstra que essa dieta foi ineficiente na prevenção da obesidade. Além disso, a mistura de CLA *cis*-9, *trans*-11 e *trans*-10, *cis*-12 CLA também causou hiperinsulinemia, hiperglicemia e resistência à insulina. Esses resultados sugerem que cautela deve ser tomada antes que suplementos sintéticos contendo CLA *cis*-9, *trans*-11 e CLA *trans*-10, *cis*-12 sejam recomendados como estratégia nutricional para a manutenção ou redução da massa corporal.

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ANEXOS

Anexo A Artigo “Protocol to cryopreserve and isolate nuclei from adipose tissue without dimethyl sulfoxide” publicado na revista *Genetics and Molecular Research.*

Anexo B Artigo “Butter naturally enriched in *cis*-9, *trans*-11 CLA improves insulin sensitivity and increases both serum HDL-cholesterol and triacylglycerol levels in rats”, publicado na revista *Lidipis in health and disease.*

Anexo C Artigo “*Cis-9, trans-11* and *trans-10, cis-12* CLA mixture does not change body composition, induces insulin resistance and increases serum HDL cholesterol level in rats” aceito para publicação na *Journal of Oleo Science* em janeiro de 2014. O artigo aceito será publicado no volume 64, número 5 de 2015.

Anexo A

GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Protocol to cryopreserve and isolate nuclei from adipose tissue without dimethyl sulfoxide

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Genet. Mol. Res. 13 (4): 10921-10933 (2014)
Received May 14, 2014
Accepted October 27, 2014
Published December 19, 2014
DOI http://dx.doi.org/10.4238/2014.December.19.14

ABSTRACT. Cryopreservation injuries involve nuclear DNA damage. A protocol for cryopreserving and isolating adipocyte nuclei is proposed. Adipose tissue samples were directly analyzed (NoCRYO-0h), or stored at -196°C for 7 days without 10% dimethyl sulfoxide (DMSO) (CRYO-WO-DMSO) or with DMSO (CRYO-W-DMSO). To determine the effect of DMSO on cryopreservation treatment, adipose tissue samples were stored at 4°C for 24 h with 10% DMSO (NoCRYO-W-DMSO-24h) and without (NoCRYO-WO-DMSO-24h). Samples were processed in isolation buffer, and nuclear integrity was measured by flow cytometry. The coefficient of variation, forward scatter, side scatter, and number of nuclei analyzed were evaluated. Pea (*Pisum sativum*) was used to measure the amount of DNA. All groups contained similar amounts of DNA to previously reported values and a satisfactory number of nuclei were analyzed. CRYO-W-DMSO

presented a higher coefficient of variation (3.19 ± 0.09) compared to NoCRYO-0h (1.85 ± 0.09) and CRYO-WO-DMSO (2.02 ± 0.02). The coefficient of variation was increased in NoCRYO-W-DMSO-24h (3.80 ± 0.01) compared to NoCRYO-WO-DMSO-24h (2.46 ± 0.03). These results relate DMSO presence to DNA damage independently of the cryopreservation process. CRYO-W-DMSO showed increased side scatter (93.46 ± 5.03) compared to NoCRYO-0h (41.13 ± 3.19) and CRYO-WO-DMSO (48.01 ± 2.28), indicating that cryopreservation with DMSO caused chromatin condensation and/or nuclear fragmentation. CRYO-W-DMSO and CRYO-WO-DMSO presented lower forward scatter (186.33 ± 9.33 and 196.89 ± 26.86, respectively) compared to NoCRYO-0h (322.80 ± 3.36), indicating that cryopreservation reduced nuclei size. Thus, a simple method for cryopreservation and isolation of adipocyte nuclei causing less damage to DNA integrity was proposed.

Key words: Adipocyte nuclei; Cryopreservation; Dimethyl sulfoxide; DNA nuclear integrity; Flow cytometry; Isolation

INTRODUCTION

Cryopreservation techniques involve the cooling of a cell or tissue up to a temperature at which all metabolic processes are arrested (Özkavukcu and Erdemli, 2002). The objective is to use ultra-low temperature to reversibly cease all biological functions of living cells and tissues. In general, cells and tissues are stored at -196°C in liquid nitrogen (Ginani et al., 2012); however, in some cases, -85°C can be used successfully (Woods et al., 2009). Nevertheless, when cryopreservation methods are considered, important factors include not only the storage temperature but also the method of reaching this temperature, as the main cause of cell death is typically not the long-term storage at low temperature, but rather the process from which cells are converted from room temperature to -60°C (Mazur, 1984, 1988). Processes used to reach low temperature can involve slow freezing or ultra-rapid freezing (Khalili et al., 2012). Both procedures may be detrimental to the cells and organelles (Fuller, 2004). The injuries induced by cryopreservation involve many different cell compartments and structures (Stolzing et al., 2012), including nuclear DNA lesions (Riesco and Robles, 2013). Thus, cryoprotectant agents are important because they can be used to improve the survival rate of living cryopreserved cells (Fuller, 2004).

Typical cryoprotectant agents are low-molecular-weight organic compounds that effectively penetrate the cells and prevent intracellular ice crystal formation. These agents include glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and propylene glycol (Meryman, 2007). DMSO is widely used alone at a 10% concentration as an effective cryoprotective agent (Pu, 2009). However, at room temperature it is considered toxic (Stolzing et al., 2012).

After cryopreservation treatment, potential damage includes DNA fragmentation, but a number of other types of lesion can occur, such as deletions, creation of abasic sites, base modifications, DNA crosslinks, or epigenetic changes (Riesco and Robles, 2013). However, these results are controversial, likely because of variations in additional factors such as the

previous state of the sample, the technique used for cryopreservation, or the cryoprotectant applied (Ribas-Maynou et al., 2014). DNA modifications produced by cryopreservation treatment should be examined to ensure the effectiveness of the technique (Riesco and Robles, 2013). Application of cryopreservation to living cells and tissues has revolutionized biotechnology, plant and animal breeding programs, and modern medicine (Fuller, 2004).

A technique for cryopreservation of adipocyte nuclei and their subsequent isolation may be a useful strategy in several research fields. The genetic material of isolated adipocyte nuclei may facilitate measurements to understand gene expression regulation or DNA integrity assessment in various fields. However, it is important that the cryopreservation and isolation methods are not responsible for damaging DNA integrity or modifying their epigenetic profile, which can affect the results obtained after cryopreservation treatment and nuclear isolation. Thus, in this study, we propose a cheaper, safer, and more practical technique for cryopreserving adipocyte nuclei and a simple method for their subsequent isolation to reduce damage to DNA integrity following these processes.

MATERIAL AND METHODS

Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-the-care-and-use-of-laboratory-animals.pdf). All procedures involving animals were approved by the Ethics Committee on Animal Experimentation of Universidade Federal de Juiz de Fora at Minas Gerais, Brazil, protocol No. 054/2012. Wistar rats (*Rattus norvegicus* Berkenhout, 1769), weighing 450-470 g, were kept in a controlled temperature environment (23 ± 2°C) with a photoperiod of 12 h (7 a.m. to 7 p.m. light and 7 p.m. to 7 a.m. dark). The animals received water and commercial chow (Nuvital; Colombo, PR, Brazil) *ad libitum* prior to euthanasia.

Adipose tissue collection and storage

To determine the effect of DMSO on cryopreserved adipocytes nuclei, the following experiment was performed. Twenty-seven adipose tissue samples were weighed after collection from the retroperitoneal fat of Wistar rats anesthetized with intraperitoneal injection of a 10 mg/kg xylazine and 90 mg/kg ketamine solution and euthanized by total exsanguination. Nine adipose tissue samples were immediately processed for nuclear isolation and used for flow cytometry analysis. This group was named No CRYOpreserved and analyzed immediately (NoCRYO-0h). Nine adipose tissue samples were deposited individually in empty cryotubes; these samples were named CRYOpreserved WithOut DMSO (CRYO-WO-DMSO). Concurrently, nine adipose tissue samples were deposited individually in cryotubes containing 1 mL 10% DMSO solution in fetal bovine serum, and named CRYOpreserved With DMSO (CRYO-W-DMSO). All samples were stored in cryotubes, and then immediately and directly transferred from room temperature (25°C) to -196°C in liquid nitrogen. After 7 days, all cryotubes were removed from the liquid nitrogen tank, placed directly at room temperature, immediately processed to nuclei isolation, and analyzed using flow cytometry as described below (Figure 1A).

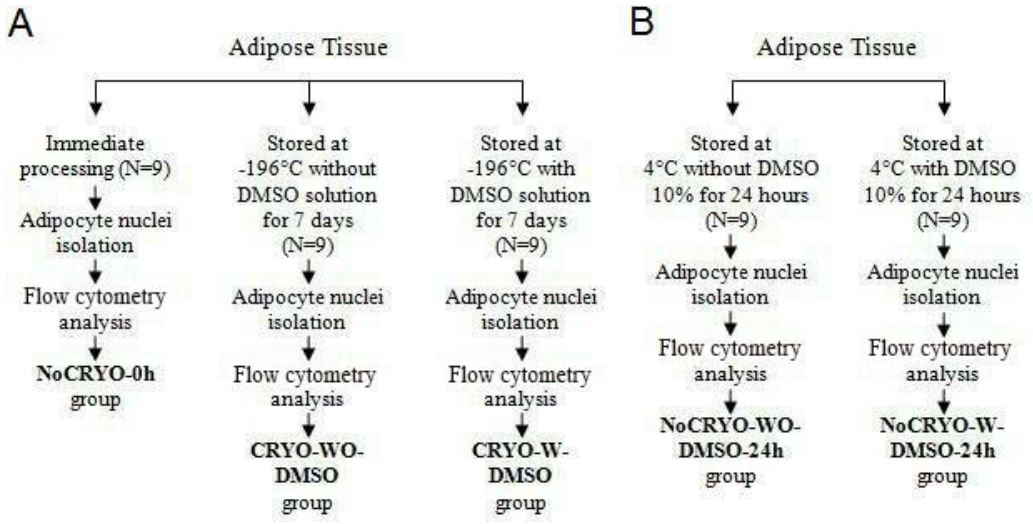

Figure 1. Flowchart showing the experimental design. **A.** Developed to assess the effect of DMSO on cryopreserved adipocyte nuclei. **B.** Developed to assess the effect of DMSO on non-cryopreserved adipocyte nuclei.

In order to determine whether the results were affected by cryopreservation in the presence of DMSO or by the presence of DMSO itself, the following experiment was performed. Eighteen adipose tissue samples were weighed after collection from the retroperitoneal fat of Wistar rats as described above. Nine adipose tissue samples were deposited individually in cryotubes containing 1 mL 10% DMSO solution in fetal bovine serum. This group was named No CRYOpreserved With DMSO for 24 h (NoCRYO-W-DMSO-24h). Nine adipose tissue samples were deposited individually in cryotubes containing 1 mL fetal bovine serum. This group was named No CRYOpreserved WithOut DMSO for 24 h (NoCRYO-WO-DMSO-24h). All cryotubes were stored at 4°C for 24 h. After this period, the samples were placed directly at room temperature, nuclei were isolated, and samples were analyzed by flow cytometry (Figure 1B).

Nuclei isolation of adipose tissue samples

The nuclei isolation method was adapted from a previous study (Dolezel et al., 2007). Briefly, each adipose tissue sample was individually placed on the center of a plastic Petri dish and 800 µL ice-cold nuclei isolation buffer was added. The nuclei isolation buffer composition was 15 mM Tris, 2 mM Na_2-ethylenediaminetetraacetic acid, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, and 0.1% (v/v) Triton X-100, at pH 7.5 adjusted with 1 M NaOH; the buffer was filtered through a 0.22-µm filter and 15 mM β-mercaptoethanol was added. Adipose tissue samples were immediately chopped in the buffer with a sharp and disposable scalpel. The homogenate was mixed by pipetting several times, avoiding air bubbles, and filtered through a 42-µm nylon mesh into a cytometry tube. In this step, we obtained a suspension of isolated adipocyte nuclei for each sample. Finally, to each of the cytometry tubes, we added 50 µL propidium iodide. Tubes were individually shaken gently and the nuclear integrity was immediately measured by flow cytometry using the FACSCanto system

(BD Biosciences; Franklin Lakes, NJ, USA). We evaluated the coefficient of variation (CV), forward scatter (FSC), side scatter (SSC), and number of nuclei analyzed (NNA). A total of 200,000 events/sample were measured. Data were analyzed using the WinMDI 2.8 software.

Determination of DNA amounts

Three adipose tissue samples were collected from retroperitoneal fat of Wistar rats as described above. Each sample collected was immediately processed in nuclei isolation buffer and analyzed by flow cytometry concurrently with a pea leaf (*Pisum sativum*), which was used as an internal standard. In addition, flow cytometry analysis of the NoCRYO-0 h, CRYO-WO-DMSO, and CRYO-W-DMSO groups and the NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h groups were always executed together with 3 samples containing only *P. sativum* prepared in nuclei isolation buffer. In all cases, the amount of DNA was determined as follows: mean fluorescence intensity of adipocyte nuclei G1 peak x 9.09 pg (pea DNA amount)/mean fluorescence intensity of pea nuclei G1 peak.

Statistical analysis

All data are reported as means ± standard error. Statistical significance ($P < 0.05$) of experimental observations among the NoCRYO-0h, CRYO-WO-DMSO, and CRYO-W-DMSO groups was determined using the Kruskal-Wallis test followed by Dunn's test. Statistical significance ($P < 0.05$) of experimental observations between the NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h groups was determined using the Mann-Whitney test. Analyses were performed using Prism 5.0 (GraphPad Software, Inc.; Palo Alto, CA, USA).

RESULTS

FLOW CYTOMETRY ANALYSIS OF ADIPOCYTE NUCLEI

Weights of adipose tissue samples in the NoCRYO-0h, CRYO-WO-DMSO and CRYO-W-DMSO groups were 0.177 ± 0.012 g, 0.231 ± 0.016 g, and 0.265 ± 0.022 g, respectively. There were no significant differences among these values. Weights of adipose tissue samples in the NoCRYO-WO-DMSO-24 h and NoCRYO-W-DMSO-24 h groups were 0.205 ± 0.020 g and 0.221 ± 0.011 g, respectively, both also statistically equal to each other.

Flow cytometry analysis for the NoCRYO-0h, CRYO-WO-DMSO, and CRYO-W-DMSO groups are shown in Table 1. The CV of CRYO-W-DMSO group was significantly higher than the CVs of the CRYO-WO-DMSO and NoCRYO-0 h groups. In addition, there was no significant difference between the CVs of the NoCRYO-0 h and CRYO-WO-DMSO groups (Figure 2A-C). FSC signals for CRYO-WO-DMSO and CRYO-W-DMSO were not significantly different; however, both results were statistically lower than those for NoCRYO-0 h FSC. In contrast, there was no significant difference between the SSC signal of CRYO-WO-DMSO and NoCRYO-0 h groups. However, the SSC of CRYO-W-DMSO was statistically higher than that of CRYO-WO-DMSO and NoCRYO-0 h (Figure 2D-F). For NNA, there was no significant difference between the NoCRYO-0 h and CRYO-W-DMSO groups. In addition, the CRYO-WO-DMSO NNA was similar to the CRYO-W-DMSO NNA and was significantly higher than the No-CRYO-0 h NNA.

Table 1. Flow cytometry analysis of adipocyte nuclei from the retroperitoneal fat of Wistar rats.

Parameter	Group		
	NoCRYO-0 h	CRYO-WO-DMSO	CRYO-W-DMSO
CV[1]	1.85 ± 0.09%[a]	2.02 ± 0.02%[a]	3.19 ± 0.09%[b]
FSC[2]	322.80 ± 3.36[a]	196.89 ± 26.86[b]	186.33 ± 9.33[b]
SSC[3]	41.13 ± 3.19[a]	48.01 ± 2.28[a]	93.46 ± 5.03[b]
NNA[4]	806 ± 60[a]	4457 ± 1244[b]	2019 ± 462[ab]

Adipose tissue samples were collected, processed immediately with the nuclei isolation buffer and analyzed by flow cytometry (NoCRYO-0 h) (N = 9) or collected, stored at -196°C without 10% DMSO solution (CRYO-WO-DMSO) (N = 9) or with DMSO (CRYO-W-DMSO) (N = 9) for 7 days, processed with the nuclei isolation buffer, and analyzed. All data are reported as means ± SE. Statistically significant differences were determined by Kruskal-Wallis test followed by the Dunn test ($P < 0.05$). Different letters indicate significant difference. [1]Coefficient of variation; [2]Forward scatter; [3]Side scatter; [4]Number of nuclei analyzed.

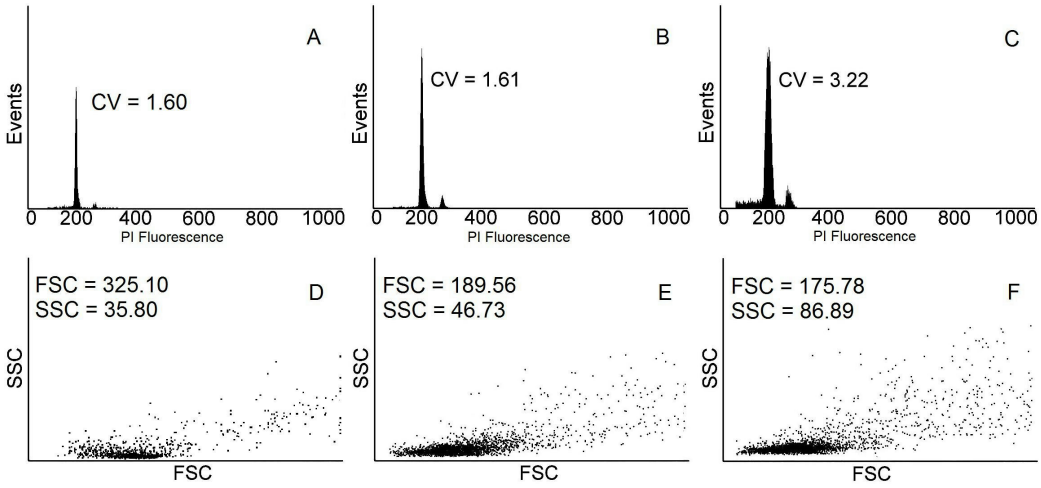

Figure 2. Representative results of flow cytometry analysis of isolated adipocyte nuclei from the retroperitoneal fat of Wistar rats. Adipose tissue samples were collected, processed immediately in nuclei isolation buffer, and analyzed by flow cytometry (NoCRYO-0 h) (N = 9) or collected, stored at -196°C without 10% DMSO solution (CRYO-WO-DMSO) (N = 9) or with DMSO (CRYO-W-DMSO) (N = 9) for 7 days, processed with the nuclei isolation buffer, and analyzed. The fluorochrome propidium iodide was used. (**A**) and (**D**) NoCRYO-0 h sample; (**B**) and (**E**) CRYO-WO-DMSO sample; (**C**) and (**F**) CRYO-W-DMSO sample. CV = coefficient of variation; FSC = forward scatter; SSC = side scatter.

Flow cytometry analysis results for NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h are shown in Table 2. The CV of NoCRYO-W-DMSO-24 h was significantly higher than the CV of NoCRYO-WO-DMSO-24 h (Figure 3A and B). FSC signals for NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h were not significantly different. There was no significant difference between the SSC of the NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h groups (Figure 3C and D). According to the NNA for each sample, there was no significant change between the two experimental groups.

Table 2. Flow cytometry analysis of non-cryopreserved adipocyte nuclei from the retroperitoneal fat of Wistar rats stored at 4°C for 24 h.

Parameter	Group	
	NoCRYO-WO-DMSO-24 h	NoCRYO-W-DMSO-24 h
CV[1]	2.46 ± 0.03%[a]	3.80 ± 0.01%[b]
FSC[2]	295.32 ± 2.45[a]	318.76 ± 8.93[a]
SSC[3]	56.43 ± 1.82[a]	55.91 ± 3.75[a]
NNA[4]	4492 ± 616[a]	3664 ± 605[a]

Adipose tissue samples were collected, stored at 4°C for 24 h with fetal bovine serum (NoCRYO-WO-DMSO-24 h) (N = 9) and with 10% DMSO in fetal bovine serum (NoCRYO-W-DMSO-24 h) (N = 9), processed with the nuclei isolation buffer, and analyzed by flow cytometry. All data are reported as means values ± SE. Statistically significant differences were determined using the Mann Whitney test ($P < 0.05$). Different letters indicate significant difference. [1]Coefficient of variation; [2]Forward scatter; [3]Side scatter; [4]Number of nuclei analyzed.

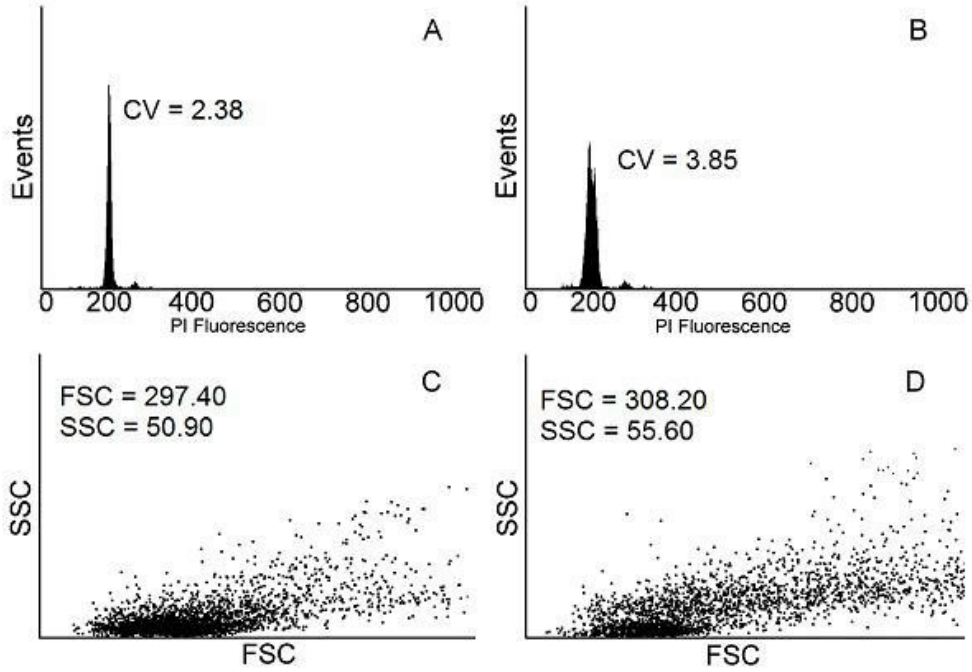

Figure 3. Representative results of flow cytometry analysis of isolated adipocyte nuclei from the retroperitoneal fat of Wistar rats. Adipose tissue samples were collected, stored at 4°C for 24 h with fetal bovine serum (NoCRYO-WO-DMSO-24 h) (N = 9) or with 10% DMSO in fetal bovine (NoCRYO-W-DMSO-24 h) (N = 9), processed with the nuclei isolation buffer, and analyzed. The fluorochrome propidium iodide was used. (**A**) and (**C**) NoCRYO-WO-DMSO-24 h sample; (**B**) and (**D**) NoCRYO-W-DMSO-24 h sample. CV = coefficient of variation; FSC = forward scatter; SSC = side scatter.

DNA amount

The 2C amount of DNA to *R. norvegicus* measured with *P. sativum* as an internal standard was 6.76 ± 0.10 pg (Figure 4). The 2C amounts of DNA for NoCRYO-0 h, CRYO-WO-DMSO, and CRYO-W-DMSO were 6.92 ± 0.01, 6.46 ± 0.05, and 6.69 ± 0.09 pg, respec-

tively. The 2C DNA amounts for NoCRYO-W-DMSO-24h and NoCRYO-WO-DMSO-24 h were 6.74 ± 0.01 and 6.59 ± 0.01 pg, respectively.

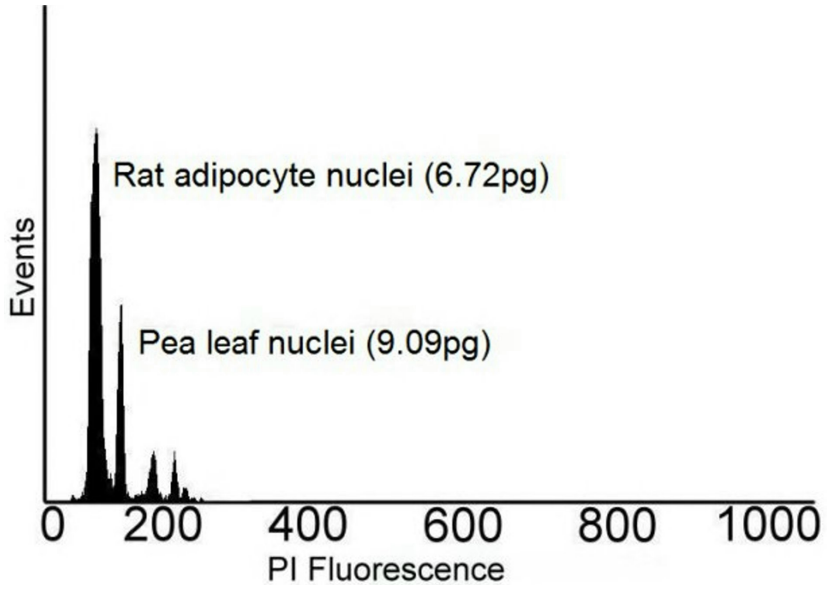

Figure 4. Histogram representing the measurement of DNA amount for rat (*Rattus norvegicus*) using pea (*Pisum sativum*) as an internal standard. Adipose tissue samples were collected from the Wistar rat retroperitoneal fat, adipocyte nuclei were immediately isolated together with *P. sativum* nuclei using isolation buffer, and analyzed by flow cytometry. The fluorochrome propidium iodide (PI) was used. The DNA amount was determined as follows: mean fluorescence intensity of adipocyte nuclei G1 peak x 9.09 pg (pea DNA amount)/mean fluorescence intensity of pea nuclei G1 peak.

DISCUSSION

For effective cryopreservation, the physicochemical characteristics of the specific cell type must be taken into account when predicting the response to freezing (Özkavukcu and Erdemli, 2002). Thus, it is relevant to consider the composition of adipose tissue. Adipocytes from white adipose tissue are characterized morphologically as having a unilocular lipid droplet that occupies 95% of the cell volume and determines the cell size, which varies from 20 to 200 μm (Lee et al., 2013). More than 95% of the total lipids stored within adipocytes are triglycerides (Lee et al., 2013), and the fat fraction range for white adipose tissue is 90-93% (Hu et al., 2010). According to a previous study, adipose depots cells were characterized by high lipid and low water contents, with values of approximately 90% and 10%, respectively (Yang et al., 2013). The low water content in adipose tissue appears to be indispensable for preserving the nuclear integrity of the adipose tissue samples stored without a cryoprotectant agent. This hypothesis is supported by the observation that water concentration in tissues or cells is one of the main factors affecting physical changes during the cooling and warming processes, as water content influences ice crystal formation, which can significantly damage tissues and cells (Stolzing et al., 2012).

Water exhibits anomalous behavior between 4°C and 0°C. In this temperature in-

terval, it expands rather than contracting (Osamu and Stanley, 1988). However, this volume increase is not sufficient to disrupt the cell membrane. As an example, a cell with a 20-μm radius has a volume of 3.45 x 10^4 μm^3. Water expansion between 4°C and 0°C is on the order of 2%; therefore, the new volume and radius may be 3.52 x 10^4 μm^3 and 20.3 μm, respectively. The elasticity of the membrane is extremely high (Diz-Muñoz et al., 2013), supporting the 0.3-μm increase in radius. Thus, the temperature decrease until 0°C can no longer damage the cell structure.

The critical moment is when the temperature achieves values below 0°C, when there is formation of ice crystals, which is an under-recognized phenomenon because water nucleation is not well understood (Morris and Acton, 2013). When a large amount of water in the cell cytoplasm is subjected to a rapid temperature decrease to temperatures well below 0°C, immediate formation of ice crystals occurs in the entire cell volume containing water. Ice crystal formation depends on the presence of water molecules for ice nucleation. Many of these crystals that form have tips, which, as they grow, can perforate the cell membrane and damage cellular compartments. However, ice crystals need to establish an action-reaction pair to exert a force against the cell's structure. If the water content in the cytoplasm is low, the action-reaction pair is not established, and when ice crystals exert pressure to perforate cell compartments, there may be displacement of the mass center of the system and no damage to the cell occurs.

Water content in adipose tissue was approximately 10% (Yang et al., 2013); thus, when this water percentage exhibits nucleation, the force required to perforate the nuclei is very small. Although ice crystals were formed, the nuclei were preserved. Importantly, the weights of adipose tissue samples were statistically equal, minimizing the interference of sample mass during the freezing and thawing processes, which may have affected the integrity of adipocyte nuclei. Therefore, similar adipose tissue masses indicate that the effects resulted from adipocyte nuclei treatments with and without DMSO as well as the cryopreservation and isolation protocols used.

CV values obtained using flow cytometry analysis supported this hypothesis because cryopreserved nuclei, even in the absence of a cryoprotectant agent, showed DNA peaks with low CVs, which occur because of the intact nuclei released from cells (Dolezel et al, 2007). Furthermore, low CV values indicate less heterogeneity in the DNA amount of nuclei released from adipose tissue. Propidium iodide, the fluorochrome used in flow cytometry analysis, intercalates into double-stranded DNA without base-dependent bias, making it suitable for estimating DNA amount (Dolezel et al., 2007). High heterogeneity in DNA amount suggests a lower quality of adipocyte nuclei because it represents higher dispersion in the DNA amount, which may be because of structural and numerical chromosomal aberrations as well as the formation of micronuclei (Otto et al., 1981). It was previously shown that treatment with a mutagen drug increased the CV in a dose-dependent manner, as measured by flow cytometry (Otto et al., 1981).

Nuclear DNA amounts can be analyzed with high precision with the CV of DNA peaks generally ranging from 1 to 5% (Dolezel et al., 2007); these values are considered acceptable, whereas CVs below 3% are considered to be good (Dolezel et al., 2007). Thus, it is possible to characterize both methods of cryopreservation without and with 10% DMSO as acceptable. The CRYO-WO-DMSO CV was significantly lower than the CRYO-W-DMSO CV and was statistically equal to the NoCRYO-0 h CV, indicating the higher quality of adipocyte nuclei that had been cryopreserved without DMSO as the cryoprotectant agent.

In the NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h groups, the CV was significantly higher in the former than for non-cryopreserved nuclei treated without DMSO. Therefore, considering or not cryopreservation treatment, DMSO presence was always associated with an increased CV, indicating that the presence of DMSO itself increased the heterogeneity in the DNA amount of nuclei released from adipose tissue and, consequently, decreased the quality of adipocyte nuclei. This observation is supported by studies that showed the effect of DMSO on increasing the DNA fragmentation percentage and DNA damage (Filipak et al., 2007). Moreover, only groups treated without DMSO, NoCRYO-0 h, CRYO-WO-DMSO, and NoCRYO-WO-DMSO-24 h, had CV values lower than 3%, which is considered good (Dolezel et al., 2007).

The amounts of DNA obtained from the NoCRYO-0 h, CRYO-WO-DMSO, and CRYO-W-DMSO groups were very similar to the 2C values of 6.10 pg (Vinogradov, 1988) and 6.72 pg (Peterson et al., 1994), which were also determined using flow cytometry for *R. norvegicus*, supporting that the protocol proposed was not responsible for DNA loss. The amount of DNA obtained from the NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h groups was also similar to the literature values, demonstrating that DMSO presence was not responsible for DNA loss.

Analysis of nucleus size based on FSC signals (Wlodkowic et al., 2012) revealed that the size of cryopreserved nuclei was not related to the use of DMSO solution, because in both cryopreservation treatments, the nuclei size was smaller than that observed in NoCRYO-0 h group. Therefore, the decrease in size of the cryopreserved nuclei was not positively correlated with the presence or absence of DMSO as a cryoprotectant agent. This reduction in nuclei size resulted from the low temperature to which these cell compartments were exposed (Atkinson et al., 2006). The FSC signal was statistically equivalent between NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h groups. In addition, the presence of DMSO did not affect nucleus size.

In contrast, nuclear granularity or complexity was analyzed by measuring the SSC signal (Wlodkowic et al., 2012). We found that SSC was significantly increased in CRYO-W-DMSO compared to CRYO-WO-DMSO, whereas CRYO-WO-DMSO SSC was statistically equivalent to NoCRYO-0 h SSC. This indicates increased chromatin condensation and/or nuclear fragmentation (Wlodkowic et al., 2012) in nuclei cryopreserved with DMSO. This result clearly demonstrates the effect of DMSO on cryopreserved adipocyte nuclei. If this damage was caused by the cryopreservation treatment used, it would also be observed in adipocyte nuclei that had been cryopreserved without DMSO, as the CRYO-WO-DMSO and CRYO-W-DMSO groups were subjected to the same cryopreservation conditions. Some studies showed that cryopreservation itself caused significant changes in the DNA methylation pattern (Hao et al., 2002a,b; Kaczmarczyk et al., 2010), while another study showed that DNA methylation was not affected by cryopreservation treatment (Klaver et al., 2012). In this study, cryopreservation treatment itself was not responsible for increasing the SSC signal, as this parameter was increased only in the group cryopreserved in DMSO. Therefore, changes in SSC occurred because of cryopreservation treatment in the presence of DMSO and did not result from the cryopreservation treatment itself. Increased chromatin condensation in cryopreserved nuclei with DMSO agrees with the results of previous studies reporting that DMSO affects the cellular epigenetic profile by inducing DNA hypermethylation (Hao et al., 2002a; Kawai et al., 2010). This may be related to chromatin condensation and thus heterochromatin expansion. Analysis of the results of SSC for non-cryopreserved nuclei treated with and without DMSO showed

that DMSO itself was not associated with increased chromatin condensation, as the SSC NoCRYO-W-DMSO-24 h group was statistically equivalent to the SSC NoCRYO-WO-DMSO-24 h group. Thus, increased chromatin condensation resulted from cryopreservation in the presence of DMSO, as the SSC of NoCRYO-W-DMSO-24 h group was not increased compared to that of the NoCRYO-WO-DMSO-24 h group, and the SSC of the CRYO-W-DMSO group was increased compared to those of the CRYO-WO-DMSO and NoCRYO-0 h groups.

Based on the number of nuclei analyzed, there are two potential explanations for these results. The first and the most important is related to the nuclei isolation method. It was very difficult to ensure that all samples in isolation buffer were chopped identically, contributing to higher or lower nuclei release from the adipose tissue. This can also explain the high values of the standard error in this parameter. The second reason is regarding the amount of adipose tissue in each sample. Although these values were not statistically different, they were also not identical, which may have contributed to the differences in this parameter among groups. Considering all experimental groups, the number of nuclei analyzed in each group was a low percentage of the 200,000 events, which occurred due to a methodological choice described as follows. The cytometer was configured to count events over a wide interval of fluorescence intensities. This enabled determination of the amount of background debris produced by each cryopreservation treatment. Based on the NNA results, debris formation was not stimulated by cryopreservation without or with DMSO or by DMSO presence in experiments without cryopreservation treatment, as the number of nuclei analyzed was statistically equivalent between the CRYO-WO-DMSO and CRYO-W-DMSO groups and between the NoCRYO-W-DMSO-24 h and NoCRYO-WO-DMSO-24 h groups.

Surfactants were present in the isolation buffer, and they are a chemical class that can cause DNA damage (Yam et al., 1984). Thus, application of nuclei isolation buffer as proposed in this study was validated by determining the DNA content of non-cryopreserved nuclei using pea as an internal standard. The DNA amount measured in adipocytes (6.76 ± 0.10 pg) was very similar to the 2C values of 6.10 pg (Vinogradov, 1998) and 6.72 pg (Peterson et al., 1994), both also determined using flow cytometry for *R. norvegicus*. This shows that the nuclei isolation buffer was not responsible for DNA loss and demonstrated that the buffer was effective for isolating adipocyte nuclei.

In this study, we proposed a simple method for cryopreserving adipocyte nuclei without using DMSO as cryoprotectant agent, and their subsequent isolation. The use of this technique is dependent upon the specific circumstances. A useful application of the proposed technique is related to the cryopreservation of adipocyte nuclei followed by a simple method for isolating these nuclei. Isolated adipocyte nuclei can be used in studies examining gene expression regulation with numerous protocols. Thus, it is possible to validate methods to quantify nuclear factor-kB (Napetschnig and Wu, 2013), protein kinase A (Prusov et al., 2013), proteins of the Notch pathway (Fiúza and Arias, 2007), and intracellular receptors (Re et al., 2010) in these isolated nuclei. In addition, isolated nuclei transcriptome studies can be conducted based on RNA synthesis and quantification (Grindberg et al., 2013).

Furthermore, our flow cytometry results revealed more injuries in adipocytes nuclear DNA when DMSO was used, indicating that DMSO should not be used in studies evaluating the structure of cryopreserved nuclear DNA, such as when identifying DNA fragmentation using the TdT dUTP nick-end labeling (TUNEL) technique (Wlodkowic et al., 2012), because the presence of DMSO may cause changes in DNA, affecting the results. In addition, nuclei cryopreserved with DMSO presented a significantly increased SSC signal, indicating

increased chromatin condensation. This result suggests that, when possible, it is better to use alternative cryoprotectant agents to DMSO in cell culture studies, mainly because increased chromatin condensation can silence genes, thereby modifying cell physiology.

In conclusion, we suggest a protocol for the cryopreservation and isolation of nuclei from adipose tissue from Wistar rat retroperitoneal fat stored using an ultra-rapid freezing technique in liquid nitrogen without 10% DMSO solution. The absence of DMSO as a cryoprotective agent is advantageous because the presence of DMSO appears to cause nuclear DNA damage of adipocytes. Furthermore, cryopreservation in the presence of DMSO was associated with increased chromatin condensation and/or nuclear fragmentation. Moreover, the absence of DMSO and ultra-rapid freezing makes the proposed technique cheaper, easier, more practical, and safer.

ACKNOWLEDGMENTS

The authors would like to thank Jacy Gameiro from the Parasitology, Microbiology, and Immunology Department of Universidade Federal de Juiz de Fora for technical support. Research supported by Universidade Federal de Juiz de Fora (UFJF) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes).

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Anexo B

LIPIDS IN HEALTH AND DISEASE

RESEARCH Open Access

Butter naturally enriched in *cis*-9, *trans*-11 CLA prevents hyperinsulinemia and increases both serum HDL cholesterol and triacylglycerol levels in rats

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Abstract

Background: Evidence from *in vitro* and animal studies indicates that conjugated linoleic acid (CLA) possesses anti-diabetic properties, which appear to be attributed to *cis*-9, *trans*-11 CLA, the major CLA isomer in ruminant fat. However, there is a shortage of studies addressing CLA from natural source. The present study aimed to evaluate the effects of butter naturally enriched in *cis*-9, *trans*-11 CLA on parameters related to glucose tolerance, insulin sensitivity and dyslipidemia in rats.

Methods: Forty male Wistar rats were randomly assigned to the following dietary treatments (n = 10/group), for 60 days: 1) Normal fat-Soybean oil (NF-So): diet containing 4.0% soybean oil (SO); 2) High Fat-Control Butter (HF-Cb): diet containing 21.7% control butter and 2.3% SO; 3) High Fat-CLA enriched Butter (HF-CLAb): diet containing 21.7% *cis*-9, *trans*-11 CLA-enriched butter and 2.3% SO; and 4) High fat-Soybean oil (HF-So): diet containing 24.0% SO. HF-Cb and HF-CLAb diets contained 0.075% and 0.235% of *cis*-9, *trans*-11 CLA, respectively.

Results: HF-CLAb-fed rats had lower serum insulin levels at fasting than those fed with the HF-Cb diet, while the PPARγ protein levels in adipose tissue was increased in HF-CLAb-fed rats compared to HF-Cb-fed rats. Furthermore, R-QUICK was lower in HF-Cb than in NF-So group, while no differences in R-QUICK were observed among NF-So, HF-CLAb and HF-So groups. Serum HDL cholesterol levels were higher in HF-CLAb-fed rats than in those fed NF-So, HF-Cb and HF-So diets, as well as higher in NF-So-fed rats than in HF-Cb and HF-So-fed rats. HF-CLAb, HF-Cb and HF-So diets reduced serum LDL cholesterol levels when compared to NF-So, whereas serum triacylglycerol levels were increased in HF-CLAb.

Conclusion: Feeding rats on a high-fat diet containing butter naturally enriched in *cis*-9, *trans*-11 CLA prevented hyperinsulinemia and increased HDL cholesterol, which could be associated with higher levels of *cis*-9, *trans*-11 CLA, vaccenic acid, oleic acid and lower levels of short and medium-chain saturated fatty acids from butter naturally modified compared to control butter. On the other hand CLA-enriched butter also increased serum triacylglycerol levels, which could be associated with concomitant increases in the content of *trans*-9 and *trans*-10 C18:1 isomers in the CLA-enriched butter.

Keywords: High conjugated linoleic acid enriched butter, Functional food, Rats, Insulin sensitivity, Dyslipidemia, Diabetes

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Background

Diabetes mellitus is an important cause of mortality and morbidity worldwide, with harmful effects on life expectancy and health-care costs [1]. According to the World Health Organization [2], type 2 diabetes comprises 90% of the total population with diabetes mellitus around the world, and is characterized by the body's ineffective use of insulin. It is projected that the number of people with diabetes mellitus worldwide will rise to 439 million by 2030 [3]. There is compelling evidence that diet plays an important role in the prevention of a number of non-communicable diseases, including type-2 diabetes [4]. In this context, conjugated linoleic acid (CLA) has attracted considerable attention in the scientific community due to its health-promoting properties reported in a number of *in vitro* and animal studies [5]. CLA refers to the positional and geometric conjugated dienoic isomers of linoleic acid (C18:2 n-6) [6] which are predominantly found in ruminant fat [7]. Although nearly twenty isomers have been identified in ruminant products [8], 75-90% of total CLA is represented by *cis*-9, *trans*-11 CLA (rumenic acid) [9], whereas the *trans*-10, *cis*-12 CLA isomer is normally found in very low concentrations [10].There is some evidence that the anti-diabetogenic effects reported in several studies are mediated by rumenic acid [11], the major CLA isomer in ruminant fat.

As dairy products are the major source of CLA in the human diet [7], efforts have been made to increase the milk fat CLA content, which can be achieved by including plant oils in the diet of dairy cows [12,13]. Most of the *cis*-9, *trans*-11 CLA secreted in milk is synthesized endogenously from trans-11 C18:1 (vaccenic acid) through stearoyl-CoA desaturase enzyme (also known as Δ-9 desaturase). Therefore, milk naturally enriched in *cis*-9, *trans*-11 CLA is also a rich source of vaccenic acid [14]. Endogenous synthesis of *cis*-9, *trans*-11 CLA from C18:1 *trans*-11 has also been reported in humans [15] and other species [16,17], which further contributes to increasing the *cis*-9, *trans*-11 CLA levels in the body tissues. It should also be noted that the concentrations of some minor (e.g. *trans*-C18:1 isomers other than vaccenic) and major (e.g. medium-chain saturated) fatty acids are also altered in milk fat from cows fed diets supplemented with plant oils [13], which should be taken into account when food sources naturally enriched in CLA are used in a given study.

In light of the potential anti-diabetogenic effects of *cis*-9, *trans*-11 CLA observed in previous studies and the shortage of studies addressing CLA from natural source, we investigated the effects of a diet containing butter naturally enriched in *cis*-9 *trans*-11 CLA on glucose tolerance, insulin sensitivity and dyslipidemia in Wistar rats.

Results

Food intake of HF-Cb, HF-CLAb and HF-So diets was 20.76%, 19.54% and 27.60% lower than NF-So food intake, respectively, while no difference was observed between HF-Cb, HF-CLAb and HF-So (Table 1). The energy intake observed in rats fed with the HF-Cb, HF-CLAb and HF-So diets was 15.85%, 13.95% and 11.04% higher than in NF-So-fed rats, respectively, but there was no difference among HF-Cb, HF-CLAb and HF-So (Table 1). No differences in weight gain (expressed as a percentage of initial weight) were observed among treatment groups (Table 1).

The effect of NF-So, HF-Cb, HF-CLAb and HF-So diets on body weight during all experimental period is shown in Figure 1. There were no differences among dietary treatments.

Concerning the carcass chemical composition, no differences in moisture, lipid, protein and ash contents was observed among groups (Table 1). PPARγ protein levels in adipose tissue were decreased by 58.70%, 62.35% and 41% in HF-Cb-fed rats in comparison to those fed with the NF-So, HF-CLAb and HF-So diets, respectively (Figure 2) (Additional files 1, 2, 3 and 4).

Fasting serum insulin levels increased by 21.73%, 11.60% and 23.65% in HF-Cb-fed rats in comparison to those fed with the NF-So, HF-CLAb and HF-So diets, respectively (Figure 3A), whereas there were no differences in glycemia levels among experimental groups (Figure 3B). NEFA and leptin did not differ among dietary treatments (Table 1).

HOMA index was unchanged by the dietary treatments (Table 1). However, the HF-Cb group had a lower R-QUICKI index (13.63%) than NF-So, while no difference was observed among HF-CLAb, HF-So and NF-So groups (Table 1). There were no differences in the area under the OGTT glycemic curve (AUC) among dietary treatments (Table 1). Serum cholesterol levels did not differ between HF-CLAb and NF-So groups, whereas there were no differences between HF-Cb and HF-So (Figure 4A). Serum triacylglycerol levels in HF-CLAb were increased by 58.81%, 49.54% and 131.12% when compared to NF-So, HF-Cb and HF-So groups, respectively (Figure 4B). Serum levels of HDL cholesterol were increased by 10.08%, 23.29% and 25.76% in HF-CLAb-fed rats as compared to those fed with the NF-So, HF-Cb and HF-So diets, respectively (Figure 4C). There was no difference in serum LDL cholesterol levels between rats fed with the HF-Cb and HF-CLAb diets, but values observed in these groups were 39.68% and 36.88% lower than in NF-So group, respectively, and 21.05% and 17.37% lower than in HF-So, respectively (Figure 4D). There was no difference in the LDL cholesterol:HDL cholesterol ratio between HF-Cb and HF-CLAb groups, and these values were lower than HF-So result. The LDL

Table 1 Metabolic and serum parameters in Wistar rats fed with control or naturally enriched in *cis*-9, *trans*-11 CLA butters for 60 days

	Dietary treatments			
	NF-So[1]	HF-Cb[2]	HF-CLAb[3]	HF-So[4]
Dietary intake and weight gain				
Intake (g/day/rat)	26.45 ± 1.06	20.96 ± 0.37***	21.33 ± 0.49***	19.15 ± 0.49***
Intake (Kcal/day/rat)	63.19 ± 2.52	73.21 ± 1.31**	72.01 ± 1.67**	70.17 ± 1.89*
Weight gain (%)	62.15 ± 1.90	69.31 ± 2.13	66.05 ± 2.41	59.80 ± 3.32
Body composition				
Moisture (%)	50.10 ± 1.05	50.03 ± 0.47	48.19 ± 0.44	50.83 ± 1.17
Lipid (%)	29.41 ± 1.38	28.55 ± 0.64	31.31 ± 0.50	27.14 ± 1.36
Protein (%)	17.76 ± 0.32	17.60 ± 0.21	16.96 ± 0.19	17.57 ± 0.49
Ash (%)	3.38 ± 0.05	4.13 ± 0.09	3.66 ± 0.28	3.87 ± 0.38
Insulin Sensibility Indexes and AUC				
HOMA index	1.11 ± 0.02	1.40 ± 0.10	1.39 ± 0.16	1.08 ± 0.05
R-QUICKI	0.88 ± 0.02	0.76 ± 0.03*	0.82 ± 0.02	0.81 ± 0.04
AUC	13180 ± 1505	12330 ± 1158	14390 ± 1398	14610 ± 1021
Serum metabolites				
NEFA (mmol/L)	0.375 ± 0.023	0.325 ± 0.017	0.354 ± 0.022	0.294 ± 0.025
Leptin (ng/mL)	2.21 ± 0.21	2.59 ± 0.26	2.72 ± 0.35	1.99 ± 0.20
LDL-C[5]/HDL-C[6]	1.42 ± 0.07	0.93 ± 0.04***,##	0.81 ± 0.05***,###	1.17 ± 0.06**
non-HDL-C/HDL-C	1.73 ± 0.11	1.39 ± 0.08*	1.33 ± 0.07**	1.46 ± 0.05*

Data are presented as mean values ± S.E.M (n = 10 rats/group). Statistically significant differences were determined by Anova followed by Newman-Keuls. Asterisk denotes statistically significant differences compared to NF-So (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) and number sign denotes statistically significant differences compared to HF-So (##$p < 0.01$, ###$p < 0.001$). [1]Normal Fat-Soybean oil (NF-So), diet containing 4.0% soybean oil (SO); [2]High Fat-Control Butter (HF-Cb), diet containing 21.7% control butter and 2.3% SO; [3]High CLA Butter (HF-CLAb), diet containing 21.7% butter naturally enriched in *cis*-9, *trans*-11 CLA and 2.3% SO; [4]High Fat-Soybean oil (HF-So), diet containing 24.0% SO.
[5]LDL-C: LDL cholesterol; [6]HDL-C:HDL cholesterol.

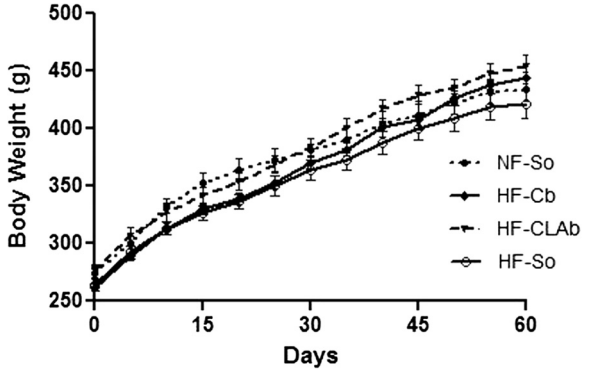

Figure 1 Effect of control or naturally enriched in *cis*-9, *trans*-11 CLA butters on body weight. Male Wistar rats fed the following dietary treatments for 60 days: Normal fat-Soybean oil (NF-So): diet containing 4.0% soybean oil (SO); High Fat-Control Butter (HF-Cb): diet containing 21.7% control butter and 2.3% SO; High Fat-CLA enriched Butter (HF-CLAb): diet containing 21.7% *cis*-9, *trans*-11 CLA-enriched butter and 2.3% SO; High fat-Soybean oil (HF-So): diet containing 24.0% SO. All data are presented as mean values ± S.E.M (n = 10 rats/group). Statistically significant differences were determined by Anova followed by Newman-Keuls. *$p < 0.05$, **$p < 0.01$.

cholesterol:HDL cholesterol ratio of high fat diet groups were lower than the value of NF-So (Table 1). There was no difference in the non-HDL cholesterol: HDL cholesterol ratio among HF-Cb, HF-CLAb and HF-So groups, while these values were lower than NF-So result (Table 1).

Discussion

In recent years, conjugated linoleic acid has received much attention as a dietary supplement [11], however few studies assess the effects of CLA from natural sources on insulin, glucose and serum lipid metabolism. In this paper, we have demonstrated dietary effects of *cis*-9, *trans*-11 CLA-enriched butter in 60-day-old Wistar rats on feed intake, body composition, insulin and glucose metabolism as well as dyslipidemia.

In this study, there were no differences in dietary intake among rats fed with *cis*-9, *trans*-11 CLA-enriched butter, control butter or high fat-soybean oil. HF-Cb, HF-CLAb or HF-So-fed rats adapted to the higher energy density of these diets by reducing their daily food intake compared to the NF-So group, as was previously reported [18]. Daily energy intake was higher in HF-Cb,

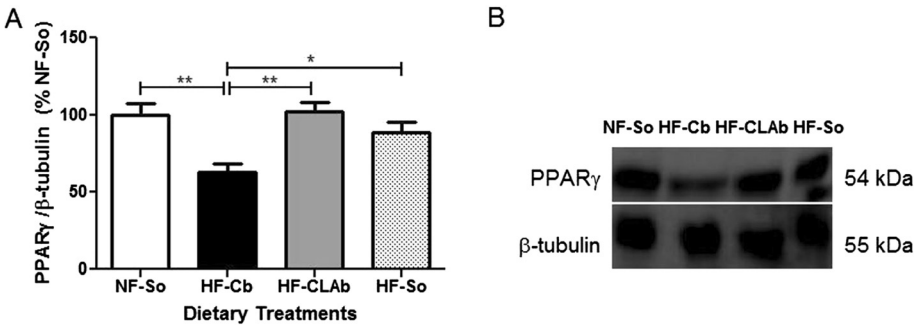

Figure 2 Analysis of PPARγ protein level in retroperitoneal adipose tissue. PPARγ levels **(A)** and representative blot for PPARγ and β-tubulin (loading control) (additional Electrophoretic blot files show this in more detail [see Additional files 1, 2, 3 and 4]) **(B)** of male Wistar rats fed the following dietary treatments for 60 days: Normal fat-Soybean oil (NF-So): diet containing 4.0% soybean oil (SO); High Fat-Control Butter (HF-Cb): diet containing 21.7% control butter and 2.3% SO; High Fat-CLA enriched Butter (HF-CLAb): diet containing 21.7% *cis*-9, *trans*-11 CLA-enriched butter and 2.3% SO; High fat-Soybean oil (HF-So): diet containing 24.0% SO. All data are presented as mean values ± S.E.M (n = 10 rats/group). Statistically significant differences were determined by Anova followed by Newman-Keuls. **p* <0.05, ***p* <0.01.

HF-CLAb and HF-So-fed rats than in the NF-So group, which can be attributed to the increased palatability of high fat diets, which is directly related to higher energetic intake [19]. High fat diets are more palatable because fat content is one of the factors that contribute to food palatability [19].

Experiments have shown that PPARγ is the master adipogenic regulator [20] and, interconnected to its role in adipocyte differentiation, PPARγ regulates insulin sensitivity by transcriptionally activating genes involved in insulin signaling, glucose uptake, and fatty acid uptake and storage [21]. HF-CLAb-fed rats presented increased levels of PPARγ in adipose tissue compared to HF-Cb-fed rats, which may be attributed to higher (213.20%) supply of *cis*-9, *trans*-11 CLA from the CLA-enriched butter diet in comparison to the control butter diet. Studies have demonstrated that *cis*-9, *trans*-11 CLA increased the expression of PPARγ, whose down-regulation may lead to insulin resistance [22]. It was demonstrated that CLA mixed with 0.286% *cis*-9, *trans*-11 CLA increased the mRNA expression of PPARγ in adipose tissue of Wistar rats, which was related to improved insulin sensitivity [23]. Besides, it was shown that depletion of PPARγ in adipose tissue causes insulin resistance, since decreased PPARγ action in mature adipocytes, leads to reduced expression of key genes required for insulin signaling in adipocytes [24]. It was previously shown that adipocyte-specific constitutive activation of PPARγ in mature adipocytes can regulate whole body insulin sensitivity [25].

Therefore, CLA-enriched butter was shown as having action mechanisms PPARγ-dependent, up-regulating its expression in adipose tissue, and preventing PPARγ reduction as was observed by a control butter diet.

Rats fed with *cis*-9, *trans*-11 CLA-enriched butter had lower fasting serum insulin levels than rats fed with control butter. Therefore HF-CLAb diet prevented the fasting hyperinsulinemia, which is a result potentially beneficial. According to the European Group for the

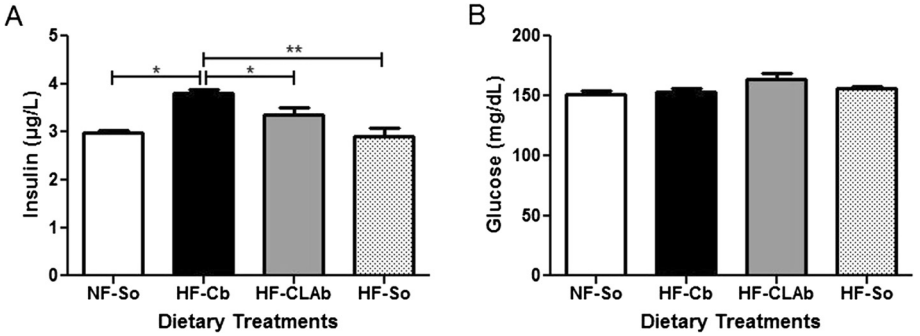

Figure 3 Effects of control or naturally enriched in *cis*-9, *trans*-11 CLA butters on serum metabolites. Insulin **(A)** and glucose **(B)** of male Wistar rats fed the following dietary treatments for 60 days: Normal fat-Soybean oil (NF-So): diet containing 4.0% soybean oil (SO); High Fat-Control Butter (HF-Cb): diet containing 21.7% control butter and 2.3% SO; High Fat-CLA enriched Butter (HF-CLAb): diet containing 21.7% *cis*-9, *trans*-11 CLA-enriched butter and 2.3% SO; High fat-Soybean oil (HF-So): diet containing 24.0% SO. All data are presented as mean values ± S.E.M (n = 10 rats/group). Statistically significant differences were determined by Anova followed by Newman-Keuls. **p* <0.05, ***p* <0.01.

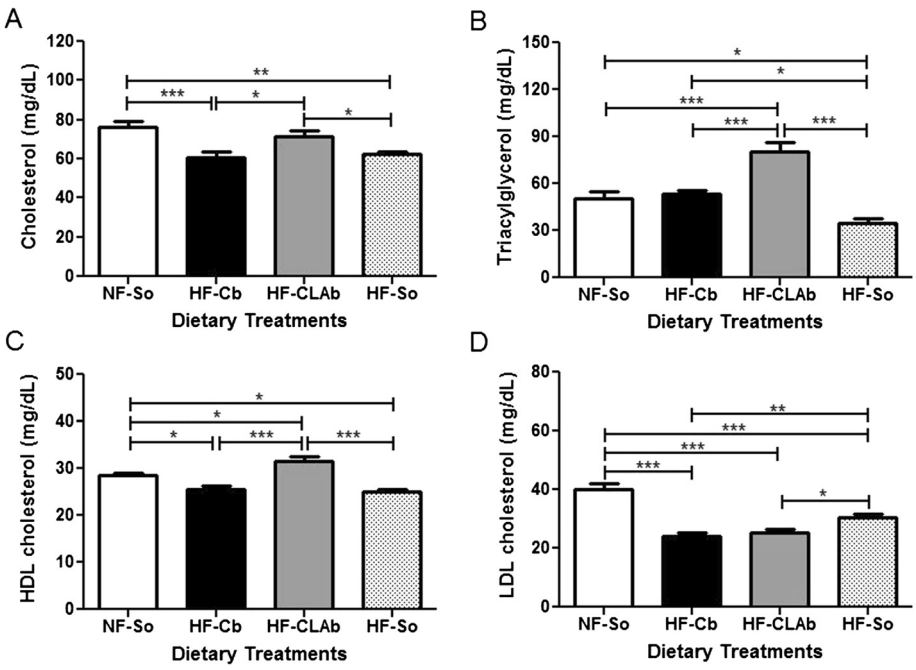

Figure 4 Effects of control or naturally enriched in *cis*-9, *trans*-11 CLA butters on lipid serum. Cholesterol **(A)**, triacylglycerol **(B)**, HDL cholesterol **(C)** LDL cholesterol **(D)** of male Wistar rats fed the following dietary treatments for 60 days: Normal fat-Soybean oil (NF-So): diet containing 4.0% soybean oil (SO); High Fat-Control Butter (HF-Cb): diet containing 21.7% control butter and 2.3% SO; High Fat-CLA enriched Butter (HF-CLAb): diet containing 21.7% *cis*-9, *trans*-11 CLA-enriched butter and 2.3% SO; High fat-Soybean oil (HF-So): diet containing 24.0% SO. All data are presented as mean values ± S.E.M (n = 10 rats/group). Statistically significant differences were determined by Anova followed by Newman-Keuls. *p <0.05, **p <0.01, ***p <0.001.

Study of Insulin Resistance, fasting insulin is the best available simple proxy for insulin resistance, which is defined by presence of fasting hyperinsulinemia [26]. Besides, it was demonstrated that a gradual increase in serum insulin in the fasting state reflects decreased insulin sensitivity [27]. HOMA index did not differ among experimental groups, however R-QUICKI index, which also denotes insulin sensitivity [28], was lower in the HF-Cb group compared to the NF-So group, while there was no difference among the NF-So, HF-CLAb and HF-So groups. Thus, R-QUICKI index shows that control butter diet induces insulin resistance compared to normal fat diet, a condition that was not observed in HF-CLAb group and may be associated to PPARγ reduced level in adipose tissue of HF-Cb-fed rats [24].

The beneficial effect of *cis*-9, *trans*-11 CLA-enriched butter on fasting insulin level might be due to the higher supply of *cis*-9, *trans*-11 CLA from the CLA-enriched butter diet in comparison to the control butter diet. It was previously shown that animals fed with a 0.25% *cis*-9, *trans*-11 CLA diet decreased serum insulin concentration at fasting [11]. As observed in Table 1, the concentrations of several fatty acids were also altered in the HF-CLAb diet as compared to the HF-Cb diet. For instance, there was a higher (269.72%) supply of vaccenic acid from HF-CLAb diet compared to HF-Cb diet, which contributed to increase the tissue level of *cis*-9, *trans*-11 CLA in HF-CLAb-fed rats [16]. Furthermore, there was a lower (32.06%) supply of short and medium-chain saturated fatty acids from HF-CLAb diet compared to HF-Cb diet, which could also have contributed to the decreased fasting serum insulin level of the HF-CLAb group, since it has been suggested that diets high in saturated fatty acids have effects on hyperinsulinemia [29-31]. Despite the changed parameters of HF-Cb-fed rats, the areas under the curves of oral glucose tolerance tests did not differ among NF-So, HF-Cb, HF-CLAb and HF-So-fed rats, therefore the experimental diets were not responsible for glucose intolerance.

Serum NEFA concentration is a risk factor for type 2 diabetes because the combination of excessive levels of non-esterified fatty acids and glucose leads to decreased insulin secretion, impairments in insulin gene expression and beta-cell death by apoptosis [32]. Previous studies showed that *cis*-9, *trans*-11 CLA reduced NEFA levels [11] however, in the present investigation, there were no differences among groups. The lack of an effect of butter enriched in *cis*-9, *trans*-11 CLA on NEFA may be attributed to altered bioavailability and bioactivity of *cis*-9, *trans*-11 CLA when inserted into the fat butter. A similar hypothesis was developed when it was observed less distinct effect of high-CLA beef compared to synthetic

CLA on the proteome of insulin-sensitive tissues [33]. Leptin is an adipokine that plays a role in glucose metabolism and insulin sensitivity [34], however in the present study there were no differences among groups. Similarly, it was shown in previous studies that *cis*-9, *trans*-11 CLA did not alter leptin levels [11,22,35].

In the present work, serum cholesterol and LDL cholesterol concentrations were not modified by the HF-CLAb diet compared to the NF-So and HF-Cb diets, respectively. Similarly, no effects of *cis*-9, *trans*-11 CLA on cholesterol and LDL cholesterol levels were also shown previously [36,37]. The high LDL cholesterol concentration in NF-So-fed rats may be due to high levels of carbohydrate (73.39% of energy) in this diet, since it was demonstrated that when dietary carbohydrate was increased from 50% to 67% of energy, the fasting triacylglycerol level rose [38], which is commonly related to increased precursors of LDL cholesterol in the blood, the very-low-density lipoproteins, and consequently increased LDL cholesterol levels [39]. Decreased total cholesterol concentration in HF-Cb or HF-So-fed rats was related to the low HDL cholesterol level in these groups, which is a risk factor for type 2 diabetes mellitus [40].

Increased triacylglycerol levels in HF-CLAb-fed rats may be due to higher (160.37%) contents of *trans*-9 and *trans*-10 C18:1 isomers in the HF-CLAb diet compared to the HF-Cb diet. It has been shown that high intake of *trans*-9 C18:1 was correlated to increased plasma concentration of triacylglycerol [41] as well as the high intake of *trans*-10 C18:1 [42]. Concerning the effect of *cis*-9, *trans*-11 CLA on the triacylglycerol level, previous studies in animals fed with this CLA isomer did not modify triacylglycerol concentration [43,44]. However, rats fed with the HF-CLAb diet had an increased HDL cholesterol level, which is a potentially beneficial result because it reduces the risk of having a cardiovascular event [45] and HDL cholesterol also has a positive effect on glycemic control [45]. The high level of HDL cholesterol in HF-CLAb-fed rats may be attributed to a higher level of *cis*-9, *trans*-11 CLA, as also reported by a previous study [46]. Similarly, it was demonstrated that high CLA enriched clarified butter increased plasma HDL cholesterol in Wistar rats [47]. However, it is possible that the higher supply of oleic acid (*cis*-9 C18:1) (27,61%) from the HF-CLAb diet compared to the HF-Cb diet may also have contributed to increased HDL cholesterol levels, since it has been suggested that oleic acid has effects on increasing HDL cholesterol [48]. Besides, there was a lower (36.91%) supply of lauric (C12:0) and myristic (C14:0) acids from HF-CLAb diet than HF-Cb diet, which could also have contributed to raised HDL cholesterol levels of HF-CLAb group, since it was demonstrated that a lauric and myristic acid-rich diet decreased HDL cholesterol concentration [49]. On the other hand, the HF-CLAb diet had higher (147.82%) levels of *trans*-9 C18:1, which has been associated with decreased levels of HDL cholesterol [50]. Therefore, we hypothesized that fatty acids related to increased HDL cholesterol level were capable of acting synergistically, prevailing over negative effects of *trans*-9 C18:1 isomers on HDL cholesterol levels, resulting in higher concentration of this lipoprotein in HF-CLAb-fed rats. However, concerning the triacylglycerol levels, it has already been demonstrated by a previous study with animals fed with butter naturally enriched in *cis*-9 *trans*-11 CLA that this diet had no effect on the plasma concentration of triacylglycerol [14]. Thus, it was possible to hypothesize that the higher contents of *trans*-9 and *trans*-10 C18:1 isomers in the HF-CLAb diet prevailed over the absence of *cis*-9 *trans*-11 CLA effects on triacylglycerol levels, resulting in a higher concentration of triacylglycerol in HF-CLAb-fed rats.

Conclusion

In conclusion, the present investigation suggests that a 60 day feeding of a diet containing butter naturally enriched in *cis*-9, *trans*-11 CLA to 60-day-old male Wistar rats has effects on insulin, HDL cholesterol and triacylglycerol metabolism. *Cis*-9, *trans*-11 CLA-enriched butter significantly raised serum HDL cholesterol and prevented fasting hyperinsulinemia, which could be attributed to higher levels of *cis*-9, *trans*-11 CLA, vaccenic acid, oleic acid and lower levels of short and medium-chain saturated fatty acids from CLA-enriched butter compared to control butter. However, CLA-enriched butter was also found to cause fasting hypertriglyceridemia, which could be associated with concomitant increases in the content of *trans*-9 and *trans*-10 C18:1 isomers in the CLA-enriched butter. Additional studies are still needed before conjugated linoleic acid from natural sources can be used in human diets as a functional food to decrease type-2 diabetes risk factors.

Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals [51]. All procedures with animals were approved by the Ethic Committee on Animal Experimentation of Federal University of Juiz de Fora at Minas Gerais, Brazil, protocol number 054/2012.

Animals

Forty (n = 40) male Wistar rats (*Rattus norvegicus Berkenhout, 1769*), 60 days old and weighing 250–300 g, were obtained from the Center of Reproduction Biology of the Federal University of Juiz de Fora,

Minas Gerais, Brazil. They were kept in a controlled temperature environment (23 ± 2°C) with a photoperiod of 12 hours (7 a.m. to 7 p.m. - light and 7 p.m. to 7 a.m. - dark). Water and the experimental diets were offered on an *ad libitum* basis to the animals throughout the study.

Production of experimental butters

Experimental butters used in the current study were produced at Embrapa Dairy Cattle (Juiz de Fora, Minas Gerais, Brazil). Standard butter and *cis*-9, *trans*-11 CLA-enriched butter were produced from milk of cows (Holstein x Gir) fed diets composed of either corn silage and concentrate containing no sunflower oil, or chopped elephant grass and concentrate supplemented with sunflower oil at 4.5% of diet dry matter, respectively. The butters were produced as described previously [52].

Dietary treatments and experimental design

After a 7 day acclimatization period in which all animals were fed a commercial chow (Nuvital, Colombo, PR, Brazil), the rats were randomly assigned to four dietary treatments (n = 10/group), for 60 days: 1) Normal fat-Soybean oil (NF-So): diet containing 4.0% soybean oil (SO); 2) High Fat-Control Butter (HF-Cb): diet containing 21.7% control butter and 2.3% SO; 3) High Fat-CLA enriched Butter (HF-CLAb): diet containing 21.7% *cis*-9, *trans*-11 CLA-enriched butter and 2.3% SO; and 4) High fat-Soybean oil (HF-So): diet containing 24.0% SO. SO was included in both HF-Cb and HF-CLAb diets in order to reach the requirements of linoleic and linolenic acids to adults rats [53].

All diets were produced according to the American Institute of Nutrition (AIN-93 M) [53]. Ingredients were carefully mixed in order to obtain a homogeneous mass which was used to produce handmade pellets. The pellets were prepared weekly, purged with nitrogen and stored at −20°C in daily portions in sealed polythene bags to minimize the oxidation of fatty acids. The composition of purified diets is presented in Table 2.

Samples of pellets (50 g) from each diet were randomly collected and analyzed for chemical composition according to reference methods [54,55]. To determine the fatty acid composition of experimental diets, total lipids were extracted according to Hara and Radin [56] using a 3:2 (vol:vol) mixture of hexane and isopropanol (4.5 mL/g of pellet) followed by a 67 g/L of sodium sulfate solution (3 mL/g of pellet). Fatty acid methyl esters (FAME) were obtained by base-catalyzed transmethylation using a freshly prepared methylation reagent (0.4 mL of 5.4 mol/L of sodium methoxide solution + 1.75 mL of methanol) according to Christie et al., [57] with modifications [58]. The mixture was neutralized with oxalic acid (1 g of oxalic acid in 30 mL diethyl ether) and calcium chloride was added to remove methanol residues. The

Table 2 Ingredient composition of experimental diets

Ingredient	% of the diet (g/100 g of diet)
Corn starch[2]	46.6 or 29.1[a,b,c,d]
Dextronized corn starch[2]	15.5
Casein[1]	14.0 or 17.3[a,b,c,d]
Sucrose[1]	10.0
Cellulose[2]	5.0
AIN-93 mineral mix[1]	3.5
AIN-93 vitamin mix[1]	1.0
L-Cystine[2]	0.18
Choline bitartrate[2]	0.25
tert-Butylhydroquinone[1]	0.01
SO[3] or Butter[4] + SO[a,b,c]	4.0 or 24.0[a,b,c,d]

[1,2]Dietary ingredients were purchased from Rhoster (Araçoiaba da Serra, SP, Brazil) and Farmos (Rio de Janeiro, RJ, Brazil); [3]Soybean oil (SO); [4]Control Butter or High CLA Butter. [a]Normal Fat-Soybean diet consisted of 46.6% corn starch, 14.0% casein and 4.0% SO; [b]High Fat-Control butter diet consisted of 29.1% corn starch, 17.3% casein and 21.7% Standard Butter + 2.3% SO; [c]High Fat-CLA enriched butter diet consisted of 29.1% corn starch, 17.3% casein and 21.7% High CLA Butter + 2.3% SO; [d]High Fat-Soybean oil diet consisted of 29.1% corn starch, 17.3% casein and 24.0% SO.

FAME were determined by gas chromatography (model 6890 N; Agilent Technologies Brasil Ltda., Barueri, Brazil) fitted with a flame-ionization detector and equipped with a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm × 0.2 μm film thickness; Varian Inc., Mississauga, ON). Operating conditions included injector and detector temperatures both at 250°C, H_2 as the carrier gas (1 mL/min), and for the flame-ionization detector (35 mL/min), N_2 as the makeup gas (30 mL/min), and purified air (286 mL/min). The initial temperature was 45°C and held for 4 min, increased by 13°C/min to 175°C and held for 27 min, and increased by 4°C/min to 215°C and held for 35 min [59]. The FAME were identified by comparison with 4 FAME reference standards (Supelco37 mix #47885-U, linoleic acid isomers mix #47791, CLA isomers mix #05632; Sigma-Aldrich, St. Louis, MO, and Nu-Chek GLC-463); minor *trans*-18:1 isomers were identified according to their elution order reported under the same chromatographic conditions [59,60]. The fatty acid composition of experimental diets was expressed as a weight percentage of total fatty acids using theoretical relative response factors described by Wolff et al., [61] (Table 3).

The *cis*-9, *trans*-11 CLA content in HF-Cb and HF-CLAb diets was calculated as follows: (dry matter content of the diet) x (fat content x 0.95) x (Concentration of *cis*-9, *trans*-11 CLA in g/100 g of total fatty acids). The 5% discount on fat content was applied to correct for the glycerol concentration in triacylglycerol molecules [62]. Based on the above-mentioned calculations, the *cis*-9, *trans*-11 CLA contents in HF-Cb and HF-CLAb diets were 0.075% and 0.235%, respectively. However, considering that about 11% of vaccenic acid (*trans*-11 C18:1) is

Table 3 Chemical composition and fatty acid profile of the experimental diets

	Dietary treatments			
	NF-So[2]	HF-Cb[3]	HF-CLAb[4]	HF-So[5]
	Chemical composition,% of diet dry matter			
Dry matter content (%)	79.1	86.8	85.4	88.4
Fat	3.11	17.6	17.4	21.1
Crude protein	13.1	16.0	16.2	14.8
Ash	2.76	2.98	3.09	2.95
Neutral Detergent Fiber (NDF)	2.76	3.55	3.26	3.89
Carbohydrate	55.4	44.8	43.4	42.7
	Energetic composition			
Carbohydrate Energy (%)	73.4	44.6	43.9	40.7
Protein Energy (%)	17.4	15.9	16.4	14.1
Fat Energy (%)	9.35	39.5	39.6	45.2
Kcal/g	2.39	3.49	3.38	3.71
	Fatty acids (g/100 g of total fatty acids)			
C4:0	n.d.[1]	3.16	2.95	n.d.
C5:0	n.d.	0.03	0.01	n.d.
C6:0	n.d.	1.69	1.37	n.d.
C7:0	n.d.	0.02	0.01	n.d.
C8:0	n.d.	1.00	0.64	n.d.
C9:0	n.d.	0.03	0.01	n.d.
C10:0	n.d.	2.07	1.14	n.d.
C10:1 *cis*-9	n.d.	0.26	0.12	n.d.
C11:0	n.d.	0.02	0.01	n.d.
C12:0	n.d.	2.37	1.25	n.d.
C12:1 *cis*-9/C13:0	n.d.	0.16	0.08	n.d.
C14:0	0.52	8.71	5.74	0.54
C15:0 *iso*	n.d.	0.20	0.25	n.d.
C15:0 *anteiso*	n.d.	0.41	0.47	n.d.
C14:1 *cis*-9	n.d.	0.83	0.46	n.d.
C15:0	n.d.	0.95	0.90	n.d.
C16:0	11.7	29.3	19.7	11.8
C16:1 *trans*-9	n.d.	0.03	0.03	n.d.
C17:0 *iso*	n.d.	0.32	0.51	n.d.
C16:1 *cis*-9 + C17:0 *anteiso*	n.d.	1.51	1.16	n.d.
C17:0	n.d.	0.49	0.51	n.d.
C17:1 *cis*-9	n.d.	0.18	0.19	n.d.
C18:0	4.25	9.02	13.9	4.23
C18:1 *trans*-4	n.d.	0.02	0.07	n.d.
C18:1 *trans*-5	n.d.	0.02	0.06	n.d.
C18:1 *trans*-6/7/8	n.d.	0.31	0.80	n.d.
C18:1 *trans*-9	n.d.	0.23	0.57	n.d.
C18:1 *trans*-10	n.d.	0.30	0.81	n.d.
C18:1 *trans*-11	n.d.	1.09	4.03	n.d.
C18:1 *trans*-12	n.d.	0.29	0.65	n.d.

Table 3 Chemical composition and fatty acid profile of the experimental diets *(Continued)*

C18:1 *trans*-13/14	n.d.	0.24	0.49	n.d.
C18:1 *cis*-9/*trans*-15	23.8	20.3	25.9	22.4
Minor *cis*-C18:1 isomers (c11 + c12 + c13)	1.43	0.83	1.03	1.45
C18:1 *trans*-16	n.d.	0.23	0.36	n.d.
C18:1 *cis*-14	n.d.	0.05	0.10	n.d.
C19:0/C18:1 *cis*-15	n.d.	0.11	0.11	n.d.
C18:2 *trans*-9 *trans*-12	n.d.	0.01	0.01	n.d.
C18:2 *cis*-9 *trans*-12	0.09	0.04	0.06	0.08
C18:2 *trans*-9 *cis*-12	n.d.	0.03	0.04	n.d.
C18:2 *cis*-9 *cis*-12	49.5	8.04	7.15	52.4
C20:0	0.36	0.18	0.20	0.35
C18:3 *cis*-6, *cis*-9 *cis*-12	n.d.	0.02	0.01	n.d.
C20:1 *cis*-11	n.d.	0.06	0.12	n.d.
C18:3 *cis*-9 *cis*-12 *cis*-15	6.16	0.96	0.89	6.58
CLA *cis*-9 *trans*-11	n.d.	0.53	1.66	n.d.
CLA *trans*-10 *cis*-12	n.d.	0.01	0.01	n.d.
CLA *trans*-11 *cis*-13	n.d.	0.01	0.02	n.d.
C21:0	n.d.	0.03	0.03	n.d.
C20:2 *cis*-11, *cis*-14	n.d.	0.02	0.02	n.d.
C22:0	0.41	0.11	0.13	0.30
C20:3 n-6	n.d.	0.05	0.04	n.d.
C20:4 n-6	n.d.	0.10	0.08	n.d.
C23:0	n.d.	0.03	0.01	n.d.
C20:5 n-3 (EPA)	n.d.	0.02	0.01	n.d.
C24:0	0.15	0.06	0.06	0.16
C22:5 n3 (DPA)	n.d.	0.06	0.06	n.d.
C22:6 n-3 (DHA)	n.d.	n.d.	n.d.	n.d.

[1]n.d.: not detected; [2]Normal Fat-Soybean oil (NF-So), diet containing 4.0% soybean oil (SO); [3]High Fat-Control Butter (HF-Cb), diet containing 21.7% control butter and 2.3% SO; [4]High CLA Butter (HF-CLAb), diet containing 21.7% butter naturally enriched in *cis*-9, *trans*-11 CLA and 2.3% SO; [5]High Fat-Soybean oil (HF-So), diet containing 24.0% SO.

endogenously converted into rumenic acid in rodents [16], the increase expected of *cis*-9, *trans*-11 CLA in tissue levels of HF-CLAb-fed rats is approximately 15% higher than the levels in HF-Cb-fed rats. The rats were provided fresh food (F_i) *ad libitum* daily (between 11 a.m and 12 p.m) and the refusals were weighed the next day (F_f), immediately before the provision of another F_i. Average food intake (grams/animal) was estimated as follows: (F_i - F_f)/5 (number of animals per cage). Individual body weight was measured every 5 days throughout the treatment period. After the treatment period, the rats were fasted for 12 hours (7 a.m. to 7 p.m.) and blood samples collected from a tail nick for glycemic determinations using the glucose oxidase method [63]. Immediately after glycemic determinations, animals were anesthetized with an intraperitoneal injection of a xylazine (10 mg/Kg)/ketamine (90 mg/Kg) solution, and euthanized by total exsanguination. Glycemic determinations were performed prior to anesthesia as it was shown to induce hyperglycemia [64]. After euthanasia, blood samples, adipose tissue samples and carcasses were analyzed for parameters related to insulin sensitivity and dyslipidemia in rats.

Analysis of carcass chemical composition

The carcasses were eviscerated, sliced, stored at −80°C, lyophilized (model Liotop L120; Liobras, São Carlos, Brazil) and minced in a knife-type mill. Carcasses were weighed before and after lyophilization to determine their dry matter contents. Moisture, ash, protein and lipid contents were determined according to reference methods [54]. Protein content was quantified using the Kjeldahl method with Foss equipment (model Kjeltec 8400, Foss, Hillerød, Denmark) and lipid content was determined using the Ankom procedure with an Ankom extractor (model XT10, Ankom Technology, New York, USA).

Analysis of PPARγ protein level by western blot

Retroperitoneal adipose tissue samples were homogenized in a lysis buffer [Tris–HCl: 50 mM, pH 7.4, $Na_4P_2O_7$: 30 mM, NP-40: 1%, Triton (1%), SDS: 0.1%, NaCl: 150 mM, EDTA: 5 mM, NaF: 50 mM, plus Na_3VO_4: 1 mM and protease inhibitor cocktail (Roche Diagnostics, Mannheim, DE)] using an Ultra-Turrax homogenizer (IKA Werke, Staufen, DE). After centrifugation (7500 × g for 5 min), the homogenates were stored at −20°C until SDS-PAGE assay. The total protein content of homogenate was determined by the BCA protein assay kit (Pierce, Illinois, USA). Contents of peroxisome proliferator-activated receptor (PPAR)γ and β-tubulin (loading control) proteins in the retroperitoneal adipose tissue samples were evaluated by incubating monoclonal primary antibodies (anti-PPARγ and anti-β-tubulin; 1:1000; from Abcam, Cambridge, UK) overnight at 4°C, followed by proper secondary antibody (1 hour; 1:7000 antibody from Sigma-Aldrich Co., Missouri, USA) and streptavidin (1 hour; 1:7000; Zymed, California, USA) incubation. The protein bands were visualized by chemiluminescence with Kit ECL Plus (GE Healthcare Life Sciences, Buckinghamshire, UK) followed by exposure in the ImageQuant™ LAS 500 (GE Healthcare Life Sciences). Area and density of the bands were quantified by Image J software (Media Cybernetics, Maryland, USA). The results were normalized by β-tubulin content and expressed as relative (%) to NF-So group.

Serum metabolites

Blood samples were collected from euthanized animals by cardiac puncture and centrifuged (5714 × g for 5 min) for serum separation. Serum insulin levels were determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Serum non-esterified fatty acids (NEFA) levels were analyzed using a colorimetric kit (Randox Laboratories, Antrim, United Kingdom), while leptin was analyzed using a Leptin ELISA kit (R&D Systems, Minneapolis, USA). Serum levels of cholesterol, triacylglycerol, HDL cholesterol and LDL cholesterol were determined by colorimetry using the BT 3000 equipment from Wiener laboratories.

HOMA and R-QUICKI

Homeostatic Model Assessment (HOMA) index was calculated as follows: [fasting insulin (ng/ml) × fasting glucose (mM)]/22.5. A high HOMA index denotes low insulin sensitivity [65], although it should be acknowledged that the HOMA model has not been validated for use in animal models [66]. The Revised Quantitative Insulin Sensitivity Check Index (R-QUICKI) is another equation to assess insulin sensitivity [28]. This index was calculated as following: [1/log fasting insulin (mU/ml) + log fasting glucose (mg/dl) + log NEFA (mmol/l)] [28].

Oral glucose tolerance test (OGTT)

After 55 days on the experimental diets, the rats were fasted for 12 hours (7 a.m. to 7 p.m) and received a 50% glucose solution (2 g/kg body weight) by oral gavage [67]. Blood samples were collected from a tail nick for glycemic determinations using the glucose oxidase method [63] at 0, 30, 60, 90, 120 and 240 minutes post gavage. Due to reasons previously described, anesthesia was not used in the OGTT. Changes in blood glucose concentration during the oral glucose tolerance test were evaluated by estimation of the total area under the curve (AUC) calculated as an incremental considering the response from the starting point that was analyzed and using the trapezoidal method [68].

Statistical analysis

The statistical analyses were performed using Prism 5.0 (GraphPad Software, Inc). Data from different dietary groups were analyzed by one-way ANOVA for overall significance followed by Newman-Keuls's post-hoc tests to identify differences between treatment groups. Results were expressed as means ± SEM (standard error mean). Treatment effects and differences between means were considered significant when $p < 0.05$.

Additional files

Additional file 1: Complete electrophoretic blot of representative bands of PPARγ level in adipose tissue of Wistar rats. Figure containing complete electrophoretic blot of representative bands of PPARγ level shown in Figure 2.

Additional file 2: Complete electrophoretic blot of representative bands of PPARγ level in adipose tissue of Wistar rats. Figure containing complete electrophoretic blot of representative bands of PPARγ level shown in Figure 2. In this file we indicate the experimental group related to each band.

Additional file 3: Complete electrophoretic blot of representative bands of β-tubulin (loading control) level in adipose tissue of Wistar rats. Figure containing complete electrophoretic blot of representative bands of β-tubulin level shown in Figure 2.

Additional file 4: Complete electrophoretic blot of representative bands of β-tubulin level (loading control) in adipose tissue of Wistar rats. Figure containing complete electrophoretic blot of representative bands of β-tubulin level shown in Figure 2. In this file we indicate the experimental group related to each band.

Abbreviations

CLA: Conjugated linoleic acid; NF-So: Normal fat-soybean oil; SO: Soybean oil; HF-Cb: High fat-control butter; HF-CLAb: High fat-CLA enriched butter; HF-So: High fat-soybean oil; FAME: Fatty acid methyl esters; PPARγ: Peroxisome proliferator-activated receptor γ; HOMA: Homeostatic model assessment; R-QUICKI: Revised quantitative insulin sensitivity check index; OGTT: Oral glucose tolerance test; AUC: Area under the curve.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MMA conducted the production of experimental diets, rodent feeding experiments, analyzed data, performed statistical analyses and helped to draft the manuscript. SCPDL and CMS conducted the production of

experimental diets and helped draft the manuscript. JOAC, LGR, EPSC, PCL, EGM and JG provided technical support, helped to interpret data and draft the manuscript. MASG and FCFL assisted with cow feeding and milk collection, production and analysis of experimental butter and diets and helped to draft the manuscript. RMGG oversaw all aspects of the experiments, helped to interpret data and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements
Authors are thankful to Embrapa Dairy Cattle, Coordination of Improvement of Higher Education Personnel (CAPES), National Counsel of Technological and Scientific Development (CNPq), Foundation for Research Support of the Minas Gerais State (FAPEMIG) and Federal University of Juiz de Fora (UFJF) for the financial grants to carry out this work.

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Received: 21 July 2014 Accepted: 4 December 2014
Published: 22 December 2014

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doi:10.1186/1476-511X-13-200
Cite this article as: de Almeida *et al.*: Butter naturally enriched in *cis*-9, *trans*-11 CLA prevents hyperinsulinemia and increases both serum HDL cholesterol and triacylglycerol levels in rats. *Lipids in Health and Disease* 2015 **13**:200.

Anexo C

Journal of Oleo Science
Copyright ©2015 by Japan Oil Chemists' Society
J-STAGE Advance Publication date : Aaaaaa 00, 2015
doi : 10.5650/jos.ess14222
J. Oleo Sci.

Cis-9, *trans*-11 and *trans*-10, *cis*-12 CLA Mixture does not Change Body Composition, Induces Insulin Resistance and Increases Serum HDL Cholesterol Level in Rats

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Abstract: Synthetic supplements of conjugated linoleic acid (CLA) containing 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers have been commercialized in some places for reducing body fat. However the safety of this CLA mixture is controversial and in some countries the CLA usage as food supplement is not authorized. Changes in insulinemic control and serum lipids profile are potential negative effects related to consumption of CLA mixture. The present study aimed to evaluate the effects of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA on prevention of obesity risk as well as on potential side effects such as insulin resistance and dyslipidemia in Wistar rats. Thirty male Wistar rats were randomly assigned to the following dietary treatments (n=10/group), for 60 days: Normolipidic Control (NC), diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C), diet containing 24.0% SO; High Fat-synthetic CLA (HF-CLA), diet containing 1.5% of an isomeric CLA mixture (Luta-CLA 60) and 22.5% SO. Luta-CLA 60 (BASF) contained nearly 60% of CLA (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA at 50:50 ratio). The HF-CLA diet contained 0.3% of each CLA isomer. HF-CLA diet had no effect on dietary intake and body composition. HF-CLA-fed rats had lower levels of PPARγ protein in retroperitoneal adipose tissue, hyperinsulinemia compared to HF-C-fed rats, hyperglycemia compared to NC-fed rats while no differences in glycemia were observed between NC and HF-C groups, increased HOMA index and higher levels of serum HDL cholesterol. Thus, feeding rats with a high fat diet containing equal parts of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers had no effect on body composition and induced insulin resistance. Despite HF-CLA-fed rats had increased serum HDL cholesterol levels, caution should be taken before synthetic supplements containing *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are recommended as a nutritional strategy for weight management.

Key words: *cis*-9, *trans*-11 CLA; *trans*-10, *cis*-12 CLA; obesity; insulin resistance; dyslipidemia

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Accepted January 6, 2015 (received for review October 4, 2014)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

1 INTRODUCTION

The prevalence of overweight and obesity is increasing at an alarming rate[1]. According to World Health Organization[2], once considered a problem only in high income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries. In 2008 a total of more than half a billion adults were obese worldwide[3] and it is projected that this number will rise to 1.12 billion by 2030[1]. Furthermore, overweight and obesity are major risk factors for a number of chronic diseases, since visceral adipose tissue is an independent risk factor for insulin resistance, type 2 diabetes mellitus[4], hypertension, subclinical inflammation and dyslipidemia, all factors leading to atherosclerosis[5].

There is compelling evidence that diet plays an important role in the prevention of a number of non-communicable diseases, including obesity and type-2 diabetes[6]. In this context, conjugated linoleic acid (CLA) has attracted considerable attention in the scientific community due to its health-promoting properties reported in a number of *in vitro* and animal studies[7]. CLA refers to the positional and geometric conjugated dienoic isomers of linoleic acid (C18:2 n-6)[8] which are predominantly found in the lipid fraction of meat, milk and other dairy products from ruminant[9]. Although nearly twenty isomers have been identified in ruminant products[10], *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are the more biologically active[11] and they are the major CLA isomers in the commercial preparations[12].

Diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA or containing *trans*-10, *cis*-12 CLA alone have been shown as having anti-obesity properties[8, 13], such as decreased body fat mass, increased lean body mass[12] and reduction of energy intake[11]. Synthetic supplements of CLA containing 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers have been commercialized in some places for reducing body fat[14]. However the safety of CLA isomer mixture to assist in weight loss is not unanimity in the scientific community and the CLA isomer mixture usage as food supplement is not authorized in some countries[15, 16]. Hyperinsulinemia associated with insulin resistance[8] which may be responsible for increasing type 2 diabetes risk, decreased serum HDL cholesterol and increased serum LDL cholesterol, are side effects which have been already reported as related to CLA isomer mixture consumption[15].

Thus, use of mixture of active CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 as dietary supplement is controversial and mechanisms of action for CLA effects are not yet fully understood[11]. More studies are needed to elucidate these mechanisms of action, providing valuable information on the efficacy, specificity, and potential side effects of CLA isomer mixture, before its use as a dietary strategy for weight loss. In light of potential anti-obesity properties and side effects of conjugated linoleic acid, we investigated the effects of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA on preventing obesity risk as well as on insulin resistance and dyslipidemia in Wistar rats.

2 EXPERIMENTAL

2.1 Ethics statement

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals[17]. All procedures with animals were approved by the Ethic Committee on Animal Experimentation of Federal University of Juiz de Fora at Minas Gerais, Brazil, protocol number 053/2012.

2.2 Animals

Thirty (n = 30) male Wistar rats (*Rattus norvegicus Berkenhout, 1769*), 60 days old and weighing 250-300 g, were obtained from the Center of Reproduction Biology of the Federal University of Juiz de Fora (UFJF), Minas Gerais, Brazil. They were kept in a controlled temperature environment (23 ± 2℃) with a photoperiod of 12 hours (7 a.m. to 7 p.m. - light and 7 p.m. to 7 a.m. - dark). Water and the experimental diets were offered on an *ad libitum* basis to the animals throughout the study.

2.3 Dietary treatments and design experimental

After a 7 days acclimatization period in which all animals were fed a commercial chow (Nuvital, Colombo, PR, Brazil), the rats were randomly assigned to three dietary treatments (n = 10/group), for 60 days: Normolipidic Control (NC), diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C), diet containing 24.0% SO; High Fat-enriched synthetic CLA (HF-CLA), diet containing 1.5% Luta-CLA 60 and 22.5% SO. Luta-CLA 60 (BASF AG, São Paulo, Brazil) is composed of 30% *cis*-9, *trans*-11 CLA, 30% *trans*-10, *cis*-12 CLA, 27.4% C18:1 *cis*-9/*trans*-15, 4.06% C16:0, 3.64% C18:0, 1.23% C18:2 *cis*-9, *cis*-12, 0.06% C14:0.

All diets were produced according to the American Institute of Nutrition (AIN-93M)[18]. Ingredients were carefully mixed in order to obtain a homogeneous mass which was used to produce handmade pellets. The pellets were prepared weekly, purged with nitrogen and stored at −20℃ in daily portions in sealed polythene bags to minimize the oxidation of fatty acids. The composition of purified diets is presented in Table 1.

Samples of pellets (50 g) from each diet were randomly collected and analyzed for chemical composition according to reference methods[19, 20]. To determine the fatty acid composition of experimental diets, total lipids were extracted according to Hara and Radin[21]. Fatty acid methyl esters (FAME) were obtained according to Christie[22] with

Table 1 Composition of purified experimental diets.

	NC[1]	HF-C[2]	HF-CLA[3]
	% of the diet (g/100g of total diet)		
Corn starch[4]	46.6	29.1	29.1
Dextronized corn starch[4]	15.5	15.5	15.5
Casein[5]	14.0	17.3	17.3
Sucrose[5]	10.0	10.0	10.0
Cellulose[4]	5.0	5.0	5.0
AIN-93 mineral mix[5]	3.5	3.5	3.5
AIN-93 vitamin mix[5]	1.0	1.0	1.0
L-Cystine[4]	0.18	0.18	0.18
Choline bitartrate[4]	0.25	0.25	0.25
tert-Butylhydroquinone[5]	0.01	0.01	0.01
Soybean oil	4.00	24.00	22.5
CLA mix[6]	–	–	1.50

[1] Normolipidic Control; [2] High Fat-Control; [3] High Fat-CLA; [4, 5] Dietary ingredients were purchased from Farmos (Rio de Janeiro, RJ, Brazil) and Rhoster (Araçoiaba da Serra,SP,Brazil), respectively; [6] Luta-CLA 60 (BASF AG, São Paulo, Brazil) composed of 60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA.

modifications[23] and determined by gas chromatography (model 6890 N; Agilent Technologies Brasil Ltda., Barueri, Brazil) fitted with a flame-ionization detector and equipped with a CP-Sil 88 fused silica capillary column (100 m × 0.25 mm × 0.2 μm film thickness; Varian Inc., Mississauga, ON). Operating conditions included injector and detector temperatures both at 250℃, H_2 as the carrier gas (1 mL/min), and for the flame-ionization detector (35 mL/min), N_2 as the makeup gas (30 mL/min), and purified air (286 mL/min). The FAME were identified by comparison with 4 FAME reference standards (Supelco37 mix #47885-U, linoleic acid isomers mix #47791, CLA isomers mix #05632; Sigma-Aldrich, St. Louis, MO, and Nu-Chek GLC-463); minor *trans*-18:1 isomers were identified according to their elution order reported under the same chromatographic conditions[24, 25]. The fatty acid composition of experimental diets was expressed as a weight percentage of total fatty acids using theoretical relative response factors described by Wolff[26]. The chemical composition and the fatty acid profile of experimental diets are presented in Table 2.

The CLA isomers contents in HF-CLA diet was calculated as follows: (dry matter content of the diet) x (fat content x 0.95) x (Concentration of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in g/100g of total fatty acids). The 5% discount on fat content was applied to correct for the glycerol concentration in triacylglycerol molecules[27]. Based on the above-mentioned calculations, the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA contents in HF-CLA diet were 0.3% and 0.3%, respectively.

The rats were provided (between 11 a.m. and 12 p.m.) fresh food (F_i) *ad libitum* daily and the refusals were weighed the next day (F_f), immediately before the provision of another F_i. Average food intake (grams/animal) was estimated as follows: (F_i - F_f)/5 (number of animals per cage). Individual body weight was measured every 5 days throughout the treatment period. After the treatment period, the rats were fasted for 12 hours (7 a.m. to 7 p.m.) and blood samples collected from a tail nick for glycemic determinations using the glucose oxidase method[28]. Immediately after glycemic determinations, animals were anesthetized with an intraperitoneal injection of a xylazine (10 mg/Kg)/ketamine (90 mg/Kg) solution, and euthanized by total exsanguination. Glycemic determinations were performed prior to anesthesia as it was shown to induce hyperglycemia[29]. After euthanasia, it was determined the liver weight, blood and adipose tissue samples and carcasses were analyzed for parameters described below.

2.4 Analysis of carcass chemical composition

The carcasses were eviscerated, being the gastrointestinal contents and the brain removed while all fat depots, including the perivisceral fat, remained in the carcasses, which were sliced, stored at −80℃, lyophilized (model Liotop L120; Liobras, São Carlos, Brazil) and minced in a knife-type mill. The carcasses were weighed before and after lyophilization to determine their dry matter content. Moisture, ash, protein and lipid contents were determined according to AOAC[19]. Protein content was quantified using

Table 2 Chemical composition and fatty acid profile of experimental diets.

	NC[1]	HF-C[2]	HF-CLA[3]
	Chemical composition, % of diet dry matter		
Dry matter content (%)	79.1	88.4	88.3
Fat	3.11	21.07	20.9
Crude protein	13.1	14.83	15.2
Ash	2.66	2.95	2.95
Neutral Detergent Fiber (NDF)	2.76	3.89	3.00
Carbohydrate	55.4	42.75	44.5
	Energetic composition		
Carbohidrate Energy (%)	73.4	40.7	41.7
Protein Energy (%)	17.3	14.1	14.3
Fat Energy (%)	9.25	45.2	44.0
Kcal/g	2.39	3.71	3.77
	Fatty acid profile (g/100 g of total fatty acids)		
C14:0	0.14	0.13	0.12
C16:0	10.9	10.8	10.6
C18:0	4.44	4.06	4.06
C18:1 *cis*-9/*trans*-15	23.5	22.4	23.5
C18:2 *cis*-9 *cis*-12	49.8	52.4	49.8
C20:0	0.41	0.35	0.30
C20:1 *cis*-11	0.04	0.03	0.03
C18:3 *cis*-9 *cis*-12 *cis*-15	6.11	6.58	5.81
CLA *cis*-9 *trans*-11	n.d.[4]	n.d.	1.70
CLA *trans*-10 *cis*-12	n.d.	n.d.	1.77
C22:0	0.45	0.30	0.21
C24:0	0.04	0.05	0.07

[1] Normolipidic Control, diet containing 4% of soybean oil (SO); [2] High fat-Control, diet containing 24% SO; [3] High fat-enriched synthetic CLA, diet containing 1.5% Luta-CLA 60 (BASF AG, São Paulo, Brazil) and 22.5% SO. Luta-CLA 60 is composed of 60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA; [4] n.d.: not detected.

the Kjeldahl method[19] with Foss equipment (model Kjeltec 8400, Foss, Hillerød, Denmark) and lipid content was determined using the Ankom procedure with an Ankom extractor (model XT10, Ankom Technology, New York, USA).

2.5 Analysis of PPARγ protein level by Western blot

Retroperitoneal adipose tissue samples were homogenized in a lysis buffer [Tris-HCl: 50 mM, pH 7.4, $Na_4P_2O_7$: 30 mM, NP-40: 1%, Triton (1%), SDS: 0.1%, NaCl: 150 mM, EDTA: 5 mM, NaF: 50 mM, plus Na_3VO_4: 1 mM and protease inhibitor cocktail (Roche Diagnostics, Mannheim, DE)] using an Ultra-Turrax homogenizer (IKA Werke, Staufen, DE). After centrifugation (7500 × g for 5 min), the homogenates were stored at −20℃ until SDS-PAGE assay. The total protein content of homogenate was determined by the BCA protein assay kit (Pierce, Illinois, USA). Contents of peroxisome proliferator-activated receptor (PPAR) γ and β-tubulin (loading control) proteins in the retroperitoneal adipose tissue samples were evaluated by individually incubating monoclonal primary antibodies (anti-PPARγ and anti-β-tubulin; 1:1000; from Abcam, Cambridge, UK) overnight at 4℃, followed by proper secondary antibody (1 hour; 1:7000 antibody from Sigma-Aldrich Co., Missouri, USA) and streptavidin (1 hour; 1:7000; Zymed, California, USA) incubation. The protein bands were visualized by chemiluminescence with Kit ECL Plus (GE Healthcare Life Sciences, Buckinghamshire, UK) followed by exposure in the ImageQuant™ LAS 500 (GE Healthcare Life Sciences).

Area and density of the bands were quantified by Image J software (Media Cybernetics, Maryland, USA). The results were normalized by β-tubulin content and expressed as relative (%) to the normolipidic control group.

2.6 Serum metabolites

Blood samples were collected from euthanized animals by cardiac puncture and centrifuged (5714 × g for 5 min) for serum separation. Serum insulin levels were determined using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). Serum non-esterified fatty acids (NEFA) levels were analyzed using a colorimetric kit (Randox Laboratories, Antrim, United Kingdom), while leptin was analyzed using a Leptin ELISA kit (R&D Systems, Minneapolis, USA). Serum levels of cholesterol[30], triacylglycerol[31], high-density lipoprotein (HDL) cholesterol[32] and low-density lipoprotein (LDL) cholesterol[33] were determined by colorimetry using the BT 3000 equipment from Wiener laboratories.

2.7 HOMA and R-QUICKI

Homeostatic Model Assessment (HOMA) index was calculated as follows: [fasting insulin (ng/mL) × fasting glucose (mM)]/22.5. A high HOMA index denotes low insulin sensitivity[34], although it should be acknowledged that the HOMA model has not been validated for use in animal models[35] Revised Quantitative Insulin Sensitivity Check Index (R-QUICKI) is another equation to assess insulin sensitivity[36]. This index was calculated as following: [1/log fasting insulin (mU/mL) + log fasting glucose (mg/dL) + log NEFA (mmol/L)][36].

2.8 Oral glucose tolerance test (OGTT)

After 55 days on the experimental diets, the rats were fasted for 12 hours (7 a.m. to 7 p.m.) and received a 50% glucose solution (2 g/kg body weight) by oral gavage[37−40]. Blood samples were collected from a tail nick for glycemic determinations using the glucose oxidase method[28] at 0, 30, 60, 90, 120 and 240 minutes post gavage. Due to reasons previously described[29], anesthesia was not used in the OGTT. Changes in blood glucose concentration during the oral glucose tolerance test were evaluated by the estimate of the total area under the curve (AUC).

2.9 Statistical analysis

Results were expressed as means ± SEM (standard error mean). The statistical analyses were performed using Prism 5.0 (GraphPad Software, Inc). Data from different dietary groups were analyzed by one-way ANOVA for overall significance followed by Tukey's post-hoc tests to identify differences between treatment groups. Treatment effects and differences between means were considered significant when $p < 0.05$.

3 RESULTS

Food intake of HF-C and HF-CLA diets was 28.55% and 26.69% lower than NC food intake, respectively, while no difference was observed between HF-C and HF-CLA (Table 3). The energy intake observed in rats fed with the HF-C and HF-CLA diets was 11.05% and 15.68% higher than in NC-fed rats, respectively, but there was no difference between HF-C and HF-CLA (Table 3). No differences in initial and final body weight and weight gain (expressed as a percentage of initial weight) were observed among groups (Table 3).

The effect of NC, HF-C and HF-CLA diets on body weight during all experimental period is shown in Fig. 1. On the 10th day, body weight of rats fed with HF-C and HF-CLA diets were both decreased in comparison to those fed with the NC diet (Fig. 1).

According to carcass chemical composition, no differences in moisture, lipid, protein and ash contents was observed among treatment groups (Table 3). PPARγ protein levels in adipose tissue were decreased by 38.06% and 29.80% in HF-CLA-fed rats in comparison to those fed with the NC and HF-C diets, respectively (Fig. 2).

Fasting serum insulin levels were increased by 25.63% in HF-CLA-fed rats in comparison to those fed with HF-C diet, (Fig. 3A). Glucose concentration was increased by 8.90% in HF-CLA-fed rats in comparison to those fed with the NC diet, while no differences in glycemia were observed between NC and HF-C groups (Fig. 3B).

There was no difference in serum NEFA levels between rats fed with the HF-CLA and HF-C diets, but values observed in high fat control group was 21.60% lower than in NC (Table 3). Serum concentrations of leptin did not differ among dietary treatments (Table 3).

HOMA index was increased by 29.85% and 32.74% in HF-CLA-fed rats in comparison to those fed with the NC and HF-C diets, respectively (Fig. 4A). R-QUICKI index was unchanged by the dietary treatments (Fig. 4B). The area under the OGTT glycemic curve (AUC) did not differ among dietary treatments (Table 3).

Serum cholesterol levels of rats fed with the HF-C and HF-CLA diets were both decreased by 18.27% and 11.96% compared to those fed with the NC diet, respectively (Fig. 5A), as well as serum triacylglycerol levels of rats fed with the HF-C and HF-CLA diets were both decreased by 31.29% and 26.73% compared to those fed NC diet (Fig. 5B). Serum levels of HDL cholesterol were increased by 10.08% and 23.29% in HF-CLA-fed rats as compared to those fed with both the NC and HF-C diets, respectively (Fig. 5C). There was no difference in serum LDL cholesterol levels between rats fed with the HF-CLA and HF-C diets, but values observed in these groups were 28.32% and 23.60% lower than in NC group, respectively (Fig. 5D). Liver weights of HF-C and HF-CLA groups was 23.20% and 21.88% lower than NC value, respectively,

Table 3 Metabolic and serum parameters in Wistar rats fed with mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA for 60 days.

	Dietary Treatments		
	NC	HF-C	HF-CLA
	Dietary intake and body and liver weights		
Intake (g/day/rat)	26.45 ± 1.06	18.90 ± 0.51***	19.39 ± 0.36***
Intake (Kcal/day/rat)	63.19 ± 2.52	70.17 ± 1.89*	73.10 ± 1.34**
Initial body weight (g)	274.50 ± 5.18	264.30 ± 4.75	259.20 ± 5.67
Final body weight (g)	433.90 ± 10.11	421.00 ± 12.17	425.10 ± 9.61
Weight gain (%)	62.15 ± 1.90	59.30 ± 3.54	64.05 ± 1.83
Liver weight (g)	17.50 ± 0.39	13.44 ± 0.52***	13.67 ± 0.44***
	Body composition		
Moisture (%)	50.10 ± 1.05	50.83 ± 1.18	49.76 ± 0.82
Lipid (%)	29.41 ± 1.39	26.14 ± 1.09	28.46 ± 0.48
Protein (%)	17.76 ± 0.32	17.57 ± 0.49	18.21 ± 0.36
Ash (%)	3.23 ± 0.09	3.87 ± 0.38	3.43 ± 0.16
	Serum metabolites and AUC		
NEFA (mmol/L)	0.375 ± 0.023	0.294 ± 0.025*	0.328 ± 0.021
Leptin (ng/mL)	2.21 ± 0.21	1.98 ± 0.19	1.80 ± 0.16
AUC	13180 ± 1505	14610 ± 1021	17400 ± 2007

Data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. Asterisk denotes statistically significant differences compared to NC (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). [1] Normolipidic Control (NC), diet containing 4.0% soybean oil (SO); [2] High Fat-Control (HF-C), diet containing 24% SO; [3] High Fat-CLA (HF-CLA), diet containing 1.5% Luta-CLA 60 (BASF AG, São Paulo, Brazil) and 22.5% SO. Luta-CLA 60 is composed of 60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA.

while no difference was observed between liver weights of high fat diets (Table 3).

4 DISCUSSION

Due to the substantial rise in obesity prevalence and chronic diseases related to overweight condition, such as type 2 diabetes, it would be advantageous to identify potential therapeutic nutrients to assist in obesity prevention[12]. In this context interest in CLA has been increasing due to its potential anti-obesity effects[12]. However the usage of 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA as food supplement is not consensus and more studies are necessary to investigate the role of CLA isomers in obesity prevention and side effects related to CLA consumption, mainly associated with insulin resistance and dyslipidemia[15, 16]. Thus, in this paper, we have demonstrated dietary effects of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA in 60-day-old Wistar rats on prevention of obesity risk, as well as insulin sensitivity and profile of serum lipids.

In this study, there was no difference in dietary intake between rats fed with the HF-CLA diet and those fed with the HF-C diet. Effect of CLA on dietary intake remains controversial[41]. Several studies reported that CLA had little or no effect on food intake[42–44] while others have reported a reduction in food intake[45, 46]. Food and energy intake of HF-C and HF-CLA groups differed from the values of the NC group due to the high fat content of HF-C and HF-CLA diets. HF-C and HF-CLA-fed rats adapted to the higher energy density of HF-CLA and HF-C diets by reducing their daily food intake compared to the NC group, an effect that has been previously reported[47]. Daily energy intake was higher in HF-C and HF-CLA-fed rats than in the NC group, which may be attributed to the increased palatability of high fat diets, which is directly related to higher energetic intake[48]. High fat diets are more palatable because fat content is one of the factors that contribute to food palatability[48].

No differences among groups of dietary treatments were observed for body weight gain. In another study, an equi-

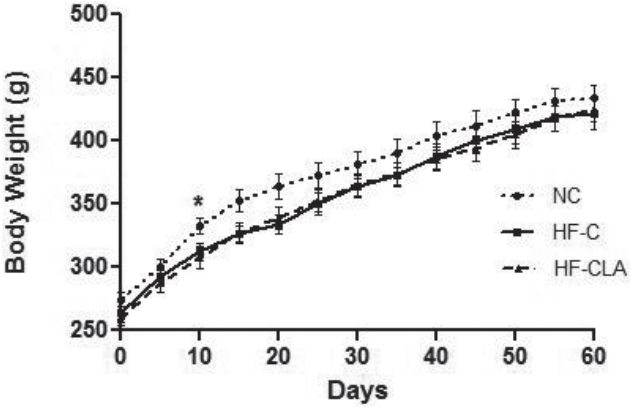

Fig. 1 Effect of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on body weight. Male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. *p<0.05.

molecular mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA was added to the diet of Wistar rats to reach 0.5% of active isomer *trans*-10, *cis*-12 CLA, and no differences in final body weight after 6 weeks were found between control and CLA-treated rats[49]. Similarly, no differences in body weight gain was observed between control group and animals fed with a diet containing 0.25% *cis*-9, *trans*-11 and 0.25% *trans*-10, *cis*-12 CLA[12]. In the present investigation, there was no effect of the dietary treatments on body composition. Similar results were observed in a previous study in which Wistar rats were fed with a diet containing 1% each CLA isomer[50].

Despite the lack of CLA effects on body composition, the levels in adipose tissue of PPARγ, the master adipogenic regulator[11], was lower in HF-CLA group than in NC and HF-C groups, which at first may seem contradictory since body composition was unchanged. However, differences in adipose tissue PPARγ levels is not necessarily associated with changes in body composition since PPARγ is not the only protein involved in adipogenesis[11] and, interconnected to the PPARγ role in adipocyte differentiation, this protein also regulates insulin sensitivity by transcriptionally activating genes involved in insulin signaling, glucose uptake, and fatty acid uptake and storage[51]. It has been demonstrated that depletion of PPARγ in adipose tissue causes insulin resistance, since PPARγ decreased action in mature adipocytes, leads to reduced expression of key genes required for insulin signaling in adipocytes[52]. In fact, the anti-diabetic drug family known as thiazolidinediones, mediate their insulin-sensitizing effects by directly activating PPARγ[51]. It was previously shown that adipocyte-specific constitutive activation of PPARγ in mature adipocytes can regulate whole body insulin sensitivity[53]. Effects of conjugated linoleic acid on PPARγ has been shown to be isomer-specific, with *trans*-10, *cis*-12 CLA down-regulating and *cis*-9, *trans*-11 CLA up-regulating its expression in the adipose tissue[54]. Therefore, the present study suggests that the effect on PPARγ of *trans*-10, *cis*-12 CLA prevailed over the effect of *cis*-9, *trans*-11 CLA, consequently HF-

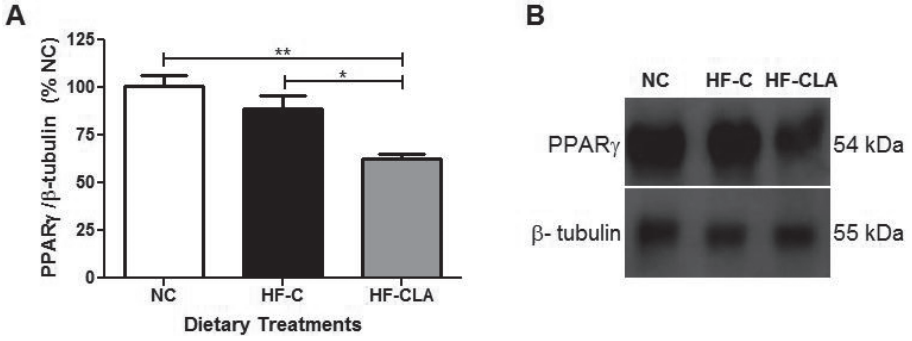

Fig. 2 Analysis of PPARγ protein level in retroperitoneal adipose tissue. PPARγ levels (A) and representative blot for PPARγ and β-tubulin (loading control) (B) of male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. *p<0.05, **p<0.01.

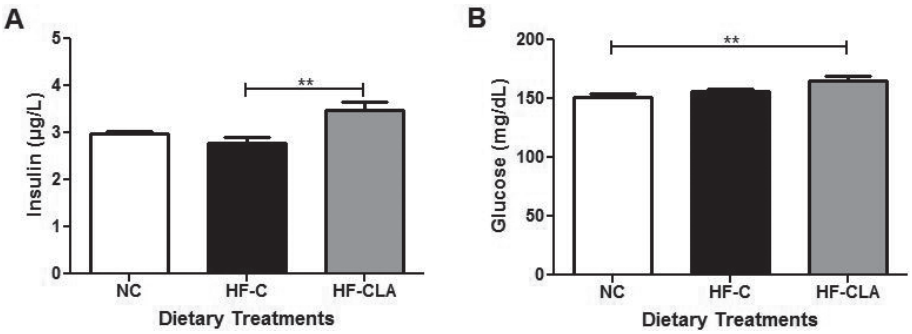

Fig. 3 Effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on serum metabolites. Insulin (A), glucose (B) of male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10 *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. ** $p<0.01$.

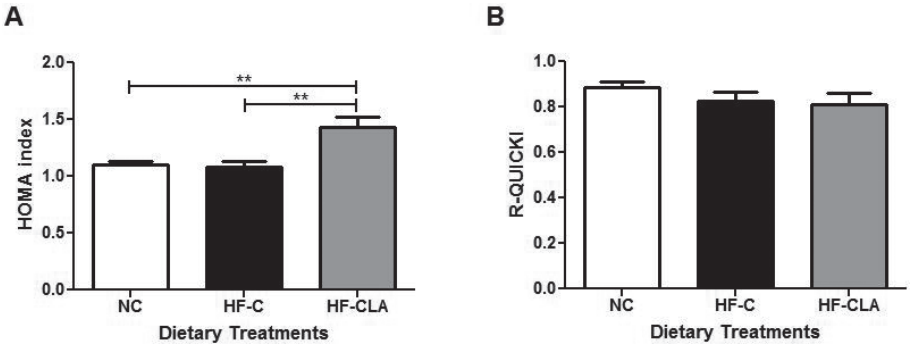

Fig. 4 Effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on indexes of insulin sensibility. HOMA index (A) and R-QUICKI (B) of male Wistar rats fed the following dietary treatments for 60 days: Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10 *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. ** $p<0.01$.

CLA-fed rats presented reduced PPARγ levels in adipose tissue.

Rats fed with the diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA had higher fasting serum insulin levels than rats fed with the high fat control diet. The fasting hyperinsulinemia is an important parameter since it was demonstrated that a gradual increase in serum insulin in the fasting state reflects decreased insulin sensitivity[55]. HF-CLA-fed rats presented hyperglycemia compared to NC-fed rats, while no differences in glycemia were observed between NC and HF-C groups, and higher HOMA index than rats fed with the NC or HF-C diets, which denotes low insulin sensitivity in the HF-CLA group[34]. These results are according to previous study which showed that animals fed with diet containing the mixture of 0.25% *cis*-9, *trans*-11 and 0.25% *trans*-10, *cis*-12 CLA had hyperinsulinemia and insulin resistance, demonstrated by increased HOMA index[12]. On the other hand areas under the curves of oral glucose tolerance tests did not differ among groups of dietary treatments. Thus it is possible to hypothesize that despite the same amount of glucose was presented by NC, HF-C and HF-CLA groups during the OGTT, higher insulin levels may have been required by HF-CLA-fed rats[12, 56].

Concerning the R-QUICK, which also denotes insulin sensitivity[36], there were no differences among groups of dietary treatments. Possibly it occurred because this index considers serum NEFA levels, which was unchanged by HF-CLA diet compared to NC or HF-C diets. Similarly, it was shown in previous study that mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA did not modify serum NEFA levels[12]. Serum NEFA concentration is a risk factor for type 2 diabetes because the combination of excessive levels of non-esterified fatty acids and glucose leads to decreased insulin secretion, impairments in insulin gene expression and beta-cell death by apoptosis[57]. Serum NEFA

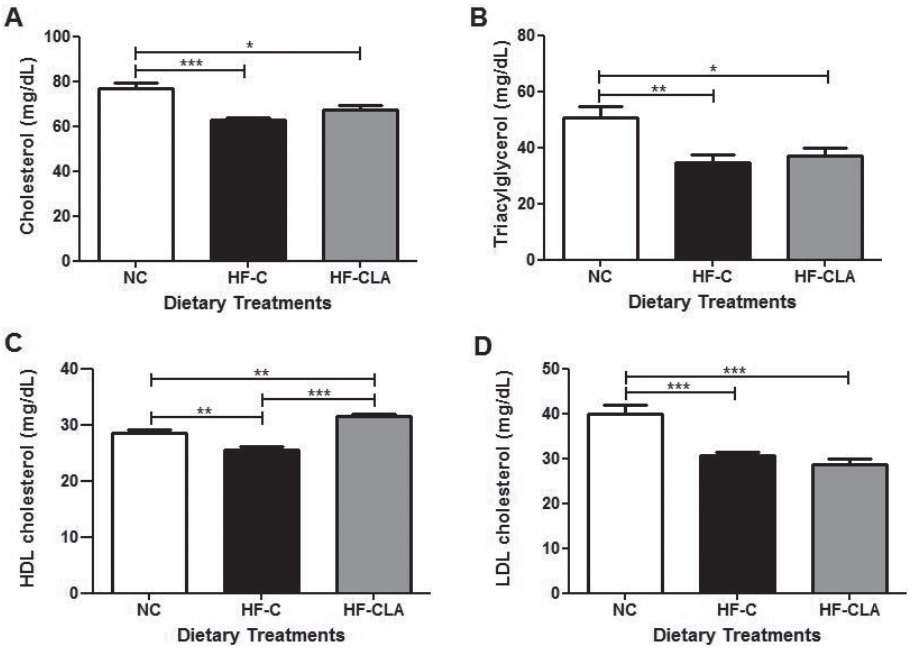

Fig. 5 Effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture on serum lipids. Cholesterol (A), triacylglycerol (B), HDL cholesterol (C) LDL cholesterol (D) of male Wistar rats fed the following dietary treatments for 60 days Normolipidic Control (NC): diet containing 4.0% soybean oil (SO); High Fat-Control (HF-C): diet containing 24% SO; and High Fat-enriched synthetic CLA (HF-CLA): diet containing 1.5% Luta-CLA 60 (60% CLA with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10 *cis*-12 CLA) and 22.5% SO. All data are presented as mean values ± S.E.M (n=10 rats/group). Statistically significant differences were determined by Anova followed by Tukey. **p*<0.05, ** *p*<0.01, *** *p*<0.01.

levels increased in NC-fed rats may be related to high levels of total cholesterol and triacylglycerol in this group, as was previously demonstrated[58]. Leptin is an adipokine that plays a role in glucose metabolism and insulin sensitivity[59]. In the present study there were no differences in serum leptin level among groups. Probably because body fat was unchanged among experimental groups and circulating leptin is highly correlated with adiposity, therefore individuals exhibiting higher serum leptin were indeed found to be more obese[12]. Similarly, it was shown previously that mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA did not modify plasma leptin level[60].

Concerning the serum lipid concentration, HF-C and HF-CLA-fed rats presented lower levels of total cholesterol, triacylglycerol and LDL cholesterol compared to NC-fed rats. It may be due to high levels of carbohydrate (73.39% of energy) in NC diet, since it was demonstrated that when dietary carbohydrate was increased from 50% to 67% of energy, the fasting triacylglycerol level rose[61], which is commonly related to increased precursors of LDL cholesterol in the blood, the very-low-density lipoproteins, and consequently increased LDL cholesterol levels[62]. All these changes in NC-fed rats contributed to high total cholesterol demonstrated by this group. Rats fed with the diet containing the mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA had favorable changes in serum lipoprotein profile compared to HF-C-fed rats, since the diet containing CLA isomers was responsible for high levels of HDL cholesterol while serum total cholesterol, triacylglycerol and LDL cholesterol concentrations were not modified compared to high fat control diet. Similarly, it was shown that animals supplemented with a diet containing 1% mixture with a 50:50 ratio of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA, presented plasma total cholesterol and LDL cholesterol unchanged compared to those values of control group[63]. Concerning the effect of CLA on the triacylglycerol level, previous studies in animals showed that triacylglycerol concentration was not modified by *cis*-9, *trans*-11 CLA (64-66) or by *trans*-10, *cis*-12 CLA supplementation[66]. The high level of HDL cholesterol in HF-CLA-fed rats may be attributed to *cis*-9, *trans*-11 CLA, as also reported by a previous study[67]. The increased HDL cholesterol level is potentially a beneficial result for two main reasons. Firstly, it possesses properties that have the potential to inhibit the development of atherosclerosis and thus reduce the risk of having a cardiovascular event[68]. Secondly, HDL cholesterol also increases the uptake of glucose by skeletal muscle[69] and stimulates the synthesis and secretion of insulin from pancreatic β cells[70] and may thus have a beneficial effect on glycemic control[68]. However, despite these

beneficial properties of HDL cholesterol, the increase of this molecule in HF-CLA-fed rats was not capable of preventing hyperinsulinemia, hyperglycemia or insulin resistance in this group. Thus, it is possible to hypothesize that the negative effects on insulin metabolism from decreasing PPARγ level in adipose tissue prevail over the potential positive effects related to increased HDL cholesterol level on glycemic and insulinemic control.

The liver weight of HF-CLA-fed rats did not differ of HF-C-fed rats liver weight. Similarly, it was previously demonstrated that liver weight was unchanged by *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA mixture[71]. The high liver weight of NC-fed rats may be an indicative of hepatic fat deposition due to high levels of carbohydrate in this diet. Similarly, it was previously reported that a carbohydrate-rich diet induced hepatic fat deposition[72].

The action mechanisms involved in effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA is not well understood yet[73]. The contradictory findings among rodent studies may be due to differences in experimental design, such as CLA isomer combination versus individual isomers, CLA dose and duration of treatment, gender, weight, age and metabolic status of the animals.

5 CONCLUSION

In conclusion, the present investigation suggests that a 60 day feeding of a diet containing mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA to 60-day-old male Wistar rats has effects on insulin, glucose and cholesterol metabolisms. Although diet containing *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA significantly raised serum HDL cholesterol, the mixture of CLA isomers was found not to change body composition, which demonstrates that this diet was useless in preventing the obesity risk, besides it was also found to cause fasting hyperinsulinemia and insulin resistance. These results suggest that caution should be taken before synthetic supplements containing *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are recommended as a nutritional strategy for weight management.

Acknowledgements: Embrapa Dairy Cattle; Coordination of Improvement of Higher Education Personnel (CAPES); National Counsel of Technological and Scientific Development (CNPq); Foundation for Research Support of the Minas Gerais State (FAPEMIG); Roberto Alcantara Gomes Biology Institute, State University of Rio de Janeiro (UERJ); Federal University of Juiz de Fora (UFJF).

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