ROSEMARY FERREIRA

EFEITO DO ESTRESSE CRÔNICO E DE DIETA HIPERCALÓRICA SOBRE O PESO CORPORAL E METABOLISMO DE RATOS

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Odontologia, área de concentração Fisiologia Oral.

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RESUMO

O estresse crônico é um fator de risco para doenças cardiovasculares e metabólicas e tem sido relacionado ao desenvolvimento de distúrbios alimentares. O objetivo deste trabalho foi avaliar o efeito, a longo prazo, do estresse crônico moderado e imprevisível (ECMI) e da ingestão de dieta hipercalórica (DH) sobre o peso corporal e metabolismo de ratos. No capítulo 1, foi avaliado o efeito da associação entre ECMI e DH sobre o peso corporal, adiposidade, teste de tolerância à glicose (TTG) e perfil lipídico de ratos Sprague–Dawley, divididos em 4 grupos: dieta padrão (DP), dieta padrão+ECMI (DPE), DH e DH+ECMI (DHE), analisados durante sete semanas. Duas semanas após a aplicação do ECMI, os grupos DPE e DHE apresentaram aumento significativo na concentração plasmática de corticosterona que os grupos DP (2,09±0,41 vs. 19,42±2,85ng/mL) e DH (3,34±0,66 vs. 18,72±3,18ng/mL), respectivamente. Os grupos DH e DHE apresentaram aumento significativo no peso corporal final que os grupos DP (435±3 vs 463±8g) e DPE (425±5 vs 444±8g), respectivamente. O estresse induziu redução significativa no ganho de peso e na ingestão alimentar, na primeira semana do protocolo de ECMI. Os grupos ECMI e DH apresentaram aumentos significativos nas concentrações plasmáticas (mmol/L) de colesterol total (DP: 1,44±0,05; DPE: 1,54±0,05; DH: 1,53±0,09; DHE: 1,88±0,08), triglicerídeos (DP: 1,41±0,09; DPE: 1,75±0,16; DH: 1,67±0,13; DHE: 2,42±0,28) e LDL (DP: 0,46±0,06; DPE: 0,73±0,08; DH: 0,77±0,08; DHE: 0,87±0,10). No TTG, os grupos DPE e DHE apresentaram área sob a curva significantemente maior comparado aos grupos DP (13549±387 vs. 14267±344) e DH (15852±270 vs. 16476±559mg x min./dL), respectivamente. No capítulo 2 avaliamos a relação entre redução do ganho de peso corporal induzida pelo ECMI e os períodos de restrição alimentar do protocolo de estresse. Ratos Sprague-Dawley (2 meses de idade) foram divididos em três grupos: Controle, ECMI e Alimentação-Pareada (AP: alimentados com a mesma quantidade de ração ingerida pelo grupo ECMI). Os grupos ECMI e AP apresentaram redução significativa de 12 e 15% na ingestão alimentar durante o protocolo de ECMI, comparado ao controle. Imediatamente após o ECMI, ratos estressados e AP apresentaram redução significativa de 6 e 10% no peso corporal e de 19 e 14% na gordura epididimal, respectivamente, comparados ao

grupo controle. O grupo AP, mas não o grupo ECMI, apresentou redução nas gorduras mesentérica (41%), inguinal (28%) e perirrenal (40%), menor proporção gordura total/peso corporal final (0,02±0,001 vs. 0,03±0,001) e menor porcentagem de gordura na carcaça (3,39±0,44 vs. 6,29±0,51%) comparado ao controle, sem diferença entre controle e ECMI. O efeito redutor do ECMI sobre o peso corporal não pode ser totalmente explicado pela restrição alimentar durante o estresse. A longo prazo, o ECMI e o tratamento com dieta hipercalórica têm efeitos semelhantes sobre a dislipidemia em ratos. Tais efeitos são potencializados quando combinados. O efeito redutor no peso corporal, promovido pelo ECMI, sofre influência da dieta empregada, e é revertido após o estresse.

Palavras-chave: estresse crônico, dieta hipercalórica, peso corporal, adiposidade, lipídeos, rato.

ABSTRACT

Chronic stress is a risk factor for cardiovascular and metabolic diseases and has been associated to development of eating disorders. The purpose of this study was to investigate the long-term effect of chronic mild and unpredictable stress (CMS) and hypercaloric diet (HD) on body weight and metabolism of rats. In Chapter 1, we studied the effect of the association of CMS and HD, fifteen days after the end of CMS on body weight, adiposity, oral glucose tolerance test (OGTT) and lipid profile of Sprague-Dawley rats. The rats were divided into 4 groups: standard diet (SD), SD + CMS (CMS), hypercaloric diet (HD) and HD + CMS, evaluated during seven weeks. The data were analyzed by two-way ANOVA (P<0.05). Two weeks after the end of CMS, both the groups SD+CMS (2,09±0,41 vs. 19,42±2,85ng/mL) and HD + CMS (3.34±0.66 vs 18.72±3.18ng/mL) had higher plasmatic corticosterone concentration than SD groups and HD, respectively. The groups HD and HD + CMS had higher final body weight that SD groups (435±3 vs 463±8g) and SD+CMS (425±5 vs 444±8g), respectively. CMS induced lower body weight gain and lower food intake only in the first week of CMS protocol. The SD+CMS and HD groups showed increased plasma concentrations (mmol/L) of total cholesterol (SD: 1.44±0.05; SD+CMS: 1.54±0.05; HD: 1.53±0.09, HD+CMS: 1.88±0,08), triglycerides (SD: 1.41±0.09; SD+CMS: 1.75±0.16, HD: 1.67±0.13; HD+CMS: 2.42±0.28) and LDL (DC: 0.46±0.06; SD+CMS: 0.73±0.08; HD: 0.77±0.08; HD+CMS: 0.87±0.10). SD+CMS and HD+CMS groups had higher area under the curve of the OGTT than SD groups (13,549±387 vs. 14267±344 mg x min/dL) and HD (16476±559 vs. 15,852±270 mg x min/dL), respectively. In Chapter 2, we investigated the association between CMS-induced body weight loss and food restriction, utilized in the protocol of stress. Sprague-Dawley rats (2 months old) were divided into three groups: Control, CMS and pair-fed (PF: the rats were fed with the same amount of food as the CMS group ate voluntarily during the corresponding period of stress protocol). The data were analyzed by One-way ANOVA (P<0.05). CMS and PF groups showed reduction of 12 and 15% in food intake during the protocol of CMS, compared to control. After CMS, PF and stressed rats showed a reduction of 6 and 10% in the final body weight and had reduction of 19 and 14% in epididymal fat compared to the control group,

respectively. The PF group, but not the group CMS, showed lower mesenteric (41%), inguinal (28%) and perirenal (40%) fat mass, lower total fat / final body weight (0.02±0001 vs. 0,03±0001) and lower percentage of fat in the carcass analysis (3.39±0.44 vs. 6.29±0.51%) compared to the control, without difference between control and CMS. The lower body weight of CMS cannot be fully explained by food restriction during the stress. The CMS and hypercaloric diet did have similar long-term effects on dyslipidemia in rats. These effects are enhanced when combined. The reduction in body weight promoted by CMS is influenced by diet employed, and is reversed after the stress.

Keywords: chronic stress, hypercaloric diet, body weight, adiposity, lipids, rat

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1. INTRODUÇÃO

Atualmente, na sociedade moderna, o estresse tem sido apontado como fator de risco para doenças cardiovasculares e síndrome metabólica devido ao seu importante papel no desenvolvimento de disfunções metabólicas, desordens emocionais e distúrbios alimentares (Raikkonen *et al.,* 1994; Epel *et al.,* 1999). A ativação do eixo hipotálamo-hipófise-adrenal (HHA) pelo estresse, e conseqüente aumento da liberação de cortisol, tem sido relacionado à ocorrência de hiperinsulinemia, hiperglicemia, dislipidemia, hipertensão, aumento da gordura abdominal - componentes da síndrome metabólica (Bjorntorp, 1997).

Na síndrome metabólica também podem ocorrer alterações vasculares e resistência à insulina. Esta última pode ser a causa subjacente de várias alterações como diabetes, doenças coronarianas, intolerância à glicose, aterosclerose e também pode estar relacionada com a doença renal crônica, hipertensão arterial e dislipidemia (Haffner *et al,* 1992). Hirose *et al.* (2003) demonstraram que a resistência à insulina, definida pelo índice de avaliação de modelo de homeostase (Homeostasis Modal Assessment – HOMA), está associada à hipertensão arterial em japoneses de meia-idade. A resistência à insulina é a diminuição da sensibilidade dos tecidos à ação da insulina, comprometendo a habilidade da insulina em distribuir a glicose de forma adequada no fígado, no músculo esquelético e em outros tecidos periféricos. Assim, o indivíduo apresenta alterações no metabolismo da glicose, com conseqüente hiperglicemia (Rhodes & White, 2002).

O estilo de alimentação do homem também está relacionado com o desenvolvimento de doenças cardiovasculares, dislipidemias, diabetes *mellitus* (DM), diferentes tipos de câncer e obesidade (LIMA *et al.,* 2000). Os conteúdos alimentares de gorduras saturadas e de colesterol influenciam o perfil lipídico no homem e em modelos animais. O aumento do consumo de gorduras saturadas e colesterol pode promover aumentos na concentração sanguínea de colesterol, por meio da redução da síntese de receptores de apolipoproteína B no fígado, responsáveis pela captação da fração de lipoproteínas de baixa densidade (*low-density lipoprotein* - LDL) (LIMA *et al.,* 2000).

Embora o organismo possua numerosos mecanismos de proteção contra as adversidades, a evolução humana guiou o organismo para preservação e estoque de energia, o que foi essencial para os raros períodos de abundância de suprimento alimentar. Porém o

moderno desequilíbrio energético, resultante de excesso de ingestão calórica e sedentarismo, pode promover o desenvolvimento de obesidade em proporções epidêmicas (Ferranti & Mozaffarian, 2008).

A síndrome do comer noturno e a desordem de compulsão alimentar, em que os indivíduos consomem alimentos ricos em caloria parecem estar associadas à ocorrência de depressão, hiperinsulinemia, hiperglicemia, menor secreção de grelina e ao desenvolvimento da obesidade (Stunkard *et al.,* 1955; Allison & Stunkard, 2004; Allison *et al.,* 2005). Gluck *et al.,* (2004) relataram elevada concentração de cortisol em mulheres obesas com desordem alimentar, após um modelo de estresse no qual o indivíduo submerge sua mão em água gelada por dois minutos. O grupo de indivíduos portadores de compulsão alimentar apresentou maiores escores na escala de depressão, maior secreção de cortisol e maior escore de fome. Além disso, apresentaram maior desejo por alimentos calóricos, após a sessão de estresse (Gluck *et al.*, 2004). Além disso, Berlin & Lavergne (2003) observaram aumento de índice de massa corporal e menor redução do apetite e redução de pensamentos negativos em 1964 indivíduos depressivos, sugerindo que a redução no apetite está relacionada à elevada concentração de insulina e alto índice de massa corporal.

Apesar de diversos estudos investigarem a contribuição do estresse, ansiedade, depressão e obesidade nas desordens alimentares, não estão ainda totalmente esclarecidos os mecanismos pelos quais hormônios e neurotransmissores ativados nestas situações alteram o controle da ingestão alimentar e do metabolismo corporal (Frederich *et al.,* 2002; Yilmaz *et al.,* 2002; Kojima *et al.,* 2005; Bosy-Westphal *et al.,* 2005). A síndrome do estresse pós-traumático, decorrente de eventos altamente estressantes, tais como guerras, morte, separação conjugal, catástrofes naturais, tortura e abuso sexual na infância, tem sido considerada como um fator desencadeante de desordens alimentares, tais como anorexia, bulimia nervosa e obesidade (Aksary *et al.,* 2000; Stunkard *et al.,* 2003). Experiências adversas na infância promovem o desenvolvimento tanto de depressão quanto da obesidade (Stunkard *et al.,* 2003). Lemieux & Coe (1995) relataram que 50% das mulheres que sofreram abuso sexual na infância apresentavam sobrepeso e elevados níveis urinários de noradrenalina, adrenalina e dopamina.

Complementando os estudos em humanos, modelos animais têm sido utilizados para investigar os mecanismos envolvidos no desenvolvimento da obesidade e nos efeitos do estresse sobre na regulação do peso corporal e ingestão alimentar. Estes modelos incluem animais modificados geneticamente - ratos Zucker e camundongos ob (Drake *et al.,* 2004; Pelleymounter *et al.,* 1995), obesidade induzida por lesão ventromedial do hipotálamo (Parkinson *et al.,* 1990), macaco Rhesus espontaneamente obesos (Bodkin *et al.,* 1993) e obesidade induzida por dieta em ratos Sprague-Dawley (Lauterio *et al.,* 1994; Levin *et al.,* 2000; Farley *et al.,* 2003). Ratos Zucker são homozigotos para uma mutação no gene do receptor de leptina, exibem resistência à insulina e hipertensão, podem refletir aumento das ações de glicocorticóides resultantes de alterações em diversos níveis de controle da secreção deste hormônio (Frisbee, 2005). Ratos espontaneamente hipertensos (Stroke-prone spontaneously hypertensive - SHRSP) também têm sido utilizados para o estudo da síndrome metabólica. Strahorm *et al.* (2005) demonstraram que ratos SHRSP alimentados com uma dieta rica em frutose apresentaram intolerância à glicose, elevadas concentrações de triglicerídeos, ácidos graxos e redução na concentração de lipoproteínas de alta densidade (HDL) e maior adiposidade. Desta forma, o tratamento com dieta rica em frutose, semelhante à dieta típica dos Estados Unidos, tem sido considerado uma ferramenta complementar ao estudo da síndrome metabólica (Shinozaki *et al.,* 2000).

Brennan *et al.*, (1992) demononstraram que ratos submetidos a sessões repetidas de estresse por choque na cauda (2 horas/ 3 dias), e tratados com dieta rica em gordura, não apresentaram diferença nas concentrações de colesterol, ao contrário de animais tratados com dieta controle nos quais houve aumento do colesterol plasmático após a terceira sessão de estresse (Brennan *et al.*, 1992). Ratos Sprague-Dawley tratados com dietas rica em gordura, associada ou não ao suplemento de cloreto de sódio (NaCl), apresentaram aumento da pressão arterial média e na depuração da creatinina (Song *et al.*, 2004). Os grupos tratados com dieta rica em gordura apresentaram moderação na redução da expressão dos transportadores de NaCl sensível à tiazida e de canais de sódio epitelial nos túbulos distais, relacionadas ao aumento da tamanho dos rins induzido pela dieta rica em NaCl. A redução dessas proteínas é importante para adaptação para limitar a reabsorção de sódio. Song *et al.*, sugeriram que esta moderação na expressão de proteínas em animais

tratados com NaCl e dieta rica em gordura pode influenciar o aumento da pressão arterial presentes na resistência à insulina, que pode progredir em hipertensão arterial mais severa e perda da função renal (Song *et al.*, 2004).

Com relação aos efeitos do estresse no comportamento alimentar, Kuriyama & Shibasaki (2004) observaram redução da ingestão alimentar em ratos submetidos a estresse emocional e demonstraram que este efeito inibitório foi cancelado por antagonistas dos receptores tipo 1 do hormônio liberador de corticotrofina (CRH). Este achado de Kuriyama & Shibasaki (2004) está de acordo com Rybkin *et al.* (1997), os quais sugerem que a perda de peso observada em vários estudos utilizando modelos animais de estresse é dependente da liberação central do CRH, o qual ativa o eixo HHA, o sistema nervoso simpático e vias serotoninérgicas e catecolaminérgicas no sistema nervoso central, inibindo a ingestão alimentar. O efeito inibitório do CRH sobre a ingestão alimentar pode ser devido à atenuação da expressão de neuropeptídeo Y (NPY) no núcleo paraventricular e na amígdala central (Lu *et al.,* 2003). Porém, em resposta ao estresse por pressão aplicada na cauda e estresse agudo por imobilização, ratos apresentaram aumento da ingestão alimentar (Samarghandian *et al.,* 2003). Logo, observa-se que o estresse pode ter efeitos opostos sobre a ingestão alimentar e controle do peso corporal, dependendo do modelo de estresse.

Além disso, o tipo de dieta pode modular as respostas a estímulos estressores. Ratos alimentados com dietas ricas em gordura apresentam reduzida resposta simpática a estressores (Buwalda *et al.,* 2001), semelhantemente ao que ocorre no homem (Dallman *et al.,* 2004). Segundo Pecoraro *et al.* (2004) dietas hipercalóricas agem no hipotálamo de ratos reduzindo a expressão de RNA mensageiro para a síntese de CRH, resultando em diminuição da atividade do eixo HPA e aumentando os estoques de gordura abdominal (Dallman *et al.,* 2003). Portanto, parece haver relações complexas entre a resposta a estímulos estressores, ingestão alimentar e controle do peso corporal, que demandam mais estudos para serem mais bem compreendidas.

Com relação ao estudo dos efeitos do estresse crônico em animais, está bem estabelecido que ratos podem se adaptar à aplicação repetida de estímulos estressores. Porém esta adaptação não ocorre quando os animais são submetidos ao modelo de estresse crônico moderado e imprevisível (ECMI), que consiste na exposição repetida à diferentes

estímulos estressores (Katz *et al.,* 1981; Willner, 2005). A ausência de adaptação aos estímulos estressores foi confirmada por Rodriguez-Echandia (1988) que observou elevação mantida nos níveis séricos de prolactina e corticosterona, em resposta ao ECMI.

Neste modelo, a exposição crônica de ratos a uma variedade de estressores moderados induz estado de anedonia ou subsensibilidade à recompensa, evidenciados pela diminuição no consumo e preferência por solução doce e pela diminuição no desempenho da auto-estimulação do hipotálamo ventrolateral, área responsável pela resposta de recompensa. Como tais efeitos podem ser cancelados pelo tratamento com antidepressivos tricíclicos, e agonistas de receptores $5HT_{1c}$, a indução de anedonia, pelo ECMI, é um modelo com validade preditiva para estudos de mecanismos envolvidos na depressão humana (Moreau *et al.,* 1992; Moreau *et al*., 1993; Moreau *et al.,* 1995; Willner, 2005).

O modelo animal de depressão utilizado por Katz e colaboradores, na década de 80, apresentava estressores mais severos, tais como choque, privação de água e comida por 40 horas, natação na água fria, movimentação da gaiola dos ratos, inversão do ciclo claro-escuro, aumento da densidade populacional na gaiola dos ratos, distribuídos ao longo de 21 dias (Katz *et al.,* 1981). Por outro lado, o procedimento de ECMI realizado pelo grupo de Moreau e colaboradores, a intensidade dos estressores foi reduzida e as análises enfocavam as medidas hedônicas, excluindo o estresse por choque intenso na pata, imersão em água fria e por longo período de privação de água e comida.

Apesar de os efeitos do estresse sobre o metabolismo, regulação do peso corporal e ingestão alimentar terem sido amplamente estudados, há poucos estudos sobre os efeitos do estresse crônico no período pós-estresse. O estresse por restrição repetida promove redução do peso corporal que é mantido durante onze semanas após o final da aplicação do estresse (Harris *et al.,* 2006). Tamashiro *et al.* (2007) demonstraram que a perda de peso corporal em ratos submetidos ao estresse social foi recuperada 21 dias após o final do estresse, sendo acompanhado de aumento do tecido adiposo. Como os efeitos ECMI sobre o estado de anedonia em ratos são gradualmente induzidos e são mantidos durante duas semanas subseqüentes ao término do regime de estresse (Bekris *et al.,* 2005), tornou-se interessante avaliar o efeito, a longo prazo, do ECMI e da ingestão de dieta hipercalórica (DH) sobre o peso corporal e metabolismo de ratos.

OBJETIVOS

O objetivo do capítulo 1 foi investigar o efeito do ECMI e da ingestão de dieta hipercalórica (DH) até duas semanas após o final da aplicação dos estressores sobre o peso corporal e metabolismo de ratos. Para isso, estudamos os efeitos de ambos os tratamentos sobre:

- concentração plasmática de corticosterona, peso corporal, ingestão alimentar, acúmulo de gordura, teste de tolerância à glicose, concentrações de triglicerídeos, colesterol total e frações, ácidos graxos livres, índice aterogênico, insulina, índice de resistencia à insulina, leptina e adiponectina.

Baseado nos resultados encontrados no estudo descrito no capítulo 1, no capítulo 2 objetivou-se investigar os efeitos do ECMI sobre o peso corporal e ingestão alimentar imediatamente e duas semanas após o final do estresse. Objetivou-se, também, estudar a relação entre a restrição alimentar, presente no modelo de estresse, e a redução no ganho de peso em animais expostos ao ECMI. Neste estudo, analisamos os efeitos do ECMI sobre os parâmetros abaixo:

- peso corporal, ingestão alimentar, análise da composição da carcaça, acúmulo de gordura, teste de tolerância à glicose, triglicerídeos, ácidos graxos livres, insulina, e corticosterona.

CAPÍTULO 1

Ferreira R, Costa R, Tamascia ML, Neves VJ, Marcondes FK. Effects of chronic mild stress and hypercaloric diet on body weight and metabolism of rats.

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Effects of chronic mild stress and hypercaloric diet on body weight and metabolism of rats.

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ABSTRACT

The effect of chronic mild and unpredictable stress (CMS) on body weight, adiposity, oral glucose tolerance test and blood concentrations of lipids in rats fed with standard and hypercaloric diets fifteen days after the end of stress protocol was examined. Male Sprague-Dawley rats were divided into four groups: Standard diet (SD), CMS, Hypercaloric diet (HD) and HD+CMS and tested for seven weeks. Rats were submitted to CMS 7 days/week/3 weeks. Rats fed with hypercaloric diet showed increased final body weight, adiposity and plasma concentration of triglycerides, total cholesterol, LDL and higher atherogenic index compared with rats fed with SD. Two weeks after the end of CMS, stressed rats had lower final body weight and higher plasma concentration of corticosterone, leptin, triglycerides, total cholesterol, LDL and higher atherogenic index compared with the respective control groups. CMS promoted increased adiposity in rats fed with hypercaloric diet, without difference in rats fed with standard diet. These findings observed two weeks after the CMS could suggest a valid animal model to study the long-term effects of stress on body weight and metabolism of rats fed with standard or hypercaloric diet.

KEYWORDS: chronic mild stress, body weight, food intake, dyslipidemia, HOMA index, hyperglicemia, rats.

INTRODUCTION

Stressful events evoke emotional, metabolic and cardiovascular alterations. During stressful events, the activation of sympathetic nervous systems (SNS) and the hypothalamus-pituitary-adrenal (HPA) axis increases the levels of catecholamines and glucocorticoids in the blood. During chronic stress, long-term activation of SNS and HPA axis induces metabolic changes that may be related to occurrence of metabolic syndrome, characterized by insulin resistance, glucose intolerance, type II diabetes, obesity, and dyslipidemia (Grundy *et al.,* 2004, Epel *et al.,* 1999; Bjorntorp, 1997). Also, stress hormones influence the body weight regulation, feeding behavior and energy expenditure in humans and animals. Vallès *et al.* (2000) observed reduction in food intake and body weight in rats exposed to acute lipopolyssacharide injection and different durations of immobilization stress. These authors found that a two-hour period of immobilization stress promoted a reduction in food intake that was sustained over nine days. In contrast, rats submitted to acute stress by pressure applied to the tail and immobilization, showed increased food intake (Samarghandian *et al.,* 2003). Then, the effect of stress on food intake may depend on the type and duration of the stressor. Michel *et al.* (2003) and Levin *et al.* (2000) demonstrated that the effects of acute and chronic mild stress on food intake and body weight may promote either body weight gain or body weight loss, depending whether the rats are prone or resistant to diet induced obesity depending and whether animals are treated with a high-energy diet or with a chow diet. Also, Pecoraro *et al.* (2004) showed that, in rats, stress increased palatable food eating, and suggested that palatable food intake is an adaptive response to stress and moderates the HPA responsiveness to stress. Moreover, since HPA response was blunted in rats consuming high-calorie diets, it has been suggested that hypercaloric diets may have feedback effects on the stress-induced HPA axis activation (Levin, 1996; Pecoraro *et al.,* 2004).

Although chronic stress causes adaptations within the HPA axis that protect the organisms from chronic increased corticosterone and catecholamines (Armario *et al.*, 1986), prolonged exposure to a variety of mild stressors prevents this adaptation and induces anhedonia, which is a core symptom of major depression. Anhedonia, the inability

to experience pleasure, is evidenced by the loss of responsiveness to reward, expressed by decreased consumption of sucrose solutions, decreased place preference condition, and decreased performance in intracranial self-stimulation (Moreau *et al.,* 1995). These effects are gradually developed by a chronic mild and unpredictable stress (CMS) regimen, remain over the subsequent two-week period, and are reversed by antidepressant drugs (Moreau *et al.,* 1992). Therefore, the CMS protocol has been used as a valid animal model to study the mechanisms involved in depression disorder (Willner, 1997).

Other studies have shown the effects of different stress models during recovery periods. Tamashiro *et al.* (2007) demonstrated that subordinate animals regained body weight lost induced by 14-day social stress period and showed an increase in adipose tissue and leptin over a 21-day of recovery period after the social stress. Harris *et al.* (2006) observed that the stress-induced body weight loss was sustained over 11 weeks after the end of restraint stress. Taken together, these studies suggested that metabolic effects induced by stress may last during the post-stress period.

Although the effect of chronic stress on body weight and feeding behavior changes are widely discussed, the long-term consequences on body weight and metabolism have not yet been clarified. Previous results obtained in our laboratory showed that 15 days after CMS, male rats fed with control diet presented proatherosclerotic changes in the thoracic aorta, related to stress-induced high levels of seric triglycerides, total cholesterol, and low-density lipoprotein (Neves *et al.,* 2008). Since CMS also reduces body weight and may influence feeding behavior and occurrence of depression (Willner *et al.,* 2007), which is an important risk factor for metabolic disorders (Bjorntorp, 1997), the CMS model can be useful to study the long-term effects of stress on body weight, feeding behavior regulation, and metabolic outcomes. Thus, the aim of this study was to examine the effect of CMS and hypercaloric diet on body weight, adiposity, oral glucose tolerance test and blood concentrations of lipids in rats, two weeks after the end of stress protocol.

METHODS

Animals

Male Sprague-Dawley rats (2 months old, weighing about 300-350 g) were obtained from the Center for Biological Investigation of the University of Campinas (CEMIB). In order to maintain Specific Patogen Free patern only reseachers had access to animal facilities, wearing lab coat, gloves, respiratory protection and foot protection. Moreover, all rats had access to filtered water. All animals were housed in individual home cages (65 x 25 x 15cm) with the floor covered with autoclaved sawdust, in a temperature-controlled room (22°C ± 2°C). The light phase of 12h light, 12h dark cycle was between 6 a.m. and 6 p.m.. All animal procedures were approved by the Institutional Committee on Animal Research Ethics (CEEA–UNICAMP/Protocol Number 900-1) and carried out in accordance with the norms of the Brazilian Society of Laboratory Animal Science - Brazilian College of Animal Experimentation (SBCAL – COBEA).

Experimental procedure

After 1 week of adaptation in animal facilities prior to beginning the experiment, all rats were randomized and divided into four groups (n = 15 per group): 1) Standard diet (SD); 2) SD plus Chronic mild and unpredictable stress (SD + CMS); 3) Hypercaloric diet (HD); and 4) Hypercaloric diet plus CMS (HD + CMS). The SD and CMS groups were fed with a rodent standard AIN-93 diet containing 3.3 kcal/g with 17% protein, 72% carbohydrate and 11% fat (Reeves *et al.,* 1993); while the HD and HD + CMS groups were fed with a hypercaloric diet containing 4.5 kcal/g, with 17% protein, 51% carbohydrate and 32% fat (Lauterio *et al.,* 1994). Both the control and hypercaloric diets were made by HN&C Nutriexperimental according with the composition of the experimental diets in Table 1. The experimental protocol was performed for seven weeks; the hypercaloric diet was introduced in the beginning of the experimental protocol, two weeks before the CMS regimen. At week 3, 4 and 5, the CMS and HD+CMS groups were submitted to CMS protocol. During the entire experimental period, body weight and daily food and water intake were monitored weekly. Also, food efficiency (amount of weight

gained relative to caloric intake) was calculated as the gain in body weight (g) per food intake (kcal) over the period of observation (Levin *et al.,* 2003). The weekly body weight and body length were used to determine the Lee index, that is, an obesity index, by the following formula: Lee-index (g/cm) = cube root of body weight/length (Diniz *et al.,* 2006).

Table 1. Diet compositon.

Ingredient	**Standard diet (g/Kg)**	**Hypercaloric diet (g/Kg)**
Casein	200	190
Corn starch	417,5	201
Sucrose	100	271,8
Dextrin cornstarch	132	75
Soybean oil	70	118
Coconut oil	0	44.2
Cellulose	30	50
Mineral mix	30	35
Vitamin mix	10	10
Choline Bitartrate	2,5	2,5
L-Cistin	3	3
Tertbutilhidroquinone	0,014	0,014

Chronic mild and unpredictable stress protocol

The animals were submitted to a CMS protocol that consisted of the application of different stressors 7 days per week / 3 consecutive weeks (3rd, 4th and 5th weeks) of an experimental protocol lasting 7 weeks. The CMS protocol, modified from the methodology described by Moreau (1997), is presented in Table 1. Control animals were kept under standard housing conditions. The immobilization stressor was performed utilizing polyethylene tubes whose diameter fit the body of the rats throughout the entire stress protocol (Gameiro *et al.,* 2006).

Table 1. Chronic mild and unpredictable (CMS) stress protocol.

	Morning	Afternoon
Monday	6 a.m.: end of reversed light/dark cycle 8 a.m.: 1 h immobilization	1 p.m.: 1 h immobilization 6 p.m.: overnight illumination
Tuesday	8 a.m.: 1 h immobilization	2 p.m.: 1 h immobilization followed by water and food deprivation for 18 h
Wednesday	8 a.m.: access to restricted food for 2 h.	1 p.m.: 1 h immobilization followed by water deprivation for 18 h
Thursday	8 a.m.: exposure to empty water bottle for 2 h 11 a.m.: 1 h immobilization	2 p.m.: 1 h immobilization 3 p.m.: damp cage for 17 h
Friday	8 a.m.: 1 h immobilzation	6 p.m.: reversed light/dark cycle throughout the weekend.
Saturday	6 a.m. reversed light/dark cycle	6 p.m.: reversed light/dark cycle
Sunday	6 a.m. reversed light/dark cycle	6 p.m.: reversed light/dark cycle

The immobilization was performed in polyethylene tubes.

Oral glucose tolerance test (OGTT)

At the 7th week, OGTT was performed two weeks after the end of CMS protocol. All animals were fasted for 5 hours (7 a.m. to 1 p.m.) (Liang *et al.,* 2005). After the fasting, the rats were anesthetized with halotane and a small amount of blood was collected by tail bleeding. Immediately after the first tail bleeding, a glucose solution (2,0g/kg body weight) was administered by oral gavage. Additional blood samples were collected 30, 60, 90 and 120 minutes after glucose overload (Liang *et al.,* 2005). Blood glucose was immediately measured by using the Prestige LX glucosimeter. The Homeostasis Model Assessment (HOMA) was used as an index of insulin resistance and calculated with the following formula: [(glucose - mmol/L) x (insulin - mU/L)]/22.5 (Sondergaard *et al.,* 2001).

Blood and fat depot collection

At the end of week 7, two weeks after the end of CMS procedure, all animals were killed by decapitation, without previous anesthesia in order to avoid anesthetic-induced high corticosterone plasma levels (Neves *et al.,* 2008), between 8 a.m. and 9 a.m..

Trunk blood was collected and divided between heparin-coated tubes and glass tubes without heparin. The heparin-coated tubes were centrifuged (3000x*g* for 15 min at 4 ºC), and the plasma was frozen for further analyses. The blood collected in glass tubes without heparin remained at ambient temperature for 2 hours and was then centrifuged to separate the serum, which was later used to determine the seric concentration of lipids. Two depots of adipose tissue were carefully removed and weighed; Epididymal fat pad: around testis and *ductus deferens* and retroperitoneal fad pad: along the posterior wall from the kidney to the hip region (Cinti *et al.,* 2008). These depots were summed and the ratio of total fat pad weights to final body weight was calculated (Levin *et al.,* 2000).

Analytic Methods

Plasma corticosterone was assayed by Assay Designs® kit (Ann Arbor, MI, USA, catalog # 900.097) for rats. Measurements of serum leptin and adiponectin were performed with a commercial leptin enzymatic immunoassay R&D Systems (Minneapolis, MN, USA catalog #MOB00) and Phoenix Pharmaceutical Inc® kit (Belmont, CA, USA, catalog # EK-ADI-02), respectively. Plasmatic insulin was assayed by Linco® kit (St. Charles, MS, USA catalog # EZRMI-13K). Serum free fatty acid was measured by colorimetric assay, using the WAKO Kit (Richmond, VA, USA catalog # 999-34691). Plasma triglycerides (TGL) were determined by using the commercially available Laborlab ® kit (Guarulhos, SP, Brazil, catalog # 02700). Plasma total cholesterol (TC) and high density lipoprotein (HDL) were determined by using the commercially available Laborlab® kit(Guarulhos, SP, Brazil, catalog # 01400 and catalog # 02300, respectively). Plasma low density lipoprotein (LDL) was determined by Friedewald's formula: LDL = CT-HDL - (TGLx0.2) (Friedewald *et al.,* 1972; Wakabayashi and Kobaba-Wadabayashi, 2002). All analyses were performed according to the manufacturer's instructions. The atherogenic index (AI) of the rats was determined by using the formula: AI= TC - HDL/TC (Kamgang *et al.,* 2003).

Statistical analyses

Weekly food intake and body weight were analyzed by The General Linear Mixed Model for Repeated Measures, followed by the Tukey-Kramer test for multiple comparison. In these analyses, three factors were considered: diet (standard and hypercaloric), stress (control and CMS) and time. Two-way Anova followed by Tukey post hoc test for multiple comparisons of means was used to test the effects of diet, stress, and their interaction, on data obtained at the end of the experiment. In OGTT, the area under the curve was calculated using the trapezoidal rule. Differences were considered significant at $P<0.05$. The results are presented as means ± SEM values.

RESULTS

Two weeks after the end of CMS, rats submitted to CMS and fed with standard diet had higher corticosterone concentration compared with control group (2.02 *vs.* 19.42ng/mL). In addition, rats submitted to CMS and fed with hypercaloric diet presented higher corticosterone concentration compared with the respective control group (3.34 *vs.* 18.72ng/mL).

There was no difference in initial body weight among the four experimental groups. At the end of the experimental period, animals fed with hypercaloric diet had higher final body weight than those fed with standard diet. Animals submitted to CMS showed lower final body weight than their respective control groups (Figure 1). CMS or the hypercaloric diet did not induce any change in the Lee index (Table 3).

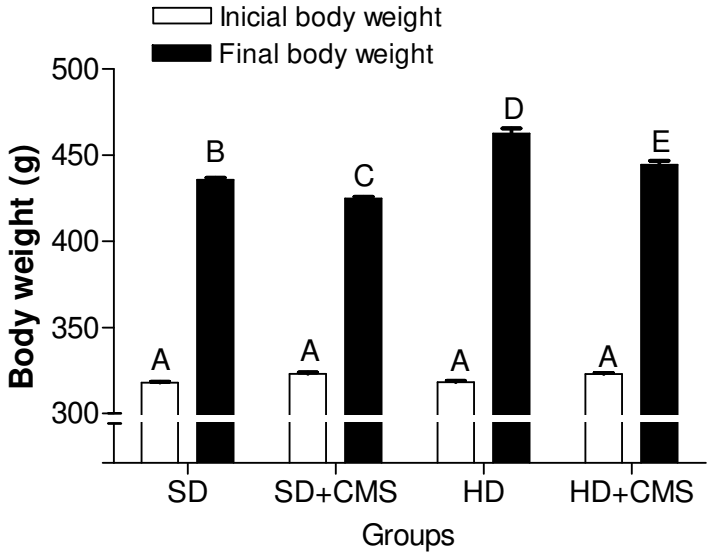

Figure 1. Initial and final body weight of rats fed with standard or hypercaloric diet submitted or not to chronic mild stress (CMS). SD: standard diet; SD+CMS: standard diet + CMS; HD: hypercaloric diet; HD+CMS: hypercaloric diet + CMS. Different letters indicate groups statistically different among them (Means ± SEM values $p<0.05$; bifactorial ANOVA and Tukey test, N = 15/groups).

Table 3. Lee index of rats fed with standard (SD) or hypercaloric diet (HD) exposed to chronic mild stress (CMS) or not, during the entire experimental protocol.

	SD	CMS	HD	HD+CMS
Week 1	0,291±0,002	0,286±0,002	0,289±0,002	0,291±0,001
Week 2	0,291±0,002	0,287±0,001	0,291±0,002	0,287±0,001
Week 3	0,295±0,002	0,284±0,001	0,292±0,002	0,285±0,001
Week 4	0,294±0,002	0,289±0,006	0,289±0,003	0,286±0,001
Week 5	0,292±0,002	0,287±0,001	0,290±0,002	0,291±0,001
Week 6	0,289±0,002	0,290±0,001	0,290±0,002	0,293±0,002
Week 7	0,290±0,001	0,291±0,001	0,289±0,002	0,292±0,002

Lee-index (g/cm) = cube root of body weight/length (Means ± SEM values $p<0.05$; bifactorial ANOVA and Tukey test, N = 15/groups)

Figure 2A represents the effect of CMS on body weight gain over seven weeks of the experimental protocol. From week 3, the body weight gain in both the HD and HD+CMS groups was higher than the SD and SD+CMS groups, respectively. Rats submitted to CMS showed lower body weight over the stress regimen (weeks 3, 4 and 5) compared with the respective controls rats, regardless of the type of diet. The HD+CMS group showed a sustained reduced body weight in the 6th week, compared with the HD group. At the end of the experimental protocol, there was not differences between stressed and control rats.

Figure 2B shows the effect of CMS on food intake, in grams, over the seven weeks of the experimental protocol. We observed that, from week 2, animals fed with the standard diet had higher food intake than animals fed with the hypercaloric diet. In week 3, animals exposed to stress had reduced food intake compared with the respective control groups, regardless of the type of diet that they were fed, without difference in weeks 4 and 5. In contrast, in week 6, one week after the end of CMS protocol, both the CMS and HD+CMS groups showed higher food intake compared with SD and HD groups, respectively. There was no difference between the control groups and the stress groups in the 7th week. Figure 2C represents the effect of CMS on food intake, in kilocalories, over the seven weeks of the experimental protocol. In the 1st and 2nd week, groups fed with the hypercaloric diet showed higher food intake, compared to groups fed with the standard

diet. In the 3rd week, the first week of the stress regimen, CMS reduced the caloric intake in rats fed with standard and hypercaloric diet. During the 4th and 5th weeks, we did not find any effects of CMS on caloric intake. In the 6th week, a week after the end of the CMS protocol, both the CMS and HD+CMS groups had increased caloric intake compared with the SD and HD groups, respectively. In week 7, there was no difference between the CMS and control groups. The food efficiency values are presented in Figure 2D. In the 1st week, rats fed with the hypercaloric diet (HD and HD+CMS groups) showed higher food efficiency than rats fed with the standard diet (SD and SD+CMS), with no difference in the 2nd week. In the 3rd week, the HD+CMS showed lower food efficiency compared with SD+CMS group, without difference between the SD and SD+CMS groups. In the 3rd and 4th week, the HD+CMS group showed higher food efficiency in relation to the control and HD group. During the post-stress period, week 6 and 7, the HD+CMS group showed higher food efficiency than their respective control group (HD).

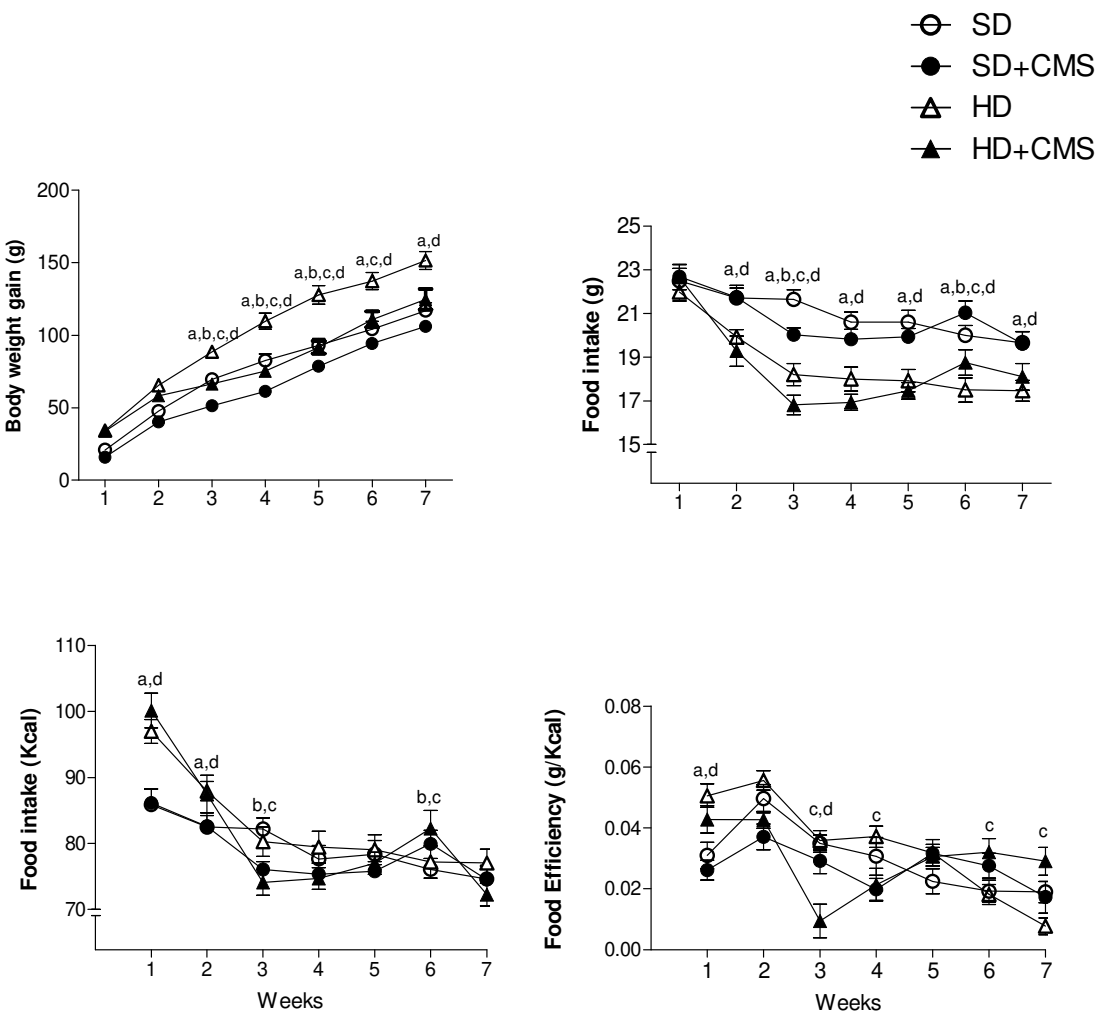

Figure 2. Effects of chronic mild stress (CMS) and hypercaloric diet on (A) body weight gain (g); (B) food intake (g), (C) caloric intake (Kcal) and (D) Food efficiency, over the 7 weeks of the experimental protocol. SD: standard diet; SD+CMS: standard diet + CMS; HD: hypercaloric diet; HD+CMS: hypercaloric diet + CMS. a: statistical difference between the HD and SD groups; b: statistical difference between the CMS and SD groups; c: statistical difference between the HD+CMS and HD groups; d: statistical difference between HD+CMS and CMS groups (Means ± SEM values $P<0.05$; bifactorial ANOVA and Tukey Kramer test, N = 15/groups).

Figure 3 shows the effect of the hypercaloric diet and CMS on body fat mass and total fat pad/ final body weight ratio measured two weeks after the end of CMS. Rats fed with the hypercaloric diet showed greater retroperitoneal, epididymal fat pad, and total fat pad weights than rats fed with the standard diet (Figure. 3A). Also, we observed that rats fed with hypercaloric diet showed higher total fat pad / final body weight ratio than rats fed with standard diet (Figure 3B). CMS effects were observed only in animals fed with hypercaloric diet, since the HD+CMS group had higher epididymal fat, higher total fat pad weights and a higher total fat pad / final body weight ratio than the HD group, without difference between SD and CMS groups.

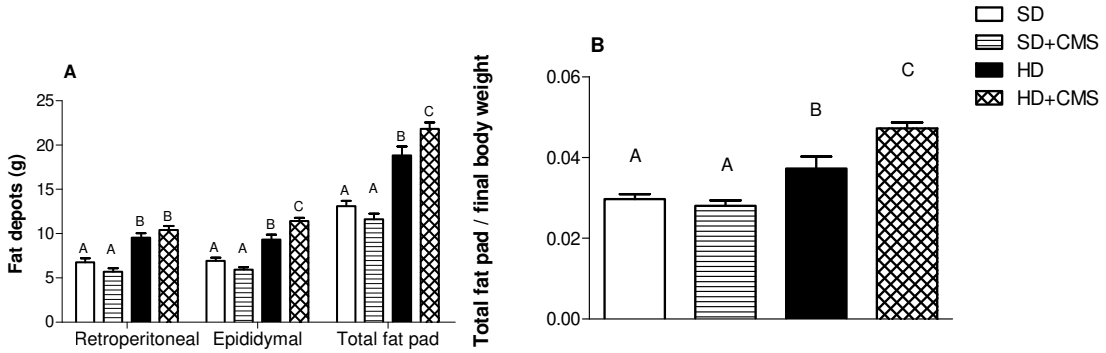

Figure 3. Effect of chronic mild stress (CMS) and hypercaloric diet on fat mass, two weeks after the end of the CMS regimen. (A) Epididymal fat pad, perirenal fat, total fat pad (g). (B) Total fat pad / final body weight ratio. SD: standard diet; SD+CMS: standard diet + CMS; HD: hypercaloric diet; HD+CMS: hypercaloric diet + CMS. Different letters indicate groups significantly different among them (Means ± SEM values $p<0.05$; bifactorial ANOVA and Tukey test, N = 15).

The effects of CMS and hypercaloric diet on the lipid profile, plasma free fatty acids, triglycerides and atherogenic index are shown in Figure 4. There was not significant difference in plasma free fatty acids and HDL among the groups (figure 4A and Figure 4B, respectively). Stressed rats fed with standard diet (SD+CMS) showed elevated plasma concentration of triglycerides (Figure 4A), total cholesterol (Figure 4B), LDL (Figure 4B) and atherogenic index (Figure 4C), compared with the respective control groups (SD). In addition, it was observed higher levels of triglycerides, total cholesterol and LDL, and atherogenic index (Figure 4A; 4B; 4C) in control rats fed with hypercaloric diet in comparison with animals fed with standard diet (SD). When chronic stress was associated with the treatment with hypercaloric diet these effects were exacerbated, since HD+CMS group showed higher plasma concentration of triglycerides, total cholesterol, and LDL in relation to all the other groups (Figure 4A; 4B).

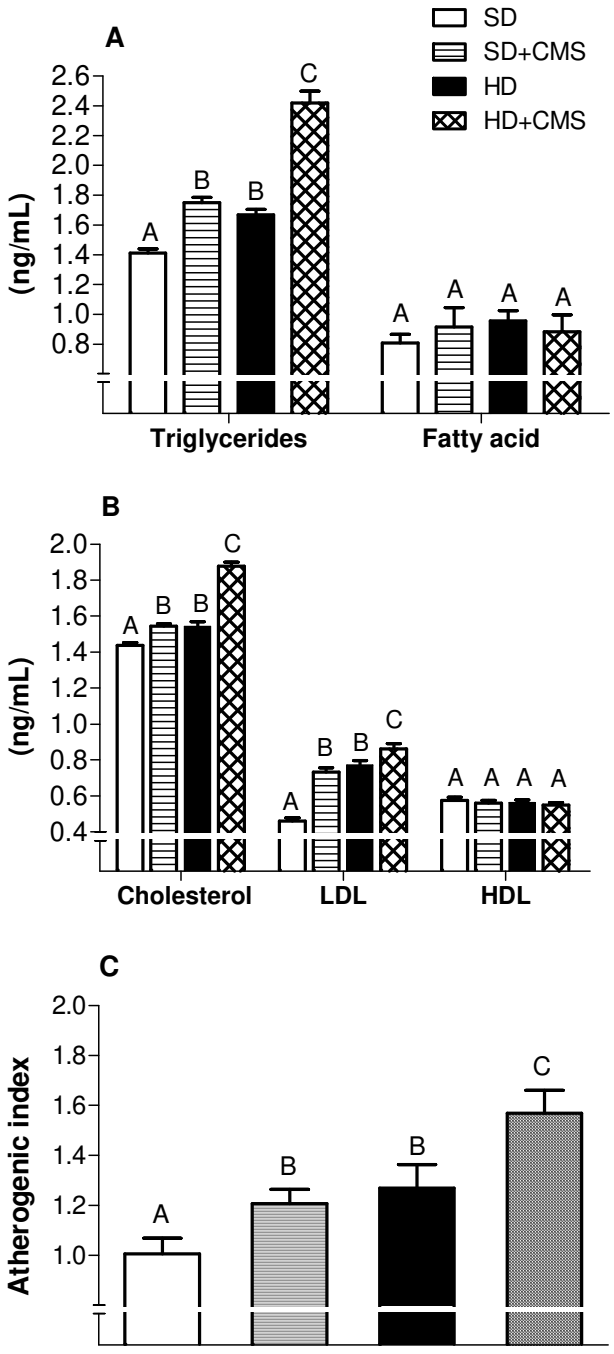

Figure 4. Effects of chronic mild stress (CMS) and hypercaloric diet on blood concentration of A) triglycerides; B) total cholesterol; C) LDL and (D) atherogenic index two weeks after the end of CMS. Different letters indicate groups significantly different among them (Means ± SEM values $p<0.05$; bifactorial ANOVA and Tukey test, N = 12).

Figure 5 represents the effect of CMS and hypercaloric diet on fasting insulin concentration and the HOMA index, measured one week after the end of the CMS protocol. Even though there was no difference on fasting glucose among the groups (SD: 90.56 ± 3.33; SD+CMS: 93.67 ± 3.19; HD: 89.44 ± 6.38; HD+CMS: 93.44 ± 5.42mg/dL), CMS increased the fasting insulin concentration, compared to respective control groups (Figure 5A). Thus, higher HOMA index in stressed rats fed with hypercaloric or standard diet was observed, compared with respective control groups (Figure 5B). Figure 5C shows the effect of CMS and the hypercaloric diet on oral glucose tolerance curves. From 30 minutes after the glucose administration, both the CMS and HD+CMS groups showed higher glucose concentrations than the SD and HD groups, respectively. CMS and HD + CMS groups presented higher area under curve values than SD (SD+CMS: 14267 ± 344 vs. SD: 13549 ± 387 mg x min / dL) and HD (HD+CMS: 16476 ± 559 vs. HD: 15852 ± 270 mg x min / dL) groups.

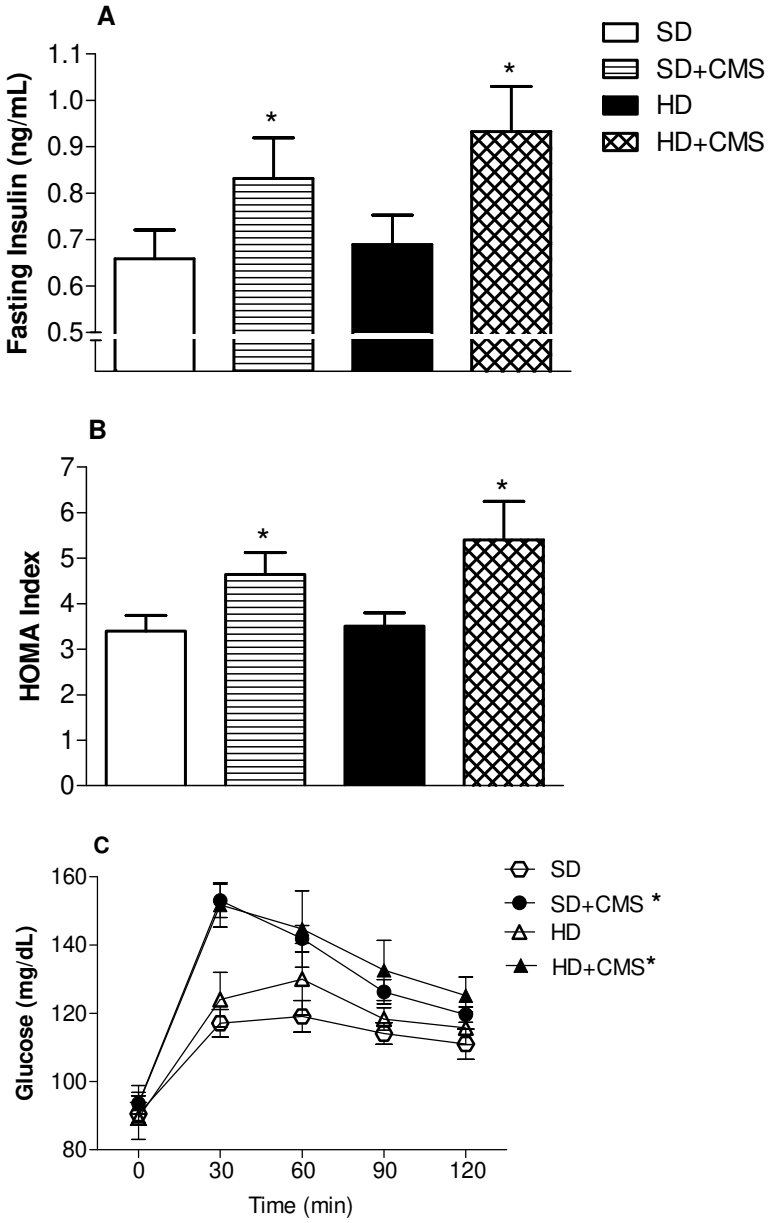

Figure 5. Effects of chronic mild stress (CMS) and hypercaloric diet on A) fasting glucose; B) fasting insulin; C) HOMA index and D) OGTT curves. SD: standard diet; HD: hypercaloric diet; CMS: standard diet + CMS; HD+CMS: hypercaloric diet + CMS. *: statistical differences between CMS and control groups (Means ± SEM values $p < 0.05$, bifactorial ANOVA and Tukey test N = 10/groups).

The effects of CMS and hypercaloric diet on plasma leptin and adiponectin concentrations are represented in Table 4. There was no significant difference in plasma leptin concentration between control rats fed with standard (SD group) and hypercaloric diet (HD group). However, HD+CMS rats showed higher plasma leptin than CMS rats (Table 4). In relation to the effect of stress, chronic mild stress reduced leptin levels in animals fed with standard diet (CMS) compared with the respective control group (SD). However, chronic stress did not change leptin levels in animals fed with hypercaloric diet since there was no difference between HD and HD + CMS groups. There were no differences in plasma adiponectin concentration among the experimental groups (Table 4).

Table 4. Leptin and adiponectin concentrations, two weeks after the end of chronic mild stress (CMS) in rats fed with standard diet (SD) or hypercaloric diet (HD), 15 days after CMS.

Groups	Leptin (mmol/L)	Adiponectin (mmol/L)
SD	4.54±0.34 (14) A	6.70±0.38 (8) A
SD+CMS	2.66±0.24 (14) B	6.15±0.34 (8) A
HD	5.76±0.82 (13) AC	6.06±0.40 (9) A
HD+CMS	6.48±0.54 (9) C	6.21±0.32 (8) A

Different letters indicate groups significantly different among them. Means ± SEM values ($p<0.05$; bifactorial ANOVA and Tukey test).

DISCUSSION

In the present study, the effect of CMS on body weight, food intake, adiposity, lipids, and carbohydrate metabolism of rats fed with standard and hypercaloric diets was examined two weeks after the end of the CMS protocol. The efficiency of the CMS regimen utilized was confirmed by the elevated corticosterone concentration in stressed rats, two weeks after the end of CMS, regardless the diet. Also, we observed lower final body weight and body weight gain in stressed rats fed with standard or hypercaloric diet during the stress regimen (weeks 3, 4 and 5). These findings are in agreement with Willner et al (1996) and corroborate other findings in the literature, which suggest that stress-induced body weight loss may be a consequence of the stress-induced increase of the HPA activity (Harris *et al.,* 1998, Smagin et al., 1999; Levin *et al.,* 2000; Kraemer *et al.,* 2002). Indeed, lower final body weight and higher plasma corticosterone observed in stressed rats show a long-lasting effects of CMS.

The lower body weight gain and the reduced final body weight observed in stressed rats in the present study may be related to alterations in feeding behavior. It was observed a reduced food intake in stressed rats fed with standard or hypercaloric diet in the 1st week of CMS. Although this effect did not last over the second and third weeks; the stress-induced lower food intake is partially in agreement with other authors. Tamashiro *et al.* (2006) observed that social stress induced lower food intake and higher corticosterone levels in subordinate rats compared with dominants rats. Harris *et al.* (2006) also reported a reduction in food intake during the 3h / 3 day – restraint stress, and until 4 days of post-stress period. However, in contrast with these authors, in the present study, stressed rats showed higher food / caloric intake, one week after the end of CMS regimen, regardless of the diets. In addition, the SD+CMS group had unchanged food efficiency in the same period. These findings suggest that hyperphagia seems to be induced after stress in order to allow body weight recovery, since stressed rats fed with stardart diet did not showed differences in body weight onde week after the end of CMS.

Levin *et al.* (2000) observed that diet-resistant rats submitted to CMS showed reduced body weight gain, without alterations in food intake, which was associated to a trend to reduce the food efficiency over the stress period. However in the present study, rats

fed with hypercaloric diet and submitted to stress had lower food efficiency during the CMS regimen that is related to the lower body weight gain, during the CMS regimen, and lower food /caloric intake in the first week. Nevertheless, HD+CMS had a sustained lower body weight one week after the end of the CMS that was associated with higher food intake and efficiency. These findings could suggest that stressed rats fed with hypercaloric diet had a prolonged effect of stress on the body weight gain. Then, our findings suggest that the lower body weight gain during the CMS is related to lower food intake, regardless the type of diet. However, during the post-stress period, stress-induced lower body weight gain observed in stressed rats fed with hypercaloric diet could not be explained by changes in the feeding behavior, since this group had an increase in food / caloric intake. The increased food efficiency observed during the post-stress period could be related to a mechanism of body weight recovery.

Hypercaloric diet did not promote alterations in the Lee index, a useful obesity index in rodents. Nevertheless, the hypercaloric diet did promote a significant increase in body weight in both HD and HD+CMS groups during the experimental period. Also, two weeks after the end of CMS we observed increased adiposity and higher final body weight in rats fed with hypercaloric diet. So our data show the effectiveness of hypercaloric diet in promoting important alterations in the body weight gain and adiposity, which could contribute to further long-lasting obesity development. Other authors demonstrated no alteration in body weight of the rats fed with a high fat diet *ad libitum* (Maegawa et al., 1986) or even smaller body weight than that of those fed with control diet (Pedersen et al., 1991). Dourmashkin et al. (2006) reported that obesity-prone rats exposure to five-day high-fat diet showed higher body weight gain, which was positively correlated with higher body fat found 4-6 weeks later. Indeed, Levin et al. (1987) demonstrated that Sprague-Dawleys rats may have different response to hypercaloric diet. These authors demonstrated that diet-induced obesity rats model, about one-half of the rats fed a diet moderately high in fat, sucrose, and energy content become obesy, and one-half of these rats become resistant to development of obesity.

Some authors have demonstrated that diet-induced obese rats have increased lipid concentration and decreased sympathetic-induced lipolysis (Hill et al., 1991), enlarged

fat cells, an increased number of α_2-adrenoceptors and fat cell proliferation (Saulnier-Blache et al., 1990; Lafontan et al., 1995). Zhao et al. (2007) suggested that the obesity in diet-induced obesity-prone rats may be associated with decreased hormone sensivity lipase gene expression and increased lipoprotein lipase gene expression, which promote adipose synthesis and inhibit lipolysis. Dourmashkin et al. (2006) concluded that the diet-induced higher body weight gain and metabolic disturbances, such as elevated leptin, insulin, triglycerides, glucose concentrations and increased lipoprotein lipase activity may be related to long-term accumulation of body fat. In agreement with these authors cited above, we observed that after a long-term hypercaloric feeding, the HD and HD+CMS groups showed higher body weight, heavier fat pad and altered lipid profile.

HD and HD+CMS were fed with hypercaloric diet consisting coconut oil, which is a saturated fat (Reiser et al., 1985). Ji and Friedman (2007) suggest rats fed with high-fat diet have a limited capacity to oxidize fatty acids causing overeating and obesity. These authors reported that diet-induced obesity prone (DIO) rats oxidize less fatty acid before they eat high-fat diet than diet-induced-obesity resistant (DR) rats. DIO rats fed with low-fat diet showed reduced liver mRNA expression of CD36, which transports fatty acids across cell membranes, and long-chain acyl coenzyme A dehydrogenase, which catalyzes the first step of mitochondrial β-oxidation of fatty acids, and reduced expression of liver carnitine palmitoyl transferase-I, the enzyme which mediates transport of long-chain fatty acids into mitochondria. Thus, DIO rats have preexisting limitations in transporting fatty acids into hepatocytes and in initiating β-oxidation of fatty acids (Ji and Friedman, 2007). Saturated fat may promote hipercholesterolemic effects (Reiser, 1973; 1985), increase LDL and triglycerides (Kangang et al., 2005) and potencializate the athresocleroes developement (Kang & Leaf, 2000). The saturated fats are stored in adipose tissue depots and are less readily mobilized by lipolytic stimuli (Mougios et al. 1995; Halliwell et al. 1996; Raclot et al. 1997). Lipids are able to stored energy reserve efficiently; also they form the cell membranes, when saturated fats are incorporated into cell membranes they are able to reduce metabolic rate and decrease β-adrenoceptor binding influencing the energy balance (Matsuo & Suzuki, 1997). In addition, saturated fatty acids, present in coconut oil, may reduce the plasma lecithin-cholesterol acyltransferase activity and increase the hepatic 3-

hydroxy-3-methylglutaryl- CoA reductase activity, consequently, may induce hypercholesteromia and higher LDL concentration in rats (Zulet et al., 1999). These literature data corroborate with the suggestion that the hypercaloric, diet, rich in coconut oil, may change the fat accretion, body weight gain and lipids concentration throughout changes in lipids metabolism.

In relation to the effects of the association between CMS and hypercaloric on adiposity, HD+CMS showed heavier fat depots compared to the HD and CMS groups. In rats, as well as in humans, the glucocorticoids are associated with increased adiposity (Tannenbaum *et al.,* 1997, Bjorntorp, P. 1997; Michel *et al.,* 2004). Dalman *et al.* (2004) demonstrated that the increase in intra-abdominal fat pad is a consequence of elevated glucocorticoid and insulin. Likewise, in our study, the elevated plasmatic concentration of corticosterone in HD+CMS was linked to increased adiposity and may suggests that glucocorticoids, as part of the overall stress response, could play an important role in the further development of obesity. In addition, the lipolytic activity of catecholamines is induced by the β_1, β_2 and β_3 subtypes of β-adrenoceptors in the adipose tissues (Hollenga, 1989). Desensibilization or down-regulation of the adrenoceptors present in the fat cells may increase the fat pad and induce obesity due to reduced catecholamine-induced lipolytic activity (Black, 2003; Bougnerer *et al.,* 1997). Such alterations in adrenoceptors could have occurred in stressed rats fed with hypercaloric diet, resulting in reduced lipolytic activity of catecholamines and increased adiposity / body weight.

We did not observe alterations in the adiposity of stressed rats fed with standard diet. This finding could be associated with the higher food intake of CMS group compared with SD group during the 6^{th} week of experimental protocol, without change in body weight gain. Nevertheless, the CMS group had significant increase in plasma triglycerides, total cholesterol and LDL concentration. Even though we did not find changes adiposity, we there was a trend to reduce the epididymal and retroperitoneal fat pads and the total fat pad. It has been demonstrated that the stress may increase catecholamine-induced lipolytic activities and triglycerides hydrolysis in various adipose depots (Lafontan *et al.,* 1997). So, dyslipidemia observed in CMS group could be related to lipolytic action of catecholamines. However this catecholamine-induced lipolytic action was not enough to decrease fat depot.

This finding could be explained by three factors: CMS could have induced stimulation of α_2-adrenoceptors by the catecholamine, which elicits preadipocityte proliferation (Valet *et al.,* 1998). Also, the higher food intake observed in the 6th week, which could be a rebound hyperphagia in response to food-restriction period during the CMS regimen. In addition, the mechanisms of body weight regulation could prevent the stress-induced reduction body fat mass. This finding is in agreement with Harris *et al.,* (1986) who observed that restricted rats recovered body fat than much earlier than body protein, suggesting that body fat and protein may have individual regulatory mechanisms that work together to control the body weight.

The principal function of the adipocytes is to store excess energy as triglycerides and release it in the form of free fatty acid and glycerol, throughout the action of lipoprotein lipase (Sethi & Vidal-Puig, 2007). Catecholamines are able to inhibit the activity of extra-hepatic lipoprotein lipase, which hydrolyzes triglycerides to VLDL to store in the adipocytes. Thus, the elevated serum triglycerides observed in the CMS and HD+CMS in this study may be associated with the inhibition of lipoprotein lipase, which reduces the withdrawal of triglycerides from the blood and the triglycerides stores in the adipocytes (Black, 2002; Brindley and Rolland, 1989). Also, higher levels of glucocorticoids stimulate secretion of hepatic triglycerides in VLDL, as demonstrated in perfused liver and monolayer cultures of hepatocytes (Bartlett & Gibbons, 1988). Furthermore, glucocorticoids decrease levels of lipoprotein lipase, which controls the hydrolysis of VLDL; this decrease has been shown to exaggerate hypertriglyceridemia (Taylor & Agius, 1988). Indeed, the elevated corticosterone release in the CMS and HD+CMS groups, reported in the present study, could reduce the number of LDL receptors in the liver (Brindley & Rolland, 1989), resulting in elevated blood LDL concentration, by delaying the lipoprotein metabolism. LDL particles shift a higher amount of cholesterol from the blood to the liver. Normally, most LDL formed after the degradation of VLDL is removed from the circulation via receptor-mediated endocytosis. The binding and degradation of LDL by rat hepatocytes are decreased by dexamethasone, a synthetic glucocorticoids, which could result in elevations in LDL levels (Goldstein & Brown, 1977). Then, a delay in this withdrawal may also delay the cholesterol release, increasing the

serum cholesterol concentration (Brindley & Rolland, 1989). Moreover, higher plasma LDL observed in rats fed with hypercaloric diet, HD and HD+CMS groups, may indicate that these lipoproteins effectively participated in the transfer of high intakes of dietary cholesterol to the peripheral tissues, an occurrence also expected due to the ingestion of the hypercaloric diet, which is rich in saturated fatty acids. Thus, the increase in blood concentrations of triglycerides, total cholesterol and LDL suggest an effect of hypercaloric diet and CMS on the hepatic lipid metabolism, which seems to be enhanced by the association between CMS and hypercaloric diet.

On the other hand, in the present study only CMS promoted relevant alteration in the carbohydrate metabolism. Rats fed with hypercaloric diet whithout exposure to stressors did not show alteration in the OGTT. Diniz et al. (2008) found no difference in the glucose tolerance test in rats fed with a high fat diet, but did find an increase in the area under the curve of animals fed with high carbohydrate diet. Since during OGTT we observed higher glucose levels in rats that had been stressed compared to control groups, these data may suggest an association between higher blood glucose and corticosterone concentrations induced by stress. This finding is in agreement with Verago et al. (2001), who observed a gradual increase in the glycemia of rats submitted to daily footshock for three consecutive days during OGTT, associated with higher corticosterone concentration. We also observed that stressed rats had higher fasting insulin concentration and unchanged fasting glycemia. Thus, rats submitted to CMS showed an increased HOMA index, which is an insulin resistance index. Indeed, the insulin resistance observed in stressed rats fed with hypercaloric diet may be associated to increased adiposity (Ebal et al., 2008). This finding corroborate with Wagenknecht et al. (2003) who reported that hepatic insulin resistance is strongly linked to abdominal fat pad. Insulin and corticosterone have a complex interaction in body weight regulation; human obesity may result from elevated glucocorticoids and insulin increasing the intake of high density calories (La Fleur et al., 2004). Although glucocorticoids inhibit energy storage, and insulin promotes energy storage, both the hormone act synergistically in abdominal fat depots (Stracks *et al*., 1995; Livingstone *et al.*, 2000, Woods *et al.*, 2003). Likewise, we found that the HD+CMS had

elevated levels of corticosterone associated with increased fasting insulin concentration, insulin resistance, and higher adiposity.

Leptin is a hormone secreted mainly by adipose tissue that acts in the energy balance regulating body weight, food intake, and lipid and carbohydrate metabolism. Physiological effects of decreased leptin concentrations on the energy homeostasis are more important than increased leptin concentration, since reduced leptin production is related to negative energy balance and low energy reserves, rather being an indicator of positive energy balance and increased energy reserves (Havel, 2004). The reduction of leptin concentration observed in CMS group compared with SD, two weeks after the end of the stress, may suggest a long-lasting unbalanced energy homeostasis induced by the CMS. Since, the leptin secretion also may be influenced by the activation of the HPA axis when rats are exposed to stress (Gamaro *et al*, 2008; Lu *et al.,* 2006), the lower leptin concentration, in the present study, seems to be associated with higher corticosterone concentration. In contrast, higher leptin concentration observed in HD+CMS group could be related to the increased adiposity induced by CMS in animal fed with hypercaloric diet. Therefore, the effect of CMS on leptin secretion may be dependent on caloric values of the diet utilized.

Lower levels of adiponectin have been linked to several components of metabolic syndrome, such as visceral body fat, dyslipidemia, insulin resistance / type 2 diabetes, which are related to cardiovascular disease and atherosclerosis. So, adiponectin also appears to have direct and indirect actions that would be considered to protect against cardiovascular disease (Cnop *et al.,* 1993; Tschritter *et al.,* 2001; Weyer *et al.,* 2003). However, in the present study, it was not found alteration in plasma adiponectin concentration among the experimental groups, even though we did observe an increased atherogenic index induced by CMS and hypercaloric diet. Nonetheless, we cannot relate the higher atherogenic index to alteration in adiponectin concentration, but this parameter is associated with dyslipidemia. Neves *et al.* (2008) suggested that CMS may contribute to the development of atherosclerosis by mechanisms related to impairment in nitric oxide production and dyslipidemia. These authors found that stressed rats had lower sensitivity to phenilephrine, and hypertrophy of the intima and tunica media in the thoracic aorta and

increased serum triglycerides, total cholesterol, very low-density lipoprotein cholesterol, low-density lipoprotein cholesterol and atherogenic index. In the present study, the association between the treatments, CMS and hypercaloric diet, also promoted higher atherogenic index. The diet-induced atherogenic index increase is in agreement with Diniz *et al.* (2004) who showed elevated atherogenic index associated with higher serum triacylglycerol, cholesterol and LDL in rats fed with high fatty acids diet, compared with rats fed with a poliunsatured fatty acids diets. Kangang et al. (2005) reported that hypercaloric diets, consisted by saturated acids, may induce glucose intolerance and type 2 diabetes and might have an atherogenic effect and could be used in a long-term study to induce type 2 diabetes mellitus (Kamgang *et al.* 2005).

In summary, since two weeks after the end of stress regimen rats submitted to CMS still presented high corticosterone concentration and lower final body weight compared to control animals, the experimental protocol used in the present study is useful to study the long-term effects of CMS. Also, since the stressed groups presented glucose intolerance and higher HOMA index compared with respective control rats, regardless the diets employed, the effect of stress seems to be more pronounced in the metabolism of glucose. And, considering lipids metabolism, both the CMS and hypercaloric diet promoted dyslipidemia, and higher atherogenic index, compared with the rats fed with standard diet but the association between both the treatments exacerbated the adipocity, dyslipidemia and atherogenic index. In conclusion stress and hypercaloric diet effects on the metabolism of glucose and lipids could be related to atherogenic effects and may related to the development of diabetes.

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CAPÍTULO 2

Ferreira R, Marcondes FK, Harris RB. Effects of chronic mild stress on body weight and food intake in rats. *Stress: The International Journal of biology of stress* – submetido em 29/01/2009.

Neste capitulo, apresentaremos os resultados referentes ao estudo realizado na Universidade de Georgia, no Departamento de Alimento e Nutrição, sob orientação de Dr. Ruth B. Harris. Este manuscrito foi submetido para publicação no *Stress: The International Journal of biology of stress*, em 31/01/2009.

Neste estudo, investigamos o efeito do ECMI sobre o peso corporal e ingestão alimentar durante a aplicação dos estressores. O acúmulo de gordura e corticosterona sérico foram estudados imediatamente e duas semanas após o término no ECMI. Neste estudo também foram realizados testes de tolerância à glicose durante a aplicação do ECMI e imediatamente após o seu término. Além disso, foi testado se a restrição alimentar é o principal fator que contribui para a perda de peso corporal observado em ratos expostos ao ECMI. Para esta finalidade foram utilizados grupos de ratos submetidos à restrição alimentar, onde os ratos foram alimentados com a mesma quantidade de ração ingerida pelo de ratos durante o protocolo de ECMI. Neste estudo também foram estudadas a composição de gordura, proteína e água, presentes na carcaça de ratos.

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Effects of chronic mild stress on body weight and food intake of Sprague-Dawley rats.

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ABSTRACT

Chronic mild and unpredcitable stress (CMS) is a prolonged period of exposure to different stressors that induces anhedonia and body weight loss in rats. We studied the effects of CMS on body weight and food intake during and for two weeks after the end of CMS. In Experiment 1, rats were submitted to CMS (7 days/week/3 weeks). Body weight and food intake was measured two-weeks pre- and post-stress, and during the CMS period. Weight loss induced by the CMS protocol was sustained for one week after the end of CMS. Basal corticosterone concentration was increased in CMS rats at the end of stress and two weeks later. Fat depots weights were reduced at the end of CMS, but not two weeks later. In Experiment 2, we tested the contribution made by food restriction to body weight loss observed during CMS. For this, we utilized control, CMS and pair-fed groups. CMS and pair-feeding induced weight loss, lowered food intake, selectively reduced fat depot weight and lowered serum triglyceride concentration. Total carcass fat was reduced only in pair-fed rats. These findings suggest that CMS produces only a temporary reduction in body weight that is not dependant on an inhibition of food intake. By contrast, CMS induces a prolonged disruption of the HPA axis.

KEYWORDS: chronic mild stress, body weight, food intake, food restriction, rats, corticosterone

INTRODUCTION

Chronic mild and unpredictable stress (CMS) consists of a prolonged exposure to a variety of mild stressors. This procedure has been reported to induce anhedonia, the inability to experience pleasure, evidenced by the loss of responsiveness to reward, measured as decreased consumption of sucrose solution, decreased place preference conditioning and sleep changes (Muscat *et al.,* 1990; Muscat *et al.,* 1992; Moreau *et al.,* 1995). Rats exposed to CMS gain weight more slowly than non-stressed controls, leading to a relative body weight loss. They also show signs of increased hypothalamus-pituitary-adrenal (HPA) axis activity, including adrenal hypertrophy and corticosterone hypersecretion (Muscat and Willner, 1992). Indeed, the exposure to mild and variable stressors prevents adaptations of the HPA axis to chronic stress that normally protects organisms from a chronic increase in glucocorticoid concentration (Armario *et al.,* 1986). Moreau *et al.* (1994) reported that animals submitted to unpredictable mild stress for a prolonged period exhibit attenuated intra-cranial-self stimulation threshold, this technique is used for the study of reward, anhedonia and motivation (Markou & Koob, 1991).

The effects of CMS have been reported to develop gradually over a two week period, remain constant for the remainer of the stress period and are then gradually reversed during a subsequent two-week post-CMS (Moreau *et al.,* 1994). This closely resembles the clinical time course of antidepressant action (Willner, 1997). In animals, the long term consequences of stress on body composition and endocrine parameters have been studied by Tamashiro *et al.,* (2007) who observed that rats submitted to social stress for 14 days had body weight loss during the period of stress, but they were hyperphagic and regained body weight over a 21-days recovery period. By contrast, Harris *et al.* (2006) observed that restraint-stress-induced body weight loss was sustained for at least 11 weeks after the end of stress. Restraint stress was associated with an increased energy expenditure and a reduced food intake on the days of restraint without any differences between stressed and control rats once stress endeds. Also, Harris *et al.,* (2004) reported that rats that had been previously exposed to repeated restraint had an exaggerated corticosterone response to a novel mild stressor. Houshyar *et al.* (2004) reported that 8-days after the end of 8-days of

morphine injections rats still display facilitated central stress responses, similar to the HPA-symptoms described in posttraumatic stresss disorder patients, which involve interrelated changes in energy balance and HPA activity (Lemieux & Coe, 1995). Penke (2001) reported that postnatal food deprivation induces a long-lasting reduction in food intake of rats due to a change in glucocorticoid receptor number in the hypothalamic paraventricular nucleus and/or corticotrophin-release factor (CRH) or vasopressin function. Other studies have demonstrated that a single stressful event in life may trigger physiological disorders that are significantly associated with anxiety, major depressive disorders and post-traumatic stress disorder in humans (Lemieux & Coe, 1995; Woodside and Staab, 2006; Scott *et al.,* 2008).

Although the effect of CMS on body weight and food intake has not been investigated in detail, repeated restraint and immobilization stress models are accepted as factors that might disturb the food intake and body weight regulation (Marti *et al.,* 1994; Smagin *et al.* 1999). The CMS model reduces the body weight in rats, even though its effect on food intake is unclear. CMS may increase or reduce the food intake, and has even been reported not to change food intake (Muscat *et al.,* 1992; Papp *et al.,* 1992). The mechanisms of the effects of stress on body weight have been widely investigated. Chotiwat & Harris (2008) demonstrated that activation of CRH-Type 1 receptors is required for the initiation of events that lead to a prolonged body weight loss in rats submitted to repeated restraint, independent of hypophagia on the days of restraint and independent of stress-induced corticosterone release. Elevated glucocorticoid concentration induced by chronic stress produces catabolic effects, accelerates lipolysis and increases thermogenesis leading to body weight loss (Tempel & Leibowitz, 1994). In contrast, in humans, high cortisol secretion in response to the Trier Social Stress Test is related to an increased intake of a hypercaloric diet (Epel *et al.,* 2001). Dallman *et al.* (2005) postulated that actions of corticosterone seem primarily to increase food intake in rats. Corticosterone in the absence of insulin increased chow intake, and in the presence of insulin increased caloric intake. Rats given a choice of lard and chow had an attenuated adrenocorticotrophin (ACTH) and corticosterone response to restraint stress suggesting that the HPA response to stress depends on dietary choice (la Fleur *et al.,* 2005).

The CMS protocol validated by Moreau and colleagues utilizes a shorter time of food and water deprivation that those utilized by Katz and colleagues, but even with this shorter time, these stressors per se may influence body weight regulation, feeding behavior and preference for sucrose (Willner *et al.,* 1987). Both acute food deprivation and chronic food restriction produce different metabolic states, resulting in reduced body weight, circulating leptin levels and may increase arcuate neuropeptide Y (NPY) and decrease the pro-opiomelanocortin (POMC) (Bi *et al.,* 2003). Belda *et al.* (2005) reported that food restriction reduces body weight gain and increases morning and evening levels of corticosterone, without alterations in serum ACTH or CRH mRNA levels in the paraventricular nucleus of the hypothalamus, suggesting that factors other than CRH and ACTH are involved in the control of adrenocortical secretion in conditions of food restriction. Jonhasson *et al.* (2008) confirmed the importance of the increased NPY, a potent orexigenic neuropeptide, and decreased POMC, anorexigenic neuropeptide, in body weight homeostasis of rats submitted to acute or chronic food restriction.

As CMS is validated model for the study of the mechanism of depressive drugs and promotes body weight loss, and its behavior alterations that may last at least two weeks (Wilmer, 1997), thus, the main objective of the present work was to study the effects of CMS on body weight and food intake immediately after and two weeks after the end of CMS. In addition, we tested food restriction is the main factor that contributes to body weight loss in rats exposed to CMS.

METHODS

Animals

Forty-two male Sprague Dawley rats weighing 300g were obtained from Harlan *Sprague-Dawley (Houston, TX) and were housed in individual wire mesh cages in a* humidity- and temperature-controlled room (23^{o}C and 55% humidity, 12:12h light-dark cycle with lights on at 7 a.m.). The rats had free access to water and rat chow (Purina Chow 5001). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia and met the guidelines described by the American Physiology.

Chronic mild and unpredictable stress protocol

The animals were submitted to a CMS protocol that consisted of the application of different stressors 7 days per week / 3 consecutive weeks (3rd, 4th and 5th weeks) of an experimental protocol lasting 7 weeks. The CMS started at day 15 (Wednesday) and finished on day 35 (Tuesday) of the experiment. The CMS protocol, modified from the methodology described by Moreau (1997), is presented in Table 1. Control animals were kept under standard housing conditions in a different animal room. The restraint stressor was performed in Perspex restraining (21.6 x 6.4 cm) tubes (Plas Labs, Lansing, MI).

Table 1. Chronic mild and unpredictable (CMS) stress protocol.

	Morning	Afternoon
Monday	8 a.m.: 1 h restraint	1 p.m.: 1 h restraint 6 p.m.: overnight illumination
Tuesday	8 a.m.: 1 h restraint	2 p.m.: 1 h restraint followed by water and food deprivation for 18 h
Wednesday	8 a.m.: access to restricted food for 2 h.	1 p.m.: 1 h restraint followed by water deprivation for 18 h
Thursday	8 a.m.: exposure to empty water bottle for 2 h 11 a.m.: 1 h restraint	2 p.m.: 1 h restraint 3 p.m.: damp cage for 17 h
Friday	8 a.m.: 1 h restraint	7 p.m.: reversed light/dark cycle
Saturday	7 a.m. reversed light/dark cycle	7 p.m.: reversed light/dark cycle
Sunday	7 a.m. reversed light/dark cycle	7 p.m.: reversed light/dark cycle

Control group was maintained in a different animal room during the entire CMS protocol.

Body weight and food intake analyses

Due to the food and water deprivation and damp bedding stressors, food intake was measured only three times a week; 1) on Monday, after a reversed light-dark cycle: the rats were fed on Friday, 8.00 a.m. and average food intake for Saturday, Sunday and Monday was calculated. 2) On Tuesday, after the overnight lights on: the rats were fed on Monday, 8.00 a.m. and food intake was measure on Tuesday, 3.00 p.m., before the food / water deprivation stressor; and 3) on Thursday, after the water deprivation period: the rats were fed on Wednesday, 10.00 a.m. and the food intake was measure on Thursday, 3.00 p.m., before the housing in damp cages. Body weight was measured twice a week, on Monday at 8.00 a.m., after the reversed light-dark cycle, and Thursday at 8.00 a.m., after water deprivation.

Experiment 1

The objective of this experiment was to compare the effects of CMS on body weight and food intake during CMS and 15 days after the end of CMS. Also, we tested the effects of CMS on glucose clearance one week after the beginning of CMS and again immediately after the end of CMS protocol. Finally, we tested the effects of CMS on fat pad weight immediately after the end of CMS and two weeks after the end of CMS.

After one week of adaptation to the environment, food intakes and body weights of the rats were measured for two weeks. The experiment was seven weeks long. The CMS protocol was applied at weeks 3, 4 and 5. During the post-CMS period, food intake and body weight were measured for two weeks. In week 3, the rats were divided into two weight-matched groups: Control and CMS, which were subdivided into two weight-matched groups: Control and CMS - killed one day after the end of CMS or Control and CMS - killed two weeks after the end of CMS.

To determine the effect of restraint stress on serum corticosterone concentration, blood samples were collected by tail bleeding from the CMS rats before restraint stress (time 0) and at the end of restraint (time 60) performed on Tuesday morning, after the overnight light on, one week after the start of CMS and again immediately after

the last restraint of the last week of CMS procedure. Serum corticosterone was measured by radioimmunoassay (MP Biomedicals, Cupertino, CA). The animals used to perform this analysis were killed on the day after the end of the CMS.

To test the effect of CMS on glucose clearance an oral glucose tolerance test (OGTT) was performed one week after the beginning of the CMS and again immediately after the end of the CMS protocol. The rats were not stressed on the day of the OGTT when the rats were still being exposed to CMS, but they were food deprived from 7.00 a.m. before starting the test at 12.00 p.m.. Control and CMS rats were food deprived for 5 hours and a small amount of blood was collected by tail bleeding. Immediately after the first tail bleed, each rat was administered a glucose solution (2.5g/kg body weight), by gavage. Additional blood samples were collected 10, 30, 60, 90, 120 and 180 minutes after glucose administration and were analyzed for glucose (Sigma Diagnostic Kit 510) and serum insulin (Rat Insulin RIA kit, Linco, St. Louis, MO Cat # RI-13k). The animals used to perform the OGTT were killed two weeks after the end of CMS.

On the day after the end of CMS, one group of controls and one group of CMS rats were killed by decapitation between 10.00 and 12.00 a.m. and food was removed from their cages at 8.00 a.m.. In the group sacrificed one day after the end of CMS, the stressed rats had been restrained at 2.00 p.m., on the day before the sacrifice, after this any stressors was applied, then, the last stressor was restraint at 2p.m.. The second group of control and CMS rats was killed two weeks after the end of CMS. Inguinal, mesenteric, epididymal and perirenal fat pads, adrenal gland and thymus were removed and weighed.

Experiment 2

The previous experiment indicated that CMS caused a reduction in body weight, but did not alter food intake. Because the weight loss and the reduced fat pad mass were not sustained during the post-stress period in Experiment 1, all analyses in Experiment 2 were performed immediately after the end of CMS.

Thirty-four male Sprague-Dawley rats were housed in the same conditions described for Experiment 1. After adaptation to the environment and two weeks of food

intake and body weight measurement, the rats were divided into three weight-matched groups: Control, pair-fed rats and CMS (n = 8 to 12). At 8 a.m., the pair-fed group received the same amount of food intake as the CMS group ate voluntarily during the corresponding period of the stress protocol. Therefore, the pair-fed group was delayed for one day during the experimental protocol. Neither the pair-fed group nor the CMS group had access to food during the 18-hour food deprivation that was applied as a mild stressor in CMS rats (Willner *et al.,* 1987).

The day after the end of the CMS protocol the animals were killed by decapitation between 10.00 and 12.00a.m.. The sacrifice of the animals was performed on a Wednesday. The stressed rats had been restrained at 2.00 p.m., on the day before the sacrifice, after this any stressors was applied and food was removed from their cages at 8.00 a.m.. Food was removed from the cages 2 hours before decapitation. Trunk blood was collected for measurement of serum corticosterone by radioimmunoassay (MP Biomedicals, Cupertino, CA), insulin (Rat Insulin RIA kit, Linco, St. Louis, MO Cat # RI-13k), glucose (Sigma Diagnostics), triglycerides (WAKO Ltype TG H Chemicals, USA Cat # 997-37492) and free fatty acids (WAKO Chemicals, USA HR Series NEFA-HR (2) kit). The gut was removed and discarded. Inguinal, mesenteric, epididymal and perirenal fat pads, adrenal glands and thymus were weighed and returned to the carcass for determination of body composition, as described previously (Harris, RB, 1991).

STATISTICAL ANALYSIS

Body weight and food intake were analyzed by a non-paired t Student test and repeated measures analysis of variance (Stat Soft, Tulsa, OK). Fat pads, thymus and adrenal gland weight, area under curve of OGTT and insulin, corticosterone concentration obtained from Experiment 1 were analyzed by two-way ANOVA with Bonferroni post hoc. The data obtained from Experiment 2 were compared by one-way ANOVA with Duncan post hoc. All data are expressed as means ± SEM, $P<0.05$ was considered significant.

RESULTS

Experiment 1

The body weight of rats exposed to CMS is shown in Figure 1. The CMS protocol was initiated on day 15 of the experiment and finished on day 35. CMS rats showed a significant body weight loss during the CMS protocol. The significant difference in body weight between control and CMS rats was sustained until day 41, one week after the end of CMS. There was no significant difference in body weights of two groups from day 41 to the end of the experiment.

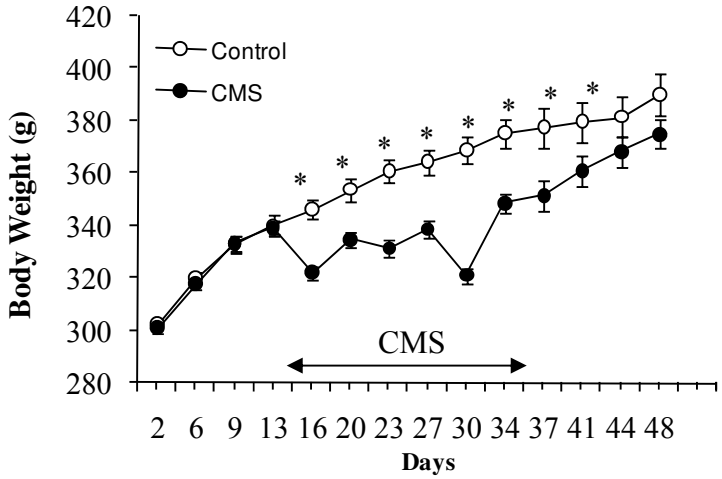


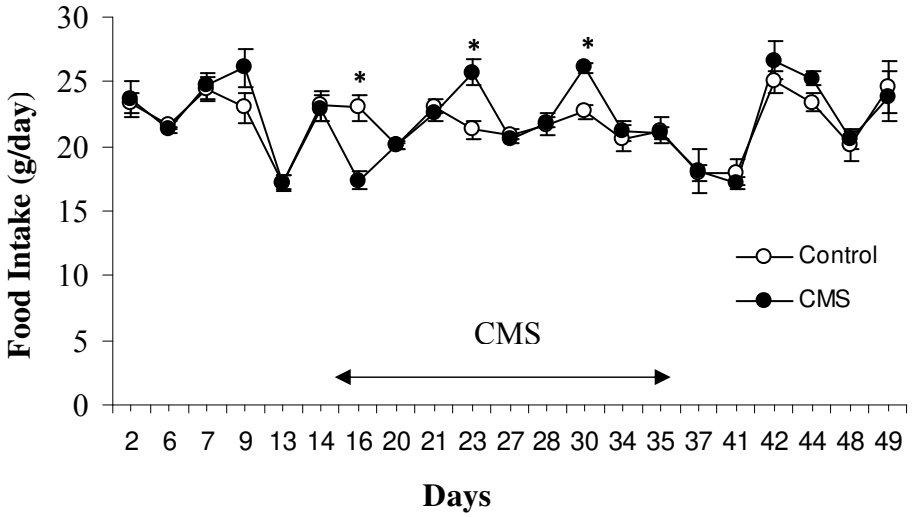
Figure 1. Effects of chronic mild stress on body weight during experiment1. Data are means ± Standard error values (n = 10). The CMS started at day 15 and finished at day 35. * Significant difference between control and CMS group. The data are means SEM (P<0.05).

Figure 2A shows food intake measured in Experiment 1. CMS reduced food intake on day 15, after the water-deprivation stressor. On days 22 and 29, food intake was higher in CMS than control rats. On the other hand, figure 2B shows no significant difference in the cumulative intake of Control and CMS groups before, during or after CMS.

Serum corticosterone concentration analyzed immediately after or two weeks after the end of the CMS is shown in Figure 3. CMS increased corticosterone in samples obtained from animals sacrificed both immediately and after two weeks after the end of stress protocol.

Figure 4 represents corticosterone concentration measured pre and post- 60-minute restraint, after the lights-on overnight, analyzed after one week of CMS and at the end of the CMS protocol. Paired t Student Test did not show a difference between pre and post 60-min of restraint.

A)



B)

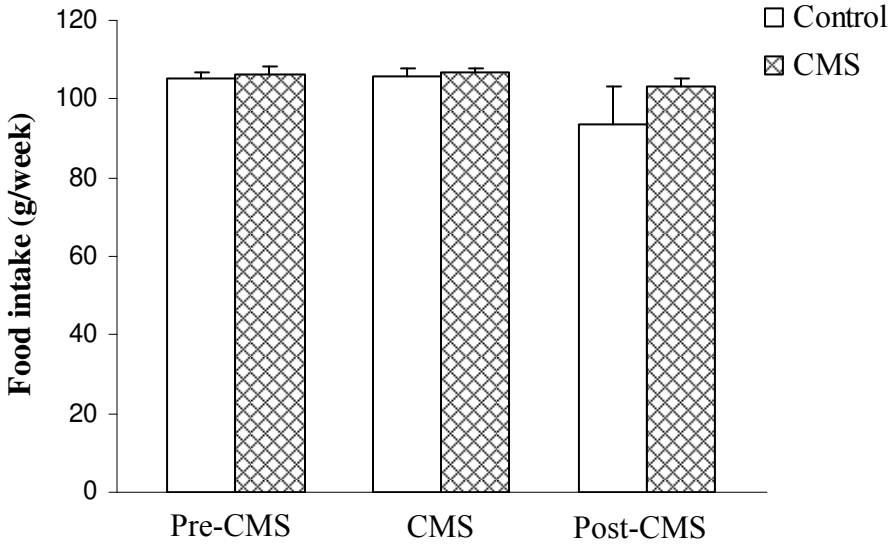


Figure 2. Effects of chronic mild stress on A) daily food intake and B) food intake for pre-stress - two weeks before, during the three of CMS protocol and post-stress - two weeks after and. The CMS started at day 15 and finished at day 35. * Significant difference between control and CMS group. The data are means SEM values (P<0.05).

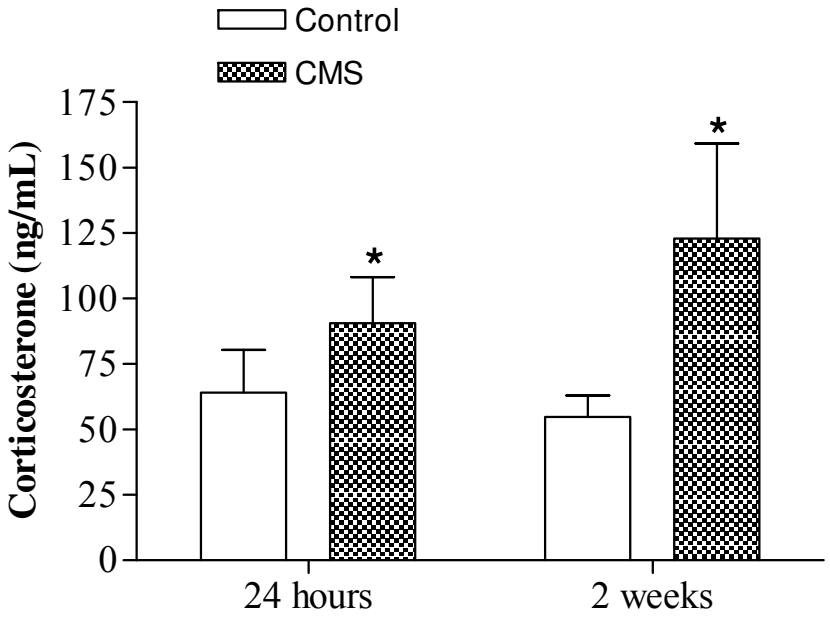


Figure 3. Effects of CMS on corticosterone concentration immediately after or two weeks after the end of the CMS. * Statistically significant differences between CMS and Control rats. The data are means SEM (P<0.05).

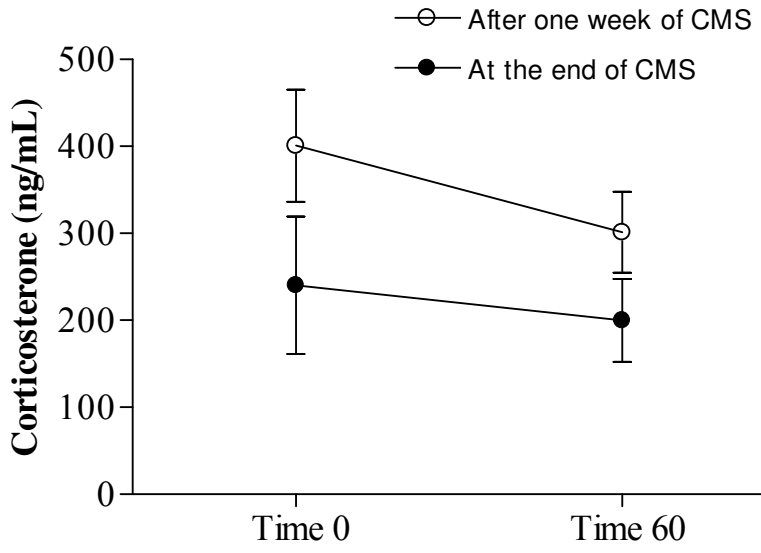


Figure 4. Effects of 1-hour restraint on corticosterone concentration in rats submitted to one week and three weeks of chronic mild stress. The data are means SEM values (P<0.05).

Inguinal, epididymal, mesenteric and perirenal fat pad, carcass, adrenal gland and thymus weights of the control and CMS groups are shown in Table 2. CMS significantly reduced the final body weight and epididymal and perirenal fat pad weights in rats sacrificed immediately after the end of the CMS period. The difference in weight of control and CMS rats sacrificed two weeks after the end of CMS was not significant. Mesenteric and perirenal fat pads were lower in CMS killed 24 hours after the end of CMS than in those killed 2 weeks after the end of the CMS protocol.

The analysis of the area under the curve of the OGTT did not show significant differences in glucose concentration in stressed rats analyzed one week after the beginning of the CMS or immediately after the end of the CMS period (data not shown).

Table 2. Body weight, inguinal, epididymal, mesenteric and perirenal fat pads, thymus and adrenal glands (mg) of rats in experiment 1.

	24-hours after CMS		**Two weeks after CMS**	
	Control	***CMS***	***Control***	***CMS***
n	11	10	10	11
Initial body weight (g)	301.8 ± 2.2	302.9 ± 5.7	303.2±3.2	298.7±2.9
Final body weight (g)	379.5 ± 8.3	351.6 ± 4.4 *	397.8 ± 7.9	379.6 ± 5.3
Inguinal fat (g)	5.3 ± 0.2	4.7 ± 0.3	5.7 ± 0.3	5.8 ± 0.2
Epididymal fat (g)	3.9 ± 0.3	3.1 ± 0.2 *	3.7 ± 0.2	3.7 ± 0.2
Mesenteric fat (g)	1.8 ± 0.1	1.8 ± 0.1	2.0 ± 0.1	2.2 ± 0.1#
Perirenal fat (g)	0.5 ± 0.04	0.4 ± 0.05 *	0.6 ± 0.04	0.7 ± 0.06 #
Fat pad/carcass	0.033 ± 0.01	0.031 ± 0.02	0.032 ± 0.01	0.035 ± 0.01
Thymus (mg)	0.30 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.33 ± 0.01
Adrenal glands (mg)	64.0 ± 3.8	66.7 ± 4.2	70.1 ± 3.1	69.5 ± 3.4

Data are means ± SEM. * statistically significant differences between CMS and Control, # statistically significant differences two weeks after CMS and 24 hours after CMS (*P<0.05*, Two-way ANOVA Graph Pad Prism 4.2).

Experiment 2

The body weights of the CMS, Pair-fed and Control groups are shown in Figure 5. There was no significant difference in the initial body weights of all the groups but final body weight was significantly lower in the pair-fed and CMS rats than for controls (Figure 5 and Table 2). CMS rats showed a significant weight loss during the entire CMS period initiated on day 15 of the experiment and finished on day 35. On day 16, only CMS rats weighed less than controls. From day 17 to the end of the experiment, both CMS and pair-fed rats weighed less than controls.

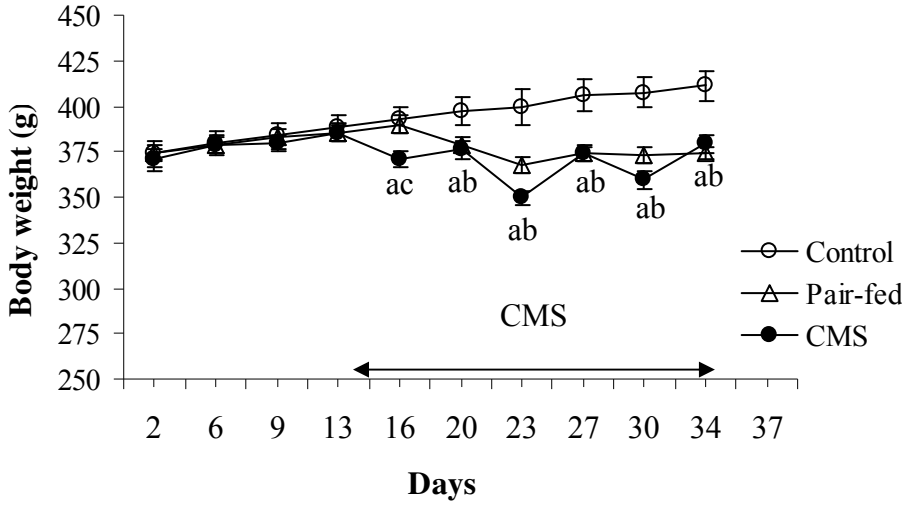


Figure 5. Effects of chronic mild stress on body weight during experiment 2. The CMS started at day 15 and finished at day 35. a: statistically differences between CMS and control groups; b: statistically differences between pair-fed and control groups; c: statistically differences between CMS and pair-fed groups. Data are means ± SEM ($P<0.05$).

Figure 6 illustrates the effect of CMS on food intake. The food intake of the pair-fed and CMS rats was lower than that of controls. There were no differences between CMS and control rats at days 23 and 29. Pair-fed rats had lower intakes on day 15, 23 and 30 compared with control rats. Pair-fed rats ate less than CMS rats due to spilling food. On the day 21, the Pair-fed group had a higher food intake compared to the CMS group. When total food intake of the groups was compared before and during the CMS, there were no observed statistical differences among the groups during the pre-stress period. However, during the CMS protocol, both Pair-fed and CMS groups had a reduced food intake compared to the control group, but there was no difference between the CMS and pair-fed groups (Figure 6B).

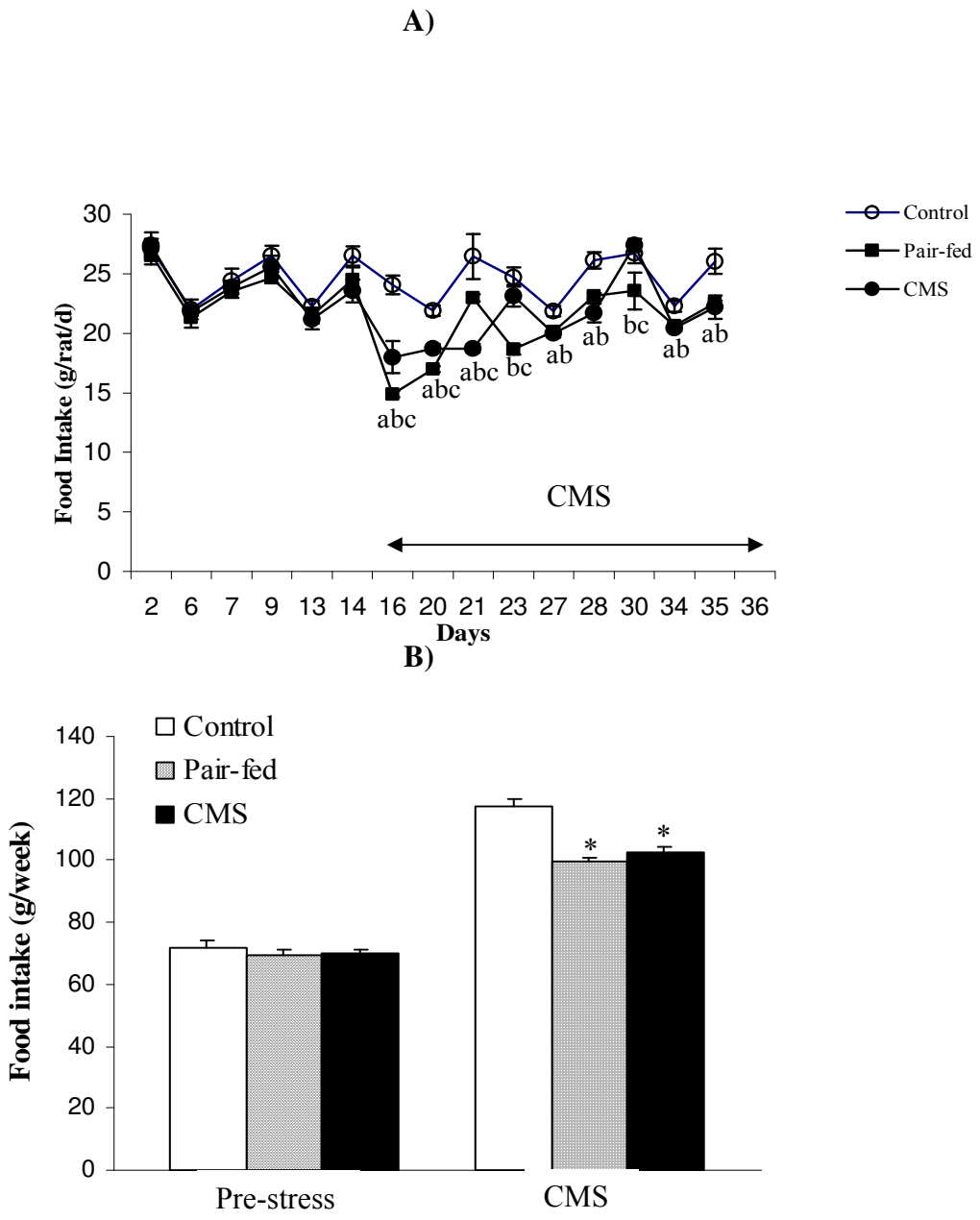


Figure 6. Effects of chronic mild stress on A) daily food intake The CMS started at day 15 and finished at day 35. a: statistically significant differences between CMS and control groups; b: statistically significant differences between pair-fed and control groups; c: statistically significant differences between CMS and pair-fed groups. B) Food intake for pre-stress - two weeks before – and during the three of CMS protocol. * Statistically different from control group. The data are means SEM values (P<0.05).

Table 3 shows the weight of the fat pads, carcasses, adrenal glands and thymus. The Pair-fed and CMS groups had a reduced final body weight and carcass weight compared with controls. The pair-fed group had smaller inguinal and mesenteric fat pads than the control and CMS groups. The epididymal fat pad was reduced in both Pair-fed and CMS groups, compared with control group. The fat pad / carcass ratio was significantly reduced in the pair-fed group, compared to the control, but was not different between the CMS and control groups.

Table 3. Body weight, inguinal, epididymal, mesenteric and perirenal fat pads, thymus and adrenal glands of Control, Pair-fed and CMS groups of rats in experiment 2.

	Control	***Pair-fed***	***CMS***
n	10	12	12
Initial body weight (g)	373.7 ± 6.8	374.7 ± 4.1	370.5 ± 6.8
Final Body weight (g)	422.4 ± 9.3	382.6 ± 7.6 *	397.8 ±4.1 *
Body weight gain (g)	48.77 ± 9.8	14.88 ± 2.1 *	7.617 ± 3.4 *
Inguinal fat (g)	6.0 ± 0.3	4.3± 0.2 *#	5.2 ± 0.2
Epididymal fat (g)	3.6 ± 0.1	2.91 ± 0.1 *	3.1 ± 0.1 *
Mesenteric fat (g)	1.7 ± 0.1	1.0 ± 0.1*#	1.5 ± 0.1
Perirenal fat (g)	0.5 ± 0.04	0.3± 0.05 *	0.4 ± 0.04
Carcass (g)	383.8 ± 8.1	361.8 ± 3.5 *	350.2 ± 4.6 *
Fat pad/carcass	0.0307 ± 0.001	0.0237 ± 0.001 *	0.0291 ± 0.001
Thymus (mg)	0.30 ± 0.02	0.30 ± 0.02	0.32 ± 0.02
Adrenal glands (mg)	73.4 ± 3.4	64.7 ± 1.9	71.7 ± 3.8

Data are means ± SEM values. * statistically significant differences compared to the Control group, # statistically significant differences compared to CMS ($P<0.05$, One-way ANOVA, Tukey post-hoc Graph Pad Prism 4.2).

Table 4 illustrates the effect of CMS and pair-fed on body composition. CMS rats did not show significant differences in the carcass fat, protein, ash or water compared with control rats. However, pair-fed rats had a lower carcass fat content, with no difference in protein, ash and water compared with control rats. Serum free fatty acid, triglycerides, insulin and glucose levels are represented in Table 5. There were no significant differences in free fatty acid, glucose, insulin and or corticosterone among the control, pair-fed and CMS groups. However, the CMS and pair-fed groups had lower serum triglycerides concentrations compared with controls.

Table 4. Body composition of experiment 2.

	Control	***Pair-fed***	***CMS***
n	8	12	10
Protein (g/rat)	82.51 ± 2.98	81.88 ± 1.97	78.54 ± 1.61
Water (g/r)	263,79 ± 4.69	256.46 ± 2.72	241,77 ± 5.54
Fat (g/rat)	23.91 ± 2,12	12,86 ± 1,75*	18,92 ± 1,60
Ash (g/rat)	16.97 ± 3.05	10,54 ± 1,27	10.99 ± 1.51

Means ± SEM values. (One-way ANOVA, Tukey post-hoc, $P<0.05$ Graph Pad Prism 4.2).

Table 5. Corticosterone, Insulin, free fat acid, triglycerides and glucose of Control, Pair-fed and CMS groups in rats of experiment 2.

	Control	***Pair-fed***	***CMS***
n	8	12	10
Corticosterone (ng/mL)	288.2 ± 89.4	180.2 ± 53.36	292.2 ± 70.2
Insulin (ng/mL)	0.61 ± 0.11	0.63 ± 0.12	0.61 ± 0.11
Free fatty acid (mEq/L)	0.28 ± 0.02	0.20 ± 0.02	0.22 ± 0.03
Triglycerides (mg/dL)	106.4 ± 26.7	56.5 ± 7.0 *	51.8 ± 5.6 *
Glucose (mg/dL)	87.92 ± 13.01	95.43 ± 5.5	87.69 ± 7.2

Means ± SEM values. One-way ANOVA, Tukey post-hoc Graph Pad Prism 4.2).

DISCUSSION

In the present study, we investigated whether the body weight loss observed in rats exposed to a CMS protocol is due to an inhibition of food intake. We found that during the stress protocol, the CMS and pair-fed rats lost body weight supporting the findings of Willner *et al.* (1996), who reported body weight loss in animals submitted to CMS. CMS causes a number of effects characteristic of depression-like behavior, such as decreased consumption of sucrose solutions, decreased place preference conditioning and decreased performance in intracranial self-stimulation (Muscat & Willner, 1992; Moreau *et al.,* 1994). Other authors also have suggested that the body weight loss is related to anhedonia (D'Aquila *et al.,* 1994, Mathews *et al.,* 1995, Cheeta *et al.,* 1996).

Changes in food intake and body weight of rats exposed to acute, repeated or chronic stress are well documented (Rybkin *et al.,* 1997; Vallès *et al.,* 2000; Gamaro *et al.,* 2003). CMS also reduces body weight and food intake (Levin *et al.,* 2000), but there is less information concerning the post-stress CMS period. In Experiment 1, we investigated if CMS inhibited food intake during the CMS period and for two weeks after CMS ended. However, we did not find a change in food intake during either the CMS or the post-stress period in Experiment 1. Some other CMS studies have reported that food intake is not altered by CMS (Muscat *et al.* 1992) and also there is evidence that despite the fact that food intake is unchanged or even increased by CMS (Papp *et al.,* 1991), the rewarding properties of food are decreased, as indicated by an attenuation of food-induced place preference conditioning and reduction of eating of sweet diets (Sampson *et al.* 1992; Willner *et al.* 1994). The failure to find a change in food intake was unexpected, since the CMS protocol includes different stressors that may cause significant alterations on feeding behavior. However, we did observe some alterations in food intake on specific days during the entire stress protocol. On day 16 of the experimental protocol, which corresponds to the measure obtained after the water deprivation, the food intake was significantly decreased in CMS rats. This specific effect could be explained by water deprivation stressor that may influence feeding behavior (Tanaka *et al.,* 2003). In contrast, we did observe increased food intake on day 22 and 29, which corresponds to the measure obtained after the food / water

depreivation, and indicates that the rats were compensating the stress-induced inhibition of food intake (Rybkin *et al.,* 1997).

In contrast to Experiment 1, CMS caused a significant inhibition of food intake in Experiment 2. The different effect of CMS on food intake found in these experiments could be explained by different individual responses to CMS. Different responses to CMS have been reported for rats of the same strain and for different batches of Sprague-Dawley rats. Nielsen *et al.* (2000) and Willner, P (1997) reported differences in sensitivity to 1 % sucrose in some batches of Wistar rats, leading to unstable patterns of consumption in repeated tests, even in control animals. Also, rats utilized in Experiment 2 were larger than experiment 1 and may have found same stressors, such as restraint, more severe than the smaller rats in experiment 1.

Body weight loss may result from a negative balance between energy expenditure and food intake. In Experiment 1, the body weight loss was associated with no change in cumulative intake. These findings may be a consequence of higher energy expenditure in stressed rats than the controls sustained over one week after the end of CMS, but recovered by the end of the experimental protocol. Harris *et al.* (1998) measured energy expenditure during 3h / 3 days of restraint stress and during the consecutive 3 days after the end of stress showing a significant increase in energy expenditure during the restraint stress that returned to control levels as soon as the stress ended. In our study, the body weight loss and unchanged food intake were observed one week after the end of CMS, suggesting that the increased energy expenditure was sustained. This data is in agreement with Michel *et al.* (2003) who suggested increased energy expenditure after 20 min of restraint in diet-induced obese rats fed with rodent chow that caused body weight loss and decreased food efficiency without a reduction in either food intake or adiposity. On the other hand, in Experiment 2, the body weight loss in pair-fed and stressed rats was associated with a lower food intake during the CMS protocol. Thus, in this experiment stressed rats showed a different feeding response to CMS and we do not have the data to determine whether there also were differences in energy expenditure of the CMS rats.

Several authors have investigated the mechanisms responsible for the stress-induced reduction in food intake and body weight utilizing different stress models (Rybkin

et al., 1997; Bell *et al.,* 2002; Vallès *et al.,* 2000). During stress the HPA axis is activated in response to the release of CRH. CRH activates the ACTH release from the pituitary gland, which promotes corticosterone and catecholamine release from the adrenal gland (Salpolsky *et al.,* 2000). It is well stablished that CRH, glucocorticoids and catecholamines have a relevant role in controlling food intake and body weight. CRH may suppress food intake by inhibiting NPY (Heinrichs *et al.,* 1993). Chronic stress induced body weight loss can also be attributed to enhanced metabolic activity. Elevated glucocorticoid and catecholamine concentrations induced by chronic stress produce catabolic effects, accelerates lipolysis and increases thermogenesis, all of which contribute to body weight loss (Holenga *et al.,* 1989; Tempel & Leibowitz, 1994). Zhou *et al.,* (1999) also suggested that the weight loss is related to changes in body composition and peripheral tissue metabolism.

We found a significant reduction in the size of fat depots immediately after the end of CMS in both the Experiment 1 and 2. Animals submitted to CMS had small epididymal and perirenal fat pads. The lower body fat and decreased body weight observed here may be ascribed to activation of their HPA and sympathetic axis, due to the catabolic effects of glucocorticoids (Smith & Vale, 2006) and to higher lipolytic activity of catecholamines is induced by the β_1, β_2 and β_3 subtypes of β-adrenoceptors in the adipose tissues (Hollenga, 1989). Indeed, we observed that pair-fed rats also had reduction in those fat pads, but they also showed reduced inguinal and mesenteric fat compared to control and CMS groups, and it may be due to CMS changing visceral fat more than subcutaneous fat. These finding suggest regional differences in the effects of the CMS and pair-feeding. In fact, Rebuffe-Scrive *et al.,* (1992) observed selectively increased visceral fat and enlarged fat cells only in mesenteric fat in stressed rats, suggesting that these regional differences could be explained by differences in glucocorticoids receptor density in the fat pad favoring the fat store preferentially in mesenteric fat depots. The different pattern of fat loss between pair-fed and CMS observed in this present work may be due to level of sympathetic activation between the groups or different sensitivity of fat depots in response to CMS and pair-fed treatments.

Since we did not observe maintained alterations in fat mass and final body weight in the Experiment 1, we analyzed the composition of the carcass only at the end of CMS in Experiment 2. CMS rats had a significantly reduced body weight and significantly smaller epididymal fat depots than control rats. Carcass fat, protein and water content also were reduced compared with controls, but the differences did not reach statistical significance.

In contrast to the CMS group, the reduced body weight of the pair-fed group was associated with significant reduction in body fat content and little change in carcass protein or water. These observations suggest that weight loss caused by food restriction (pair-feeding) and stress are produced by different mechanisms with food restriction mobilizing body fat and stress inhibiting overall growth.

In Experiment 1, rats submitted to CMS had an increased serum corticosterone concentration, immediately after the end of the stress and, this alteration was maintained for two weeks after the end of CMS. Nevertheless in Experiment 2, there was no effect of either CMS or pair-fed on corticosterone concentration. This finding could be explained by the higher levels of corticosterone in all animals in Experiment 2 than Experiment 1 and it is not unusual to have inconsistent findings related to corticosterone concentration (Azpiroz *et al.* 1999; Bielajew *et al.,* 2002; Silberman *et al.* 2002). Alternatively, others have reported that CMS rats show an exaggerated corticosterone response to novel stress in the post-CMS period with no change in basal cortiscoterone. Therefore, it is possible that we also would have found an exaggerated corticosterone release in CMS rats compared to Controls if we had exposed the animals to a novel stressor.

It is unlikely that the sustained elevation of corticosterone in Experiment 1 were responsible for the body weight loss observed during CMS because both pair-fed and CMS rats in Experiment 2 lost weight without the change in basal corticosterone. Gursoy *et al.* (2001) demonstrated that food restriction increased the corticosterone concentration and adrenal gland weight and decreased the thymus weight in pair-fed Sprague-Dawley rats when compared to *ad libitum* fed control animals. The difference between that study and ours may be the degree of food restriction that was imposed because Martin *et al.,* (2007) observed that 20 and 40% caloric restriction promoted significant increase in plasma level

of corticosterone, but García-Belenguer *et al.* (1993) observed that food restriction to 15% did not change diurnal plasma corticosterone levels and these authors suggest that the food restriction may activate the hypothalamus-pituitary-adrenal axis depending on the level of the food restriction.

In the present study we found lower serum concentrations of triglycerides in the pair-fed and CMS groups, compared with controls, without alterations in serum free fatty acid, insulin or glucose concentrations. Pair-feeding is a procedure that alters the feeding pattern, leading to a state of intermittent caloric restriction (Russell *et al.,* 2008), it also promotes body weight loss, improves insulin sensitivity and may be linked to alterations in muscle lipid metabolism (Stokkan *et al.,* 1991; Tucker & Turcotte, 2005; Escrivá *et al.,* 2007). Tucker & Turcotte (2005) showed that 60% /28 day- food restriction in hyperglycemic-hyperinsulinemic rats increased total rates of free fatty acid uptake and oxidation, increased triglyceride utilization and glucose uptake and glycogen synthesis. Our findings are in agreement with Liepa *et al.* (1980), who also observed lower serum triglyceride levels and no alterations in serum free fatty acids in food restricted rats.

In the CMS model, animals are repeatedly exposed to various unpredictable mild stressors and the resulting decreased sensitivity to reward is usually measured as a reduced consumption/preference for palatable sweet solutions (Willner *et al.* 1987). In the present work we chose the CMS model validated by Moreau *et al.* (1994), which does not include any of the severely stressful elements used by Katz and colleagues (e.g. intense footshock; cold water immersion; 48 h food/water deprivation). In the CMS validated by Moreau and colleagues, the time of food and water deprivation was reduced to 18 hours, but even with these shorter times these stressors per se may influence body weight regulation, feeding behavior and preference for sucrose. For example, some authors have demonstrated that the different time of water deprivation may change the sucrose intake. Harris *et al.* (1997), observed reduced body weight in rats submitted to 6-weeks CMS, which had lower food intake than controls, and the saccharin intake of CMS rats was dependent upon their dehydration status and could not be attributed to stress-induced anhedonia. Armario *et al.* (1987) observed a significant reduction of food intake and body weight in rats after 30 to 60-min water deprivation daily for 30 days. Grinevich *et al.,*

(2001) also demonstrated that 48 hours of water deprivation reduced the levels of CRH mRNA in rats submitted to 1-hour restraint or lipopolysacharide injection.

In summary, we observed that CMS promoted body weight loss that may be sustained for one week after the end of CMS. This body weight loss is not necessarily associated with a reduce food intake, suggesting increased energy expenditure during the CMS protocol, which lasted for one week after the end of CMS before recovering to control level. In addition, the level of food restriction present in the CMS protocol may cause important changes in the body weight, metabolism and body fat mass that are different from those caused by food restriction. In conclusion, although food restriction influences the responses to CMS, it does not explain the CMS-induced body weight and fat mass reduction observed in the present work.

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CONSIDERAÇÕES GERAIS

A obesidade, que pode ser definida como o excesso de peso associado a índice de massa corporal maior que a 30 Kg/m^2, compromete a qualidade de vida do indivíduo, além de ser um problema de saúde pública devido aos altos custos com o tratamento das doenças a ela associadas, tais como resistência à insulina, diabetes tipo II, dislipidemia e hipertensão, as quais estão envolvidas na síndrome metabólica. De acordo com a IV Diretriz Brasileira Sobre Dislipidemias e Prevenção da Aterosclerose do Departamento de Aterosclerose da Sociedade Brasileira de Cardiologia, aproximadamente 32% da população brasileira apresenta sobrepeso [Índice de Massa Corporal (IMC) > 25)], sendo esta taxa de 38% para o sexo feminino e de 27% para o sexo masculino, de acordo com os dados do Ministério da Saúde de 1993. A obesidade foi encontrada em 8% da população brasileira.

Bjontorp (1996) propõe que a hiperatividade do eixo HPA em resposta ao estresse crônico ocasionaria o acúmulo central da gordura que determina efeitos metabólicos deletérios. Na vida moderna o homem está constantemente exposto a situações estressantes que, somadas ao sedentarismo e abundância de alimentos calóricos, proporcionam sobrepeso e obesidade, que por sua vez são fatores de predisposição a doenças metabólicas e cardiovasculares.

Os modelos animais de estudo da obesidade têm sido usados ao longo de décadas, com o objetivo de compreender as conseqüências da obesidade no organismo e de sua fisiopatologia para a busca de tratamentos farmacológicos eficientes no tratamento desta epidemia e para melhorar a qualidade de vida dos pacientes. Também é vasto o número de modelos animais para o estudo do estresse. Desta forma, os estudos com animais de laboratório são ferramentas adequadas para o estudo da interação estresse-regulação do peso corporal.

No entanto, a utilização de animais de laboratório demanda uma série de cuidados para validação e reprodutibilidade dos resultados. Por exemplo, uma revisão realizada por Good (2007) relata os fatores relacionados à manutenção dos animais em biotérios, baseados no *Institutional Animal Care and Use Committee* (IACUC), que podem influenciar a instalação de obesidade em camundongos. Segundo Good (2007), modelos de

estudo da regulação do peso corporal requer vários cuidados, incluindo a habituação, regulação da temperatura ambiental, composição das dietas e principalmente manipulação por pesquisadores e técnicos bem preparados e conscientes, bem como experimentos especializados para testar a regulação do peso corporal, ingestão alimentar, exercícios físicos (Good, 2007). A idade dos animais é um fator muito importante, uma vez que estes autores relatam que alguns camundongos ganham peso na puberdade, em outros a obesidade se inicia na vida adulta, depois da maturidade sexual (Good, 2007). Embora, a revisão de Good (2007) seja referente a camundongos utilizados como modelo de obesidade, suas considerações podem ser extrapoladas para estudos da regulação do peso corporal e ingestão alimentar outros estudos com animais de laboratório.

Devido à importância dos cuidados na manutenção e manipulação dos animais de laboratório para obtenção de resultados mais acurados, no estudo apresentado no capítulo 1, realizado no Laboratório de Estresse da Faculdade de Odontologia – UNICAMP, os ratos foram mantidos em câmaras com condições ambientais controladas, nas quais somente os pesquisadores responsáveis pelo estudo tinham permissão para entrar. E estes utilizaram luvas descartáveis, máscaras respiratórias, proteção para os pés e jalecos com o objetivo de controlar o padrão sanitário e bem-estar dos animais.

Nos estudos apresentados em ambos os capítulos foram encontrados alguns resultados divergentes que podem ser decorrentes de diferentes condições de alojamento. Os efeitos do ECMI sobre a ingestão alimentar foram diferentes entre os dois estudos aqui apresentados. No primeiro capítulo, os ratos Sprague-Dawley utilizados foram mantidos em gaiolas opacas de policarbonato, cuja cama foi constituída de maravalha. No capítulo 2, animais da mesma linhagem foram mantidos em gaiolas metabólicas de metal.

O capítulo 2 foi constituído de dois experimentos, sendo o Experimento 1 realizado com o objetivo principal de investigar o efeito do ECMI sobre o peso corporal e ingestão alimentar durante o protocolo de estresse e no período pós-estresse. Enquanto que o Experimento 2 foi realizado com o objetivo se investigar se a redução do peso corporal foi decorrente da restrição alimentar presente no modelo de estresse. Um ponto crítico presente no segundo capítulo, foi o diferente perfil de ingestão alimentar apresentado pelos ratos estressados entre os Experimentos 1 e Experimentos 2. No Experimento 1, não foi

encontrada alteração na ingestão alimentar cumulativa, porém no Experimento 2, a ingestão alimentar foi reduzida durante a aplicação do CMS, reforçando os dados observados após a primeira semana da aplicação do estresse no primeiro capítulo.

O efeito diferente do ECMI sobre a ingestão alimentar encontrada nos experimentos apresentados no capítulo 2 pode ser explicado por diferentes respostas individuais ao modelo de estresse. O que está de acordo com Nielsen *et al.* (2000) que relataram diferenças na sensibilidade a 1% de sacarose, em alguns lotes de ratos Wistar, levando a padrões de consumo instável em repetidos testes, mesmo em animais controle. Além disso, a idade e peso corporal inicial dos animais também parece ter influenciado os resultados obtidos no capítulo 2.

Um estudo realizado por Pecoraro *et al.* (2006) também relataram uma série de diferenças fenotípicas em ratos Sprague-Dawley obtidos de diferentes fornecedores norte-americanos, incluindo diferenças no ganho de peso corporal, eficiência calórica, regulação da temperatura, adiposidade, secreção de insulina e atividade do eixo HPA. Pecoraro *et al.,* (2006) observaram que a taxa de crescimento dos ratos de diferentes fornecedores indicou diferenças substanciais. Ratos Sprague-Dawley provenientes do fornecedor Charles River ganharam peso corporal mais rapidamente e exibiram maior resposta ao ACTH que ratos provenientes dos fornecedores Harlan e Simonsen. A ingestão alimentar dos animais fornecidos por Charles River foi maior que ratos provenientes da Harlan, que por sua vez foi maior que aqueles provenientes da Simonsen. Ratos Sprague-Dawley fornecidos pela Charles River e Harlan apresentaram maior eficiência calórica que os ratos da Simonsen. Após ingestão da dieta rica em açúcar e gordura, os ratos provenientes da Charles River apresentaram maior adiposidade que os ratos da Harlan, os quais apresentaram maior adiposidade que os ratos da Simonsen. Em todos os ratos dos três fornecedores, a dieta de frutose e gordura aumentou a concentração de leptina, com maior concentração nos Charles River e menor nos Simonsen. A dieta hipercalórica aumentou a concentração de insulina, somente nos ratos Simonsen. Surpreendentemente, quando submetidos ao estresse repetido por restrição de 2 horas por cinco dias, os ratos Harlan não apresentaram nenhuma alteração no ACTH, mas apresentaram aumento do corticosterona. Todavia, ratos Charles River e Simonsen exibiram aumento significante de ACTH e corticosterona, sendo que nos

primeiros animais este aumento do ACTH e corticosterona foi moderado pela dieta de frutose e gordura. Nos Simonsen, somente o ACTH foi moderado pela dieta.

No estudo de Nishikawa *et al.* (2007), os autores investigaram o efeito do gênero, linhagem e idade na indução de obesidade em camundongos tratados com dieta hipercalórica durante nove meses, concluindo que as características da obesidade induzida por dieta são influenciadas pelo sexo, linhagem e idade dos camundongos. Esta influência da idade no ganho de peso dos animais de laboratório encontrados por Nishikawa *et al.* (2007) pode sugerir uma explicação para os resultados divergentes encontrados em ambos estudos, tais como na ingestão alimentar e acúmulo de gordura em animais submetidos ao CMS. Uma vez que no início dos experimentos descritos no capítulo, a idade dos animais utilizados era oito semanas, enquanto nos experimentos descritos no capítulo 2, a idade dos animais era de 10 a 11 semanas. Devido a esta diferença, os animais podem ter apresentado diferente perfil de crescimento, o que pode por sua vez influenciar a comparação do ganho de peso e acúmulo de gordura.

O modelo de estresse crônico moderado e imprevisível tem contribuído para uma adequada simulação de alguns aspectos da depressão humana em estudos com animais. A quantidade de dados já publicados utilizando o modelo validado por Willner e colaboradores, é uma ferramenta válida e útil, mas ainda existem dados conflitantes que diz respeito à metodologia do consumo de solução de sacarose (Moreau, 1997). É comum diferentes laboratórios utilizarem diferentes estressores no modelo de estresse crônico moderado e imprevisível para estudar alterações comportamentais, metabólicas e neuroendócrinas (Willner, 2005). Nos estudos descritos nos capítulos 1 e 2 houve apenas uma alteração na utilização dos estressores.

No estudo descrito no capítulo 1, foi utilizado a imobilização em tubos de polietileno com diâmetro ajustável ao corpo do animal, de acordo com seu desenvolvimento, no lugar da restrição presente no modelo validado por Moreau *et al.* (1994). Em contrapartida, no capítulo 2, a imobilização foi substituída pela restrição de movimento em tubo de acrílico, empregada correntemente naquele laboratório.

Apesar de alguns resultados divergentes encontrados nos capítulos 1 e 2, tais como redução no acúmulo de gordura induzido pelo ECMI, diferentes padrões encontrados

na ingestão alimentar, podemos concluir que a utilização de modelos de ECMI é válido para a compreensão do efeito do estresse e dieta hipercalórica sobre a regulação do peso corporal e do comportamento alimentar, e que é necessário rigoroso controle das variáveis individuais (idade, peso, linhagem) e ambientais (condições de alojamento, dieta, experiência do experimentador) durante os experimentos.

CONCLUSÕES

1. O modelo de estresse crônico constituiu um modelo eficiente para o estudo, a longo prazo, das alterações metabólicas em ratos.
2. A dieta hipercalórica empregada não induziu obesidade, mas induziu alterações no metabolismo lipídico, permitindo o estudo da interação entre estresse crônico e dieta sobre o metabolismo em ratos.
3. O efeito do ECMI sobre o ganho de peso corporal, acúmulo de gordura e ingestão alimentar, em ratos, é dependente do tipo de dieta empregada.
4. O ECMI e a dieta hipercalórica, bem como sua interação, promoveram dislipidemia e aumento do índice aterogênico, evidenciando que o ECMI e dieta hipercalórica são fatores pró-aterogênicos em ratos.
5. O ECMI induziu importantes alterações no metabolismo de carboidrato e promoveu resistência à insulina, independente da dieta ulizada.
6. Embora os períodos de restrição alimentar do protocolo de ECMI contribuam para a redução do ganho de peso corporal e do acúmulo de gordura, observados em ratos submetidos ao ECMI, eles não são suficientes para explicá-los. Outros fatores relacionados à reação de estresse parecem estar envolvidos.

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ANEXO

Universidade Estadual de Campinas
Instituto de Biologia

UNICAMP

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **900-1**, sobre **"RESPOSTAS METABÓLICAS, CARDIOVASCULARES E COMPORTAMENTAIS INDUZIDAS POR DIETA HIPERCALÓRICA E ESTRESSE CRÔNICO, EM RATOS"** sob a responsabilidade de **Profa. Dra. Fernanda Klein Marcondes** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 13 de setembro de 2005.

CERTIFICATE

We certify that the protocol nº **900-1**, entitled **"METABOLIC, CARDIOVASCULAR AND BEHAVIORAL RESPONSES INDUCED BY HIPERCALORIC DIET AND CHRONIC STRESS IN RATS"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on September 13, 2005.

Campinas, 13 de setembro de 2005.

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