FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

IMPACTO DO ESTRESSE CRÔNICO ASSOCIADO A UM MODELO DE OBESIDADE SOBRE ATIVIDADES COMPORTAMENTAIS E MARCADORES HORMONAIS E BIOQUÍMICOS

CLEVERSON MORAES DE OLIVEIRA

Orientadora: Prof. Dra. Iraci Lucena da Silva Torres

DISSERTAÇÃO DE MESTRADO

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“DEDICO ESTE TRABALHO A TODOS QUE COLABORARAM PARA SUA REALIZAÇÃO.”

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“*A mente que se abre a uma nova ideia jamais voltará ao seu tamanho original*.”

(Albert Einstein)

Resumo

Introdução: O ciclo sono-vigília, a alimentação e o metabolismo de lipídios e de glicose estão sujeitos à regulação circadiana a qual visa sincronizar a energia disponível e o gasto necessário para mudanças no meio externo de acordo com a fase claro-escuro. O desajuste do ritmo circadiano pode ser fator causal para uma relação positiva entre o trabalho noturno e obesidade. A exposição ao estresse crônico devido à liberação de glicocorticóides provoca distribuição anômala de gordura, principalmente em região abdominal que está associada à resistência a insulina, hipertrigliceridemia, e hipertensão arterial. A exposição ao estresse crônico é considerado um dessincronizador de ritmos biológicos, induz liberação de glicocorticoides esta associada a distúrbios alimentares que podem levar a obesidade. **Objetivos:** Este estudo objetiva avaliar o efeito da associação de modelos experimentais de estresse crônico e de obesidade sobre parâmetros bioquímicos, ponderais, nociceptivos e comportamentais. Adicionalmente, à avaliação do padrão temporal de glicose, insulina, grelina e corticosterona. **Métodos:** Foram utilizados 96 ratos Wistar machos com 60 dias de idade divididos em 4 grupos: dieta padrão (C), Dieta hipercalórica (dieta de cafeteria) (D), estresse crônico (estresse crônico por restrição 1h/dia/5 dias por semana, entre às 9h e às 12h) associado a dieta padrão (E), estresse crônico associado a dieta hipercalórica (DE). Após 80 dias de experimento os animais foram avaliados no campo aberto (atividade locomotora e exploratória), labirinto em cruz elevado (comportamento do tipo ansioso) e testes de formalina e tail-flick (comportamento da resposta nociceptiva). Após os animais foram mortos em 3 períodos do dia (7h -ZT0, 19h - ZT12 e 1h - ZT18) para avaliação dos níveis plasmáticos de glicose, insulina, corticosterona, e grelina. **Resultados:** Nossos resultados indicam que os animais que consumiram dieta hipercalórica apresentaram aumento na locomoção. Além disso, mostraram alterações comportamentais em relação ao comportamento do tipo ansioso em animais estressados. Adicionalmente,

os ratos estressados que consumiram a dieta hipercalórica não diferiram do grupo controle em delta de peso, peso relativo das adrenais e nos níveis de corticosterona, indicando que o estabelecimento da obesidade é influenciado pela ativação crônica do eixo HHA indexada pela hipertrofia das adrenais observada no grupo de animais submetidos a estresse crônico. No entanto em animais estressados que consumiram a dieta hipercalórica não apresentaram hipertrofia das adrenais. Também confirmamos o padrão temporal dos hormônios corticosterona e insulina, porém, nos horários avaliados não foi possível detectar a existência do padrão temporal da grelina e da glicose. Adicionalmente, os níveis de corticosterona sofreram efeito do estresse crônico e interação entre obesidade e do horário do dia. Por outro lado, o padrão temporal da insulina sofreu efeito apenas da dieta. No entanto, os níveis glicêmicos sofreram os efeitos do consumo da dieta e interação entre dieta e estresse. **Conclusão:** Os resultados desta dissertação sugerem que a exposição ao estresse crônico e à obesidade influencia o padrão temporal de biomarcadores periféricos que podem ter impactos comportamentais e repercussões metabólicas.

Palavras-chave: Estresse crônico, Dieta hipercalórica, Obesidade, Comportamento, Ritmo circadiano, ratos.

Abstract

Introduction: The sleep-wake cycle, feeding and metabolism of lipids and glucose are subject to circadian regulation, which aims to synchronize the available energy and expense required for changes in the external environment according to the light/dark phase. Such rhythms are coordinated by circadian clocks. Alterations in the circadian rhythm may be a causal factor for a positive relationship between shift work and obesity. Exposure of chronic stress due to release of glucocorticoids cause abnormal fat distribution, especially in the abdominal region that is associated with insulin resistance, hypertriglyceridemia, and hypertension. Exposure to chronic stress, which is related to biological rhythms desynchronized and induces release of glucocorticoids associated with eating disorders that can lead to obesity. **Objectives:** Since chronic stress is an important component of modern life often associated with binge eating and obesity this study aims to evaluate the effect of the combination of experimental models of chronic stress and obesity on weight, behavior and biochemical parameters, associated to evaluation of the temporal pattern of peripheral markers (glucose, insulin, ghrelin and corticosterone). **Methods:** Was used 96 male Wistar rats at 60 days old were divided into 4 groups: diet standard (C), hypercaloric diet (cafeteria diet) (HD), chronic stress (chronic stress by restricting 1h/day/5 days a week between 9am and 12pm for 80 days) associated with the standard diet (S), chronic stress associated with hypercaloric diet (SHD). After 80 days of experiment the animals were evaluated in the open field (locomotor and exploratory activities), elevated plus maze (anxiety-like behavior) and formalin tests and tail-flick (nociceptive response). After 24h the animals were killed in three periods of the day (ZT0-7h, ZT12-19h - and ZT18-1h) to evaluate plasma levels of glucose, insulin, corticosterone, and ghrelin. **Results:** Our results indicate that HD group increase in locomotion. Also, shows behavioral changes elevated plus maze test, such as anxiety. Additionally, SHD group did not differ from control group on delta weight, relative weight of the adrenals and corticosterone levels,

indicating that the establishment of obesity is influenced by chronic HPA axis activation, which is indexed by adrenal hypertrophy observed in the group of animals subjected to chronic stress. However in stressed animals who consumed the high calorie diet showed no adrenal hypertrophy.We also demonstrated the temporal pattern of plasmatic levels corticosterone and insulin; however, it was not possible to detect the temporal pattern of ghrelin and glucose levels at the times analyzed. Additionally, corticosterone levels suffered effects of obesity, chronic stress and the time of day. Furthermore, the temporal pattern of insulin was affected only by obesity. However, glucose levels suffered the effects of obesity and interaction between obesity and chronic stress. **Conclusion:** The results of this work suggest that exposure to chronic stress and hypercaloric diet may influence the temporal pattern of peripheral biomarkers that which may impact on behavior and metabolism.

Keywords: Chronic stress, Hypercaloric diet, Obesity, Behavior, Circadian rhythm, rats.

Lista de Figuras

Lista de Abreviaturas

5HT-A1	Serotonin receptor
ACTH	Hormônio adrenocorticotrópico
AgRP	Peptídeo relacionado ao agouti
CNS	Central nervous system
CP	Campo-aberto
CRF	Corticotropin releasing factor
CRH	Hormônio liberador de corticotrofina
EPM	Elevated plus maze
GC	Glicocorticóides
GH	Hormônio do crescimento
HDL	Lipoproteína de alta densidade
HHA	Hipotálamo-Hipófise-Adrenal
HPA	Hypothalamic-pituitary-adrenal
IASP	International Association for the Study of Pain
IL-1β	Interleukin-1 beta
LCE	Labirinto em cruz elevado
NSQ	Núcleo supraquiasmático
NYP	Neuropeptidio Y
OP	Open field
PFC	Prefrontal cortex

EPM	Elevated Plus Maze Test
PVN	Núcleo paraventricular
SNC	Sistema nervoso central
T3	Triiodotironina
T4	Tetraiodotironina ou Tiroxina
TFL	Tail-flick test
TNF-α	Fator de necrose tumoral - alfa
TRH	Hormônio liberador de tireotrofina
TSH	Hormônio estimulante da tireóide
ZT	Zeitgeber

Sumário

1 INTRODUÇÃO

A cronobiologia estuda os determinantes da ritmicidade temporal dos fenômenos fisiológicos e bioquímicos que se relacionam às diversas horas do dia nos seres vivos (1). Ritmos biológicos se caracterizam como estados funcionais que variam periodicamente no tempo. Estes ritmos muitas vezes refletem o funcionamento de um relógio biológico, que em mamíferos são organizados pelo núcleo supraquiasmático (NSQ) (2). Nos mamíferos, o sistema de temporização circadiano é composto por vários relógios endógenos, permitindo que as funções biológicas oscilem em sincronia com as alterações diárias no ambiente. A organização circadiana é importante para permitir que o organismo mantenha homeostasia (1). O qual pode ser definido como um conjunto de fenómenos de auto-regulação que levam à preservação da constância quanto às propriedades e à composição do meio interno e externo de um organismo (3). Estas variações diárias podem ter a periodicidade de poucos segundos, horas ou anos (4). Por outo lado, estímulos que desencadeiam estas oscilações são conhecidos como *Zeitgebers*, que podem ser de origem interna e externa (5). Estas flutuações ambientais constituem um desafio à sobrevivência. A adaptação de alguns mecanismos desenvolvidos ao longo da vida permite ressincronizar esses processos fisiológicos adequando a esses ritmos biológicos (6). Consequentemente, o rompimento do relógio circadiano pode ter efeitos significativos em nível celular, tecidual e de órgãos (7). Deste modo, diferentes tecidos e órgãos são mantidos em sincronia, de modo a operar de forma mais eficiente (8). Quando um organismo sofre uma mudança de fase (por exemplo, efeito *jet lag*), uma nova sincronização do relógio circadiano é necessária, e um estado de dessincronização interna entre

o relógio do NSQ e os osciladores periféricos ocorre (9). Eventualmente, uma relação de fase estável entre estes osciladores e do NSQ é restabelecida depois de numerosos ciclos (10).

Os hormônios desempenham um papel importante na fisiologia e no comportamento de animais. Suas ações são muitas vezes dependentes do padrão temporal em que são secretados (1, 11). Alguns ritmos neuroendócrinos são movidos por circuitos de transcrição de genes relógio, enquanto outros representam uma cascata de eventos cíclicos neuroendócrinos (1). Entre eles secreção de insulina, cortisol e melatonina apresentam um padrão temporal definido. Este padrão pode ser afetado por dessincronizadores externos e internos, entre eles o estresse crônico, padrão da alimentação, obesidade, e ciclo claro e escuro (11-13).

A exposição ao estresse ativa o eixo hipotálamo-hipofise-adrenal (HHA) com consequente liberação de glicocorticoides (GCs), hormônios catabólicos, envolvidos na distribuição anômala de gordura (14). Esta distribuição ocorre principalmente em região abdominal estando associada à resistência a insulina, hipertrigliceridemia, e hipertensão arterial (15). Em humanos a secreção noturna de hormônio adrenocorticotrófico (ACTH) e de cortisol se faz de modo pulsátil, alcançando seu nível mais baixo na primeira metade da noite, aumentando rapidamente ao aproximar-se do despertar, quando sua secreção é máxima (entre as 6 e às 10 horas da manhã) (16). Adicionalmente, o estresse crônico tem sido responsável por alterações nociceptivas (17), comportamentais (18), aprendizado (19), humor (20), memória (21, 22) e transtornos alimentares (22) que muitas vezes levam à obesidade.

A obesidade pode ser definida como um excesso de gordura corporal e pode afetar a saúde do indivíduo. Esta não é considerada apenas um transtorno, mas um conjunto heterogêneo de condições com múltiplas causas caracterizando uma síndrome (23), sendo considerada como um fator de risco para várias doenças crônicas, como: doenças cardiovasculares, dislipidemias, Diabetes Mellitus tipo 2 e alterações neuroendócrinas (24). Além disso, a obesidade é geralmente associada a alterações no limiar de dor (25), porém o mecanismo de tais alterações ainda não está bem evidenciado. Estudos sugere que as alterações endócrinas causadas pela obesidade podem ser responsáveis pelo aumento ou diminuição do limiar de dor (25), ou seja, hormônios que regulam a ingesta de alimentos e que estão relacionados com a obesidade podem exercem influência no sistema nervoso central e nocicepção (26, 27).

Considerando a relevância do tema este trabalho teve como objetivo verificar os efeitos da associação de modelos experimentais de estresse crônico e de obesidade sobre parâmetros ponderais, comportamentais e bioquímicos (glicemia), bem como avaliar os padrões temporais dos níveis plasmáticos da glicose, grelina, cortisol em ratos Wistar submetidos a estes modelos experimentais.

2 REVISÃO DA LITERATURA

2.1 BASE DE DADOS CONSULTADOS PARA REVISÃO DA LITERATURA

1 . MEDLINE - Site PubMed

http://www.ncbi.nlm.nih.gov/pubmed/

2. SciELO – Scientific Eletronic Library Online

http://www.scielo.org/php/index.php

3. Endocrinology Society

http://endo.endojournals.org/

Nesta revisão de literatura, buscamos avaliar os principais aspectos relacionados com a obesidade, estresse crônico, marcadores hormonais e o ritmo circadiano em relação as suas principais alterações fisiológicas. A estratégia envolveu as seguintes bases de dados: Endocrinology Society, SciElo e PubMed. Utilizaram-se artigos com datas de publicação entre 1930 e 2013, essas referências foram revisadas para localizar outros estudos não contemplados nesta busca.

Nos sites Endocrinology Society, SciElo e PubMed foram realizadas buscas através dos seguintes termos: Obesidade (Obesity), Estresse Crônico (Chronic stress), Ritmo Circadiano (circadian rhythm) e Marcadores Hormonais (Hormonal markers). Em relação ao termo obesidade foram encontrados 1101 artigos no site da Endocrinology Society, 513 artigos no SciElo e 172605 artigos no PubMed. Quando utilizado o termo estresse crônico (Chronic stress) foram encontrados 2623 artigos no Endocrinology Society, 3554 no site SciElo e no site PubMed 38750 artigos. Também se utilizou o termo Ritmo Circadiano (Circadian Rhythms) e foram encontrados 531 artigos no site Endocrinology

Society, 42 no site SciElo e 62248 artigos no portal PubMed. Realizou-se a busca utilizando o termo “Marcadores Hormonais” (Hormonal Markers) e foram encontrados 13 artigos no site Endocrinology Society, apenas 1 no portal SciElo e 3990 artigos no portal PubMed.

Cruzando-se na busca de artigos as quatro palavras-chave deste trabalho foram encontrados no portal Endocrinology Society 719 artigos, no site SciElo foram encontrados 7 artigos e no portal PubMed foram encontrados 1257 artigos.

Palavras-chave utilizadas: obesidade, estresse crônico, ritmo circadiano e marcadores hormonais. Para apresentar o tema, usamos a revisão sistemática esquematizada abaixo:

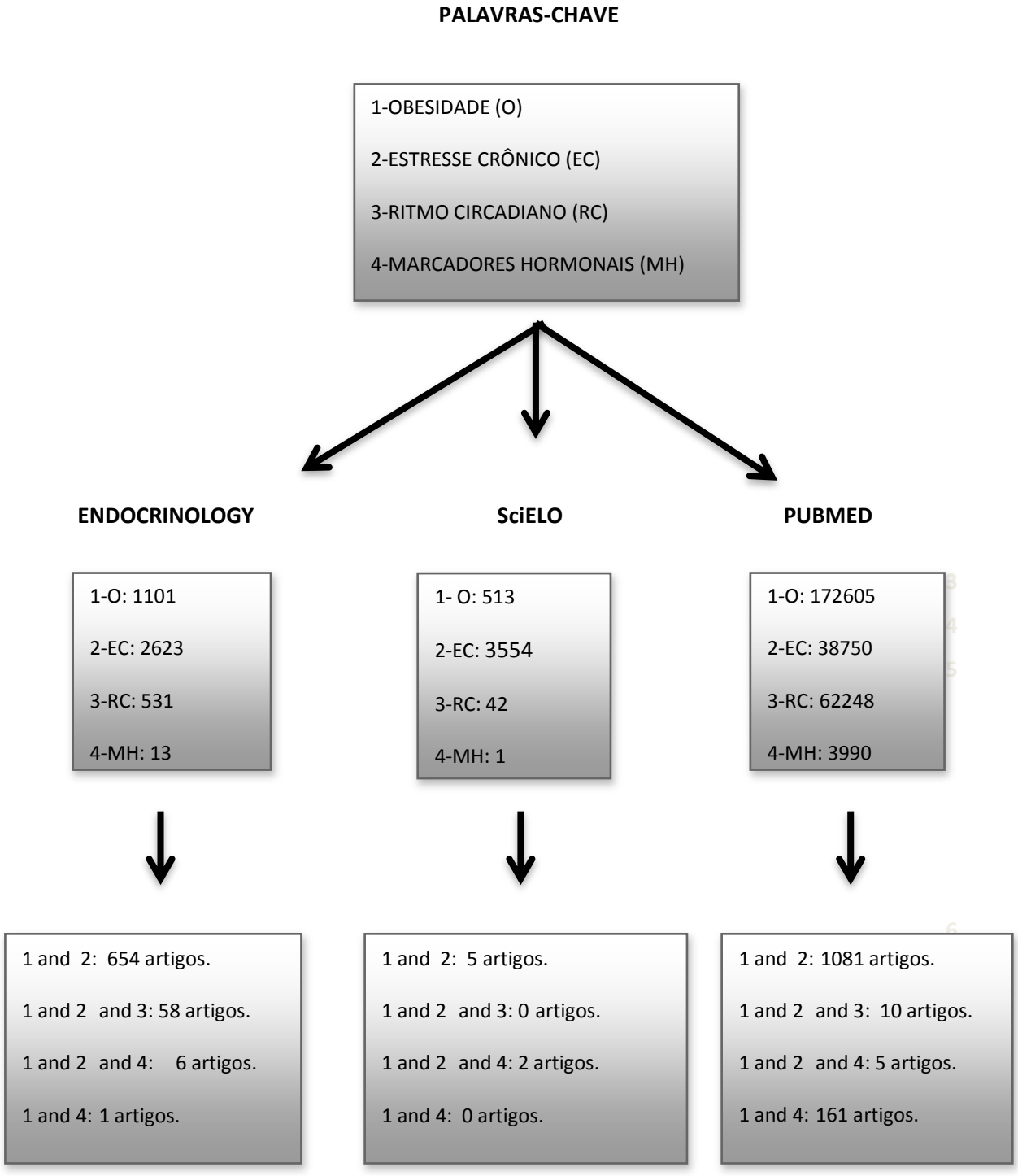

Figura 1, esquematização da seleção de dados consultada para revisão da literatura.

2.2 CRONOBIOLOGIA

Esta ciência estuda a inter-relação dos eventos biológicos relacionados à interação e ritmicidade que ocorrem em um período cíclico de cerca de 24h, conhecidos como ciclos circadianos (10, 28). O termo "circadiano" é derivado de duas palavras do latim: *circa* que significa "aproximadamente", e *diem* que significa "dia". Outros eventos biológicos que apresentam ritmos com períodos inferiores há 20 horas conhecidos como ciclos ultradianos (por exemplo: respiração, batimentos cardíacos, disparos de neurônios, etc.) e ritmos cujo período é superior a 28 horas são denominados ciclos infradianos (ciclo menstrual, ciclo sazonal climático, reprodução, etc.) (10, 29). Processos fisiológicos e comportamentais apresentam ritmos circadianos em uma ampla variedade de organismos incluindo desde as bactérias até os mamíferos (3). As variações deste ciclo têm a finalidade de preparar o organismo de uma maneira antecipada para enfrentar modificações ambientais, como por exemplo, a alternância do ciclo claro e escuro (10).

A expressão rítmica das funções fisiológicas é o resultado de interações complexas entre o ambiente externo e interno. O sistema de temporização circadiana presente nos mamíferos vem sendo amplamente estudado durante os últimos 40 anos. A cronobiologia busca explicar os ritmos biológicos e seus mecanismos ritmicos: 1) ritmicidade circadiana endógena, 2) neurofisiologia do sistema fótico, que permite a sua recepção de informações externas, 3) sincronização neuroendócrina de ritmo interno e suas respostas adaptativas (3, 4).

Em humanos o núcleo supraquiasmático (NSQ) é considerado o principal “marca-passo” por coordenar os ritmos relacionados às variações

fisiológicas e comportamentais (9). Fotorreceptores presentes na retina se comunicam levando informações sobre a exposição à luz ambiente através de células ganglionares especializadas que se projetam diretamente para o NSQ. Estas são via de saída a partir dos receptores projetados do NSQ para outras partes do sistema nervoso central (SNC) e tecidos periféricos e são dependentes dos ritmos circadianos que atuam como temporizador do SNC (30).

O NSQ em mamiferos recebe informações por aferências diretas presentes na retina, atrevés do trato retino-hipotalâmico (31). A proteína fotorreceptora melanopsina localizada as células ganglionares da retina, reage as modificações da intensidade luminosa ambiental (32). Esses estímulos são codificados e transmitidos na forma de impulsos nervosos ao NSQ que sofre ajustes de acordo com as oscilações na luminosidade externa (33).

Além disso, os ritmos circadianos são gerados por uma rede interativa de estímulos de transcrição, tradução e expressão de uma gama dos chamados “clock” genes (genes relógio) que estão presentes em quase todas as células de mamíferos (13). Diferentes tecidos apresentam especificidades relacionadas ao padrão temporal como glândula adrenal, pâncreas e tecido adiposo. Cada um destes tecidos endócrinos exibe uma função fisiológica rítmica coordenada por genes “clock” (34). A ritmicidade e pulsabilidade circadiana são mecanismos fisiológicos fundamentais que implicam na manutenção de um organismo saudável (35). Estes mecanismos geralmente envolvem um oscilador interno molecular que é ressincronizado por meio de eventos chamados de “arrastamento” ou “adiantamento” de fase, que respondem a estímulos ambientais relevantes (10, 36). Estes estímulos

exógenos que sincronizam o sistema temporal endógeno são conhecidos como “zeitgebers” ou ZTs (da língua alemã, Zeit=tempo; geber=dar). Um dos principais “zeitgeber” é o de alternância claro-escuro. Outros zeitgebers incluem temperatura, interação social e comportamento alimentar (3). Estes zeitgebers induzem alterações em componentes moleculares presentes no relógio biológico (NSQ). Assim, permitem ao organismo responder a essas flutuações ambientais, garantindo que animais e plantas realizem seus processos fisiológicos no momento apropriado do dia ou da noite (37).

Estudos recentes mostram que o padrão temporal também é responsável por controlar as alterações da temperatura corporal (38), os ciclos sono-vigília (39), os níveis séricos de lipídeos (colesterol, HDL, triglicérides) (13), o desempenho cognitivo (40), a memória de curto prazo e o comportamento (41). Sistemas hormonais também estão sobre controle circadiano e incluem os níveis de: melatonina, de hormônios do eixo hipotálamo-hipófise-adrenal (HHA) (CRH, ACTH e GCs), do eixo hipotálamo-hipófise-tireoide (TRH, TSH, T3 e T4), do eixo simpato-adrenal (epinefrina e norepinefrina) (Fig. 2) (11).

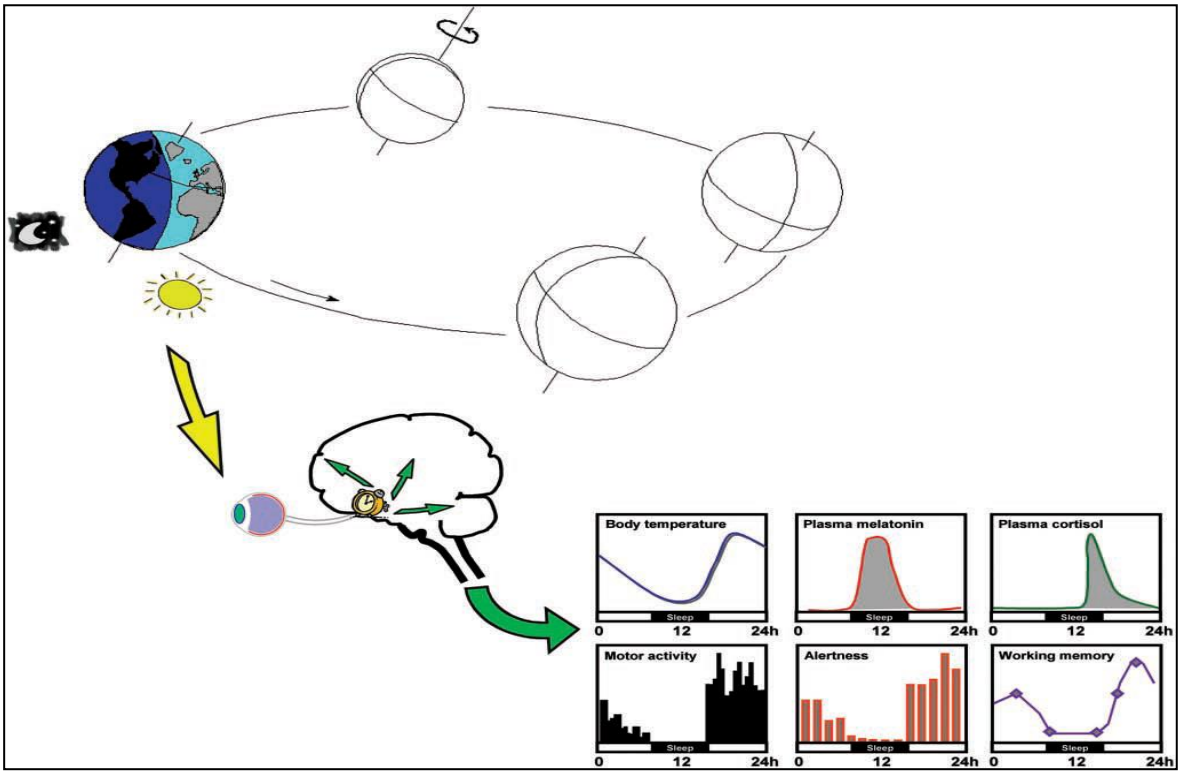

Figura 2: Esquema do ciclo claro-escuro e os principais mecanismos do sistema circadiano em humanos (42).

2.3 ESTRESSE

O conceito de estresse foi publicado por Hans Selye em “What is Stress” no ano de 1936. Neste artigo ele denomina a resposta ao estresse como a sequência de reações que alteram a integridade física e psicológica que ameaçam o estado de equilíbrio do organismo (homeostase) (43, 44). Essas reações que alteram a homeostase são desencadeadas por “estressores”. Estes podem ser definidos por eventos que provocam reações adaptativas, devido à perda da homeostasia do organismo (45).

Dois sistemas de resposta ao estresse são descritos na literatura: A) sistema vegetativo (eixo simpato-adrenal) onde há liberação de epinefrina e norepinefrina da medula da adrenal e B) há liberação de GCs produzido no

córtex da adrenal sob estímulo do eixo HHA (46, 47). A ativação aguda destas respostas promove um aumento na disponibilidade de energia e melhora do fluxo sanguíneo para os órgãos, estas reações são conhecidas como resposta "luta ou fuga" descrita por Cannon em 1920. Por outro lado, a ativação crônica destes mecanismos e a exposição crônica a GCs, como o cortisol, podem ser danosas ao organismo (15, 48, 49).

Selye e colaboradores (1949) propuseram três etapas para descrever a sequência na qual o organismo reage à atividade de seu ambiente, a "Síndrome de Adaptação Geral". As etapas se dividem em: reação de alarme, fase de adaptação com resistência ao estressor, e, eventualmente, um estágio de exaustão e morte do organismo (43, 50).

Embora o estresse seja uma resposta adaptativa, a ativação crônica do eixo HHA apresenta efeitos nocivos à saúde levando a alterações, como por exemplo: imunossupressão (51), disfunção muscular (52), morte neuronal (53), doenças cardiovasculares e psicológicas (15).

Fatores ambientais e sociais influenciam a resposta do organismo em situações de estresse que por sua vez interferem na ingestão de alimentos (54, 55). Em resposta ao estresse agudo, percebida como algo perigoso à segurança do organismo, uma resposta rápida fisiológica é frequentemente ativada, que reduz a ingestão alimentar por suprimir apetite, sendo contrárias as situações de estresse crônico. Isso depende da magnitude, tipo e gravidade dos eventos estressantes, como diferenças de acordo com idade e sexo (56, 57).

Repetidas exposições ao estresse de intensidade moderada produz uma resposta atenuada do HHA (habituação) a aquele estressor. No entanto em presença de um novo estressor, a resposta pode ser aumentada em sua intensidade (sensibilização), em ambos os casos são considerados mecanismos de adaptação (58).

A ativação do eixo HHA (Fig. 3), inicia pela estimulação dos neurônios parvicelulares no núcleo paraventricular (PVN) do hipotálamo. A liberação do hormônio liberador de corticotrofina (CRH) do PVN, por sua vez, leva à liberação de hormônio adrenocorticotrófico (ACTH) pela hipófise. O ACTH é liberado na corrente sanguínea, assim chegando ao córtex da adrenal onde promove a liberação de GCs: principalmente cortisol em humanos e corticosterona em roedores (59). A liberação de GCs é regulada por um mecanismo de *feedback* negativo, no qual o cortisol liga-se a receptores localizados na hipófise, no PVN e em diferentes áreas do sistema límbico diminuindo a secreção (60). Por outro lado, na vigência do estresse crônico, os neurônios produtores de CRH elevam seu nível de secreção, provavelmente estimulados pelas aferências provenientes de outras partes do sistema nervoso, e os GCs não conseguem bloquear a secreção de CRH, ocorrendo, então, concentrações plasmáticas elevadas de CRH, ACTH e cortisol (22, 61).

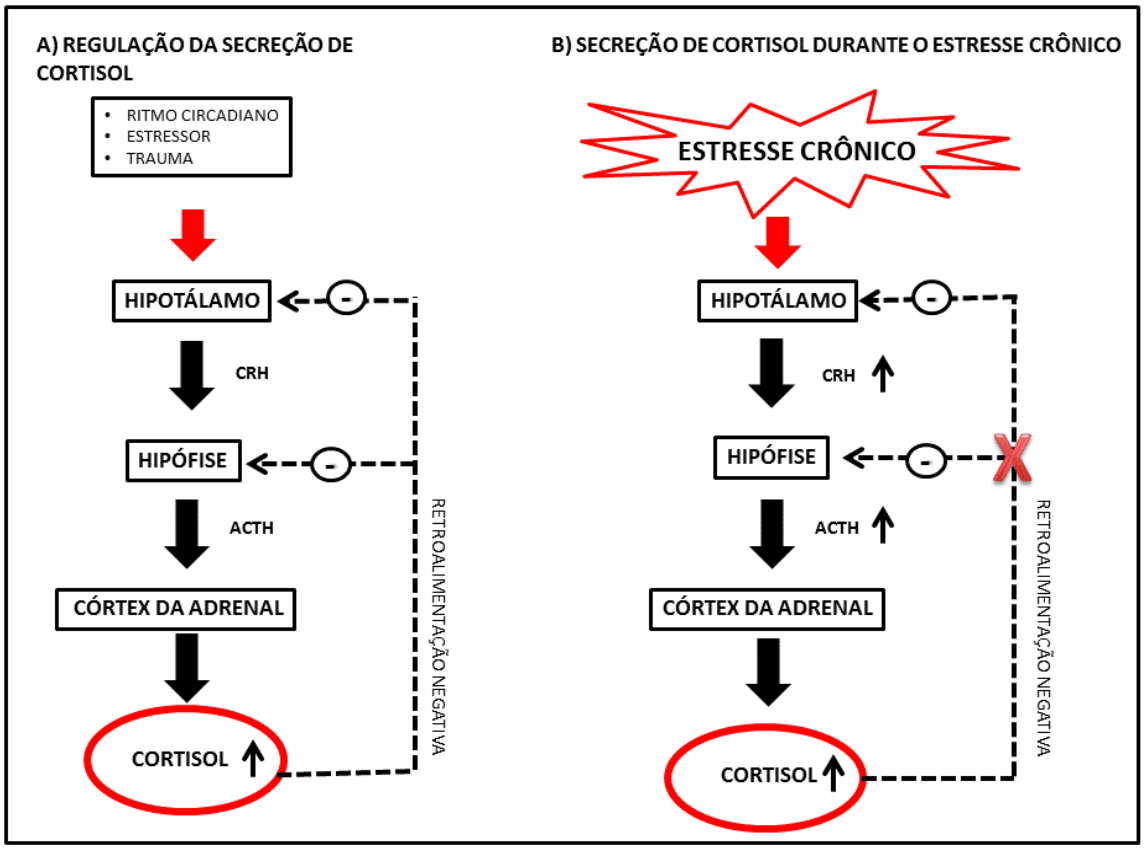

Figura 3: A) Regulação do eixo hipotálamo-hipófise – adrenal; B) Secreção de CRH, ACTH e cortisol durante o estresse crônico (62, 63).

Além disso, os GCs atuam na mobilização das reservas de energia, por exemplo, a ativação de proteólise, lipólise, inibição da captação de glicólise pelos tecidos e da imunogenese (15). Além de alterações no eixo HHA estão associadas a distúrbios alimentares e preferência por alimentos calóricos (64). Gibson e colaboradores (2006) demonstraram que humanos expostos a estresse leve consumiram alimentos ricos em gordura e açúcar, como chocolate entre outros doces (alimentos palatáveis) (65). Em estudo com ratos, além do consumo alimentar, houve redução dos níveis de corticosterona (61). A secreção de GCs tem influência sobre hormônio do crescimento (GH),

alterando o desenvolvimento corporal em ratos, assim levando a um déficit de crescimento em animais (66).

O principal hormônio GC secretado em humanos é o cortisol. Esse hormônio altera a função de numerosos tecidos mobilizando estoques de energia para atender às demandas do desafio estresse (67). Entre os muitos processos influenciados pelo cortisol, destacam-se o metabolismo da glicose (68), da gordura (69), ósseo (70), função imune (63, 67) e a capacidade de resposta cardiovascular (71).

A secreção de cortisol também está associada à fase do ritmo atividade/repouso. A secreção desse hormônio, em mamíferos, atinge os níveis máximos nas primeiras horas da manhã, diminuindo durante o dia até alcançar níveis mínimos à noite (16). Enquanto em roedores, que são animais noturnos, a corticosterona apresenta níveis máximos no início da noite (19h) e mínimos no início da manha (7h) (72).

Há evidências que estressores crônicos podem desempenhar um papel importante no aumento da susceptibilidade individual para o desenvolvimento de doenças metabólicas crônicas, como obesidade abdominal e síndrome metabólica (73). Pecoraro e colaboradores (2004) demonstraram que a ingestão de alimentos hipercalóricos e obesidade desempenham um papel importante na regulação de GCs por exercer efeito no “feedback” inibitório proveniente dos depósitos de gordura periféricos, que amenizam no eixo HHA e redes centrais de resposta ao estresse (Fig.4) (61).

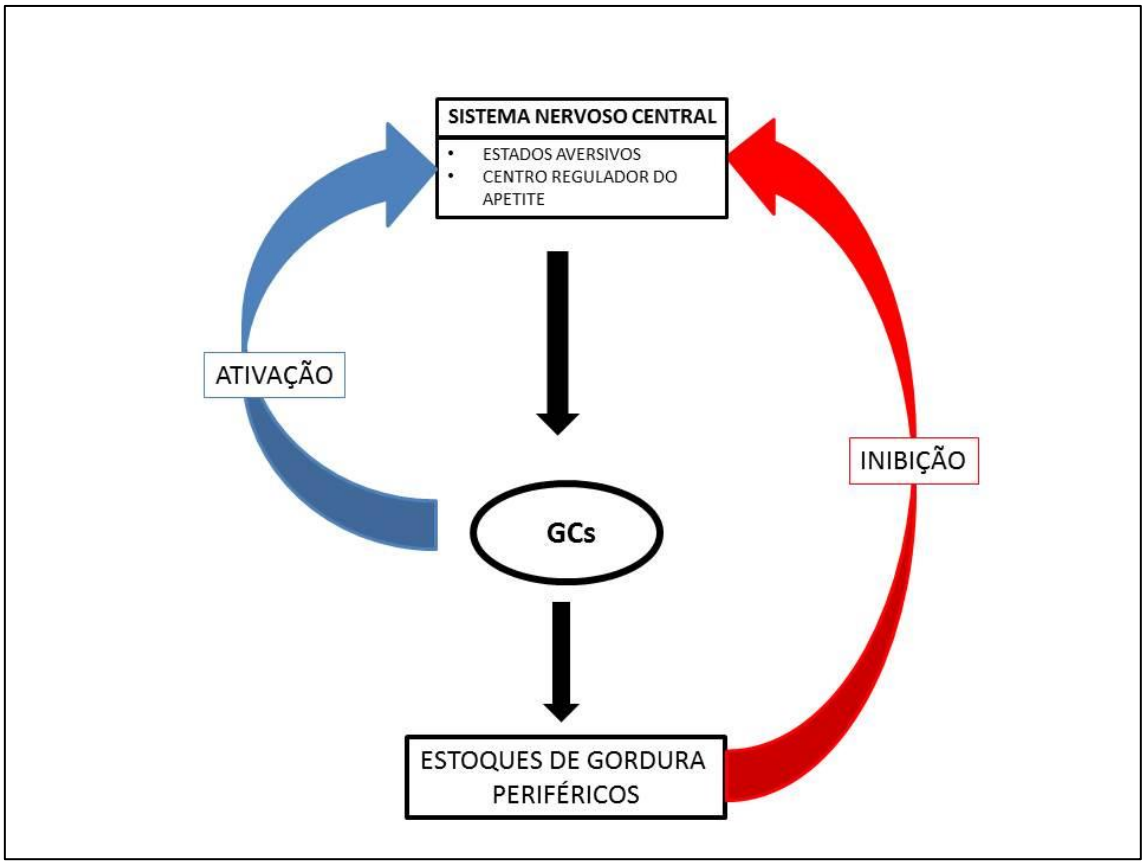

Figura 4: Novo modelo de regulação do estresse crônico (74) .

2.4 OBESIDADE

A obesidade é um problema de saúde pública que vem crescendo a cada ano (23). Estima-se que 2,6 milhões de pessoas morrem por ano como resultado das complicações geradas pelo excesso de peso (75).

A obesidade é uma condição patológica definida como o acúmulo excessivo de gordura. Alguns fatores contribuem para essa condição como, fatores genéticos, hábitos alimentares, sedentarismo e desequilíbrios neuro-hormonais (23, 76). O desenvolvimento da obesidade é decorrente de um desequilíbrio energético crônico, com o consumo de alimentos superior a demanda (77).

Dentre as várias áreas do SNC que participam do controle da ingesta de alimentos, o hipotálamo é a mais importante (78). A regulação central do processo de ingestão envolve inúmeras informações convergentes desta área, as quais têm origem neural (hedônico), endócrina, nutricional e metabólica (homeostático). O hipotálamo integra todas estas aferências e comanda as eferências inibindo ou estimulando a ingestão (Fig.5) (79-81).

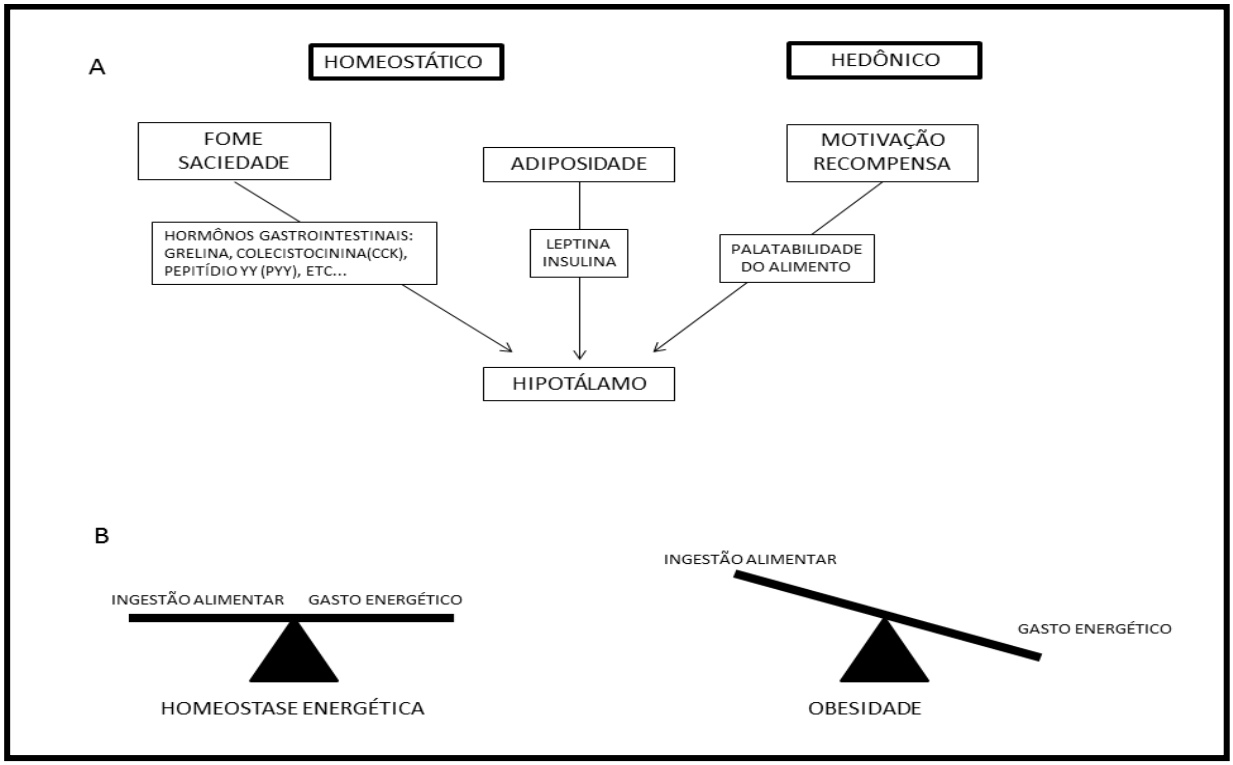

Figura 5: A- Controles homeostáticos e hedônico do consumo alimentar. B- Balanço energético normal e associado a obesidade (82-85).

O processo de obesidade envolve a interação de várias substâncias entre elas podemos destacar a grelina (86, 87), e insulina (88).

2.5 GRELINA

Grelina é um peptídeo de 28 aminoácidos, com propriedades orexígenas, que é secretado principalmente a partir do estômago e do intestino delgado proximal. Foi descrita pela primeira vez em 1999 (89).

Esse hormônio também é descrito como única substância secretada em resposta a redução do conteúdo gastrointestinal, mas suprimida pela ingestão de alimentos (90). Além disso, estimula efeitos opostos aos produzidos pela leptina (91) que por sua vez reduz o consumo de alimentos, aumenta o gasto energético, regula a função neuroendócrina e o metabolismo de glicose e de gorduras (92, 93). A grelina estimula a secreção do hormônio do crescimento (GH), mas também o apetite, a ingestão alimentar e ganho de peso. Além de estar envolvida na regulação de peso e obesidade.

Os níveis plasmáticos de grelina tipicamente seguem um ritmo circadiano com um aumento pré-prandial seguido por uma diminuição pós-prandial e retornando aos níveis basais na primeira hora após a refeição (94). A grelina estimula a secreção do neuropeptídeo Y (NPY) e *peptídeo relacionado com agouti* (AgRP) que promovem um balanço energético por meio de uma interação da ingesta alimentar e ganho de peso (95-97).

Os níveis circulantes de grelina estão alterados na obesidade, no entanto seus níveis plasmáticos são normalizados com a recuperação do peso corporal ideal (98) e são influenciados pelo estresse por restrição (99, 100), estresse social (101) e estresse crônico (102). Por exemplo, a exposição de ratos a um modelo de estresse social levou a um aumento dos níveis circulantes desse hormônio (103).

Além disso, estudos sugerem que a grelina tem também um papel crítico na integração de circuitos centrais envolvidos na ansiedade e na resposta ao estresse. Em humanos, os níveis de grelina são elevados pela ativação do eixo HHA e estão correlacionados diretamente com a magnitude da resposta ao estresse (ou seja, aqueles que possuem as maiores respostas de cortisol ao estresse apresentam um maior aumento da grelina no plasma) (104). Além disso, os níveis circulantes de grelina são reduzidos em pessoas depressivas, e pessoas com polimorfismos do gene responsável por codificar a grelina são mais propensos a transtornos depressivos (105).

2.6 INSULINA

A insulina é o principal hormônio glicorregulador que inibe a produção hepática de glicose e estimula a captação de glicose periférica pelos tecidos. A secreção de insulina no metabolismo da glicose é regulada por uma rede complexa entre diferentes órgãos e tecidos, incluindo tecido adiposo, fígado, músculo e cérebro (1, 8). Quando há um aumento de níveis de substratos energéticos no sangue as células β do pâncreas secretam insulina, levando a um aumento dos transportadores de glicose chamados de GLUT's, que estimulam a absorção destes substratos pelas células (106).

No fígado, a insulina estimula a captação da glicose pelas células. Então a glicose é transformada em glicose-6-fosfato por duas enzimas a hexoquinase IV e hexoquinase I é metabolizada até glicogênio, devido ao aumentando a atividade da glicogênio sintase e diminuindo a atividade da glicogênio fosforilase. Esta enzima tem como função estimular a degradação do

glicogênio em glicose. Além disso, a insulina inibe a gliconeogênese por inibição da transcrição do gene que expressa a fosfoenolpiruvato carboxiquinase, que é o passo limitante da velocidade na gliconeogênese hepática. Neste processo aumenta a transcrição de piruvato-quinase, produzindo moléculas de piruvato, produto final de glicólise aeróbica (107).

A manutenção de um nível constante de glicose sanguínea é essencial para a fisiologia normal, em particular, para o SNC (108). A secreção de insulina é independente dos níveis de glicose plasmáticos(109). Além disso, os níveis de insulina apresentam-se muito baixos durante a noite, enquanto sua secreção é máxima durante o dia (8). A dessincronização deste ritmo circadiano está relacionada a transtornos metabólicos relacionados à obesidade e resistência á insulina (110, 111).

No tecido adiposo, os GCs promovem a mobilização de substratos energéticos, enquanto a insulina estimula a síntese de gordura, produzindo assim uma interação antagônica, com ativação de lipases (112). Em situações de estresse, os níveis de GCs estão elevados resultantes da hiperativação do eixo HHA, estimulando a gliconeogênese e inibindo a glicólise com consequente aumento da secreção de insulina. Assim, esse efeito sinérgico dos GCs pode levar a hiperinsulinemia aumentando o risco de deposição de gordura abdominal (77).

2.7 ESTRESSE E ALTERAÇÕES COMPORTAMENTAIS

Eventos estressantes estão relacionados à ansiedade, que influenciam a resposta comportamental do organismo (113). Isto é corroborado pelo fato que

a administração de fármacos ansiolíticos como o diazepam e/ou consumo de alimentos hipercalóricos que são capazes de reverter alterações comportamentais em ratos Wistar nos testes de labirinto em cruz elevado (LCE, do inglês – *Elevated Plus Maze Test* - EPM) e de campo-aberto (CP, do inglês - *Open Field* - OP) induzidos por estresse (22, 114-116). Adicionalmente, estas alterações comportamentais dependem de diferentes fatores como, o protocolo de estresse utilizado, gênero e duração do evento estressor (117), tempo de exposição (22, 61, 62). O estresse por restrição simula os efeitos de um estresse físico e psicológico em nível de SNC interagindo com o sistema endócrino levando a alterações comportamentais e orgânicas (118).

Krolow e colaboradores (2010) demonstraram que a exposição ao estresse crônico provoca alterações comportamentais relacionadas à ansiedade ratos Wistar. Por outro lado, esse efeito foi revertido pelo consumo de chocolate (18). Outro estudo mostrou que o protocolo de 50 dias de estresse por restrição em machos e fêmeas diminuiu o crescimento dos animais e aumentou o peso das glândulas adrenais, devido à ativação crônica do eixo HHA. E também estes efeitos foram revertidos pelo consumo de chocolate (119).

Em humanos, a depressão está associada com diminuição de 5-HT1A em regiões prosencefálicas (120, 121). Evidências demonstram que a neurotransmissão serotoninérgica é sensível a diferentes modelos de estresse, entre eles o estresse por restrição (122, 123). Quando muito intenso, o estresse repetido diminui o número e a função dos receptores 5-HT1A no hipocampo, efeito mediado pelo aumento dos níveis de GCs (124, 125). Também, é importante salientar que essas alterações afetam não somente o

comportamento do animal, mas também provocam alterações neuroquímicas e neuroendócrinas (125).

2.8 NOCICEPÇÃO, ESTRESSE E OBESIDADE

A dor é considerada uma experiência subjetiva, definida como uma experiência sensorial e emocional desagradável associada a dano tecidual real ou potencial (IASP, 1986). Estas informações sensoriais são detectadas por transdutores específicos moleculares suportados pelos neurônios nociceptivos cujos corpos celulares são agrupados no gânglio da raiz dorsal ou gânglios trigêmeos (126). Além disso, percepção da dor é caracterizada como uma experiência multidimensional, diversificando-se na qualidade e na intensidade sensorial, sendo afetada por variáveis como estresse (127) e obesidade (128).

Nocicepção compreende os processos neurais que codificam e processam estímulos nocivos (129). Estes podem ser modulados por fatores externos por meio de alterações nas vias intrínsecas da dor. Síndromes dolorosas são associadas com estresse crônico, uma vez que a exposição crônica a dor, que ativa o eixo HHA (130). Esta resposta nociceptiva ao contrário depende do tipo de estressor e duração desta exposição. Durante estresse agudo, esta associada à redução da sensibilidade à dor (analgesia) (130), enquanto que, estresse crônico tem sido associado a uma diminuição do limiar de dor (hiperalgesia) (127, 131, 132) e alodinia (dor induzida por estímulos não nocivos) (127). Estudos anteriores sugerem que a hiperalgesia induzida por estresse crônico pode estar relacionada a alterações em nível central e periférico de opióides (132-134). A ausência de analgesia induzida por novidade (132), resistência à morfina (133), e diminuição no “binding” de receptores opióides em hipocampo e em córtex cerebral (134) destes animais

suportam esta teoria. Por outro lado, no estresse agudo, o sistema opióide parece ser modulado para maior atividade (135). Em estudo anterior, foi demonstrado que a hiperalgesia induzida por estresse crônico permanece por 28 dias após a interrupção do tratamento (133). É interessante salientar que a resposta analgésica ao estresse agudo por restrição foi reestabelecida 14 dias após a interrupção do estresse crônico (133). No entanto, os mecanismos subjacentes responsáveis pela longa duração da hiperalgesia induzida pelo estresse crônico ainda são desconhecidos.

A obesidade também exerce um papel na nocicepção. Estudos relatam um aumento na percepção da dor em obesos (25, 136). Esses estudos indicam inter-relações entre a nocicepção, opióides endógenos e obesidade. Desta forma, as alterações do sistema endócrino desempenham um importante papel na obesidade, que pode levar a um aumento ou diminuição no limiar da dor. Há poucas pesquisas sobre esses fatores hormonais que são relacionados ao sistema nociceptivo (como a leptina) ou efeito antinociceptivo (como a grelina, orexina A e B) (26, 27, 130).

A obesidade é hoje considerada como condição inflamatória crônica de baixo grau relacionada a estresse oxidativo (137). Evidências sugerem que ocorre um aumento de adipocinas pró-inflamatórias no tecido adiposo de obesos, como leptina, fator de necrose tumoral alfa (TNF-α), interleucinas e resistina. A literatura científica também é convincente quanto a associação da inflamação com o surgimento ou agravamento das co-morbidades da obesidade, em especial diabetes tipo 2 e doenças cardiovasculares (138, 139). Estudos sugerem que o TNF-α e interleucina (IL)-1β contribuem para o desenvolvimento de hiperalgesia e dor crônica (140, 141).

Estudos demonstram a ocorrência de condições de dor crônica e obesidade, por exemplo, a osteoartrite e dores nas costas, duas das mais comuns condições de dor crônica, geralmente coexistem com a obesidade (142, 143). Outros estudos transversais encontraram associações entre obesidade e fibromialgia (144-146), dores de cabeça crônicas (24, 147), dor abdominal (148) e artrite (149).

3 OBJETIVOS

3.1 OBJETIVO PRINCIPAL

Considerando que o estresse crônico é um importante componente da vida moderna muitas vezes associado a descontrole alimentar e consequentemente levar a obesidade. Este estudo objetiva avaliar o efeito da associação de modelos experimentais de obesidade e de estresse crônico sobre parâmetros comportamentais, hormonais e bioquímicos.

3.2 OBJETIVOS ESPECÍFICOS

Foram avaliados:

a. Os padrões temporais de níveis séricos de:

- ✓ glicose
- ✓ insulina
- ✓ grelina
- ✓ corticosterona (para controle do estresse crônico)

b. Os comportamentos

- ✓ do tipo ansioso no teste de Labirinto em Cruz Elevado.
- ✓ exploratório e locomoção, no teste de Campo Aberto.
- ✓ nociceptivo nos testes da formalina e tail flick.

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5.1 ARTIGO I

EVALUATION OF TEMPORAL PATTERNS OF OBESITY AND CHRONIC STRESS ON SERUM MARKERS IN RATS

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Running Head: Serum makers of obesity and chronic stress

Abstract

The circadian rhythm has a great importance for various physiological processes; and, the rupture of these rhythms can lead to severe damage to health. The sleep-wake cycle, feeding and metabolism of lipids and carbohydrates are subject to circadian regulation, which lead to synchronize the available energy and expense required for changes in the external environment according to the phase light and dark. Mostly circadian clocks coordinate such rhythms. The misalignment of the circadian rhythm may be a causal factor for a positive relationship between shift work and obesity. Exposure to chronic stress nowadays leads to the release of glucocorticoids. Consequently, chronic activation of hypothalamus- pituitary -adrenal (HPA) axis is linked some metabolic and physiologic disorders. Moreover, in modern societies consume of fast food or hypercaloric diet is associated some pathologies as diabetes and obesity. Considering that alteration of circadian rhythmicity described as potential biological alterations associated with diseases, the aim of this study was evaluated the effect of chronic stress associated to obesity on temporal pattern of serum markers (glucose, insulin, corticosterone, and ghrelin levels). Was used 96 male Wistar rats at 60 days old were divided into 4 groups: diet standard (C), hypercaloric diet (cafeteria diet) (HD), chronic stress (chronic stress by restricting 1h/day/5 days a week between 9am and 12pm) associated with the standard diet (S), chronic stress associated with hypercaloric diet (SHD). After 80 days of experiment the animals were killed in three periods of the day (ZT0-7h, ZT12-19h - and ZT18-1h) to evaluate plasma levels of glucose, insulin, corticosterone, and ghrelin. This study suggests that exposure to chronic stress and/or diet hypercaloric leads to metabolic and physiological changes of clinical importance. In addition, this study showed circadian pattern on corticosterone and demonstrated effect of stress and interaction between diet and ZT. Also, insulin levels demonstrated circadian pattern and showed effect of diet. In contrast, our results don’t showed rhythmicity in glucose serum. In contrast, glucose levels have changed

by diet intake and interaction between diet and stress. On the other hand, the ghrelin levels don’t showed circadian pattern or was affected by stress and diet. This study demonstrated those chronic stress exposure and hypercaloric diet intakes are able to influences circadian rhythm. Therefore, these factors are possible dysynchronizers endogenous, associated some pathological alterations.

Keywords: Chronic stress, Hypercaloric diet, Obesity, hypothalamic-pituitary-adrenal axis, Circadian rhythm, Insulin, Ghrelin, Glycemic levels, rats.

Introduction

The circadian rhythms need to be synchronized daily by external stimuli, for example in mammals the principal synchronizer is the light/dark cycle (1). The synchronism with the environment depends of synchronizers are called *Zeitgeber* (“time giver”). Study showed that glucocorticoids (GCs) induce changes in circadian rhythm (2). It is involves of change in peripheral clocks becomes potential target signals that establishing the connection between the pacemaker suprachiasmatic nucleus (SCN) and peripheral oscillators. This is supported by the observation that GCs hormones and food-induced neutralizing phase shift of the oscillators peripheral (3-5). Previous study showed relationships between of chronic stress and consumption of hypercaloric diet influences endocrine system as insulin, cortisol and ghrelin hormones in the diary circadian rhythm (6).

The chronic stress is related to modulate a variety of physiological changes. The activation of hypothalamic-pituitary-adrenal (HPA) axis releases corticotrophin hormone (CRH), adrenocorticotrophic hormone (ACTH) and GCs (cortisol in humans, corticosterone in rats and mice), in higher proportion (7). The rats present the secretion peak of corticosterone in early evening (8). GCs entrain the circadian rhythm by phase-shifting the expressions of several clock genes in peripheral organs for signals establishing the link between the SCN pacemaker and peripheral oscillators (9). Moreover, exposure to chronic stress is also associated with the development of abdominal obesity in humans (10).

Furthermore, the consumption of hypercaloric foods has increased worldwide due high rates of stress generated by our modern society. This consumption is responsible for many physiological and metabolic disorders like diabetes (11), obesity (12) and cardiovascular diseases (13). Meal times have recently been shown to be a dominant *Zeitgeber* for be able to interference of circadian Rhythm (14). Nocturnal rodents mainly eat during the night, but if food is only available during the day, various

physiological and metabolic functions are entrained to adjust to this feeding time (2, 15-17).

In addition, the insulin rhythm may be an important mediator of the neuroendocrine control of metabolism. The function performed by insulin in the regulation glucose, seems to be scheduled based on circadian instead of representing a phenomenon of feedback (3). In humans the levels of insulin secretion are very low during the night with maximum secretion at day (18) and the secretion of insulin is independent of glucose levels or feeding (19).

It is important to note that the ghrelin is a stomach-derived hormone that regulates food intake and neuroendocrine function (20). Studies "*in vitro"* and "*in vivo"* indicate that a key function of ghrelin is to signal stress to the brain (21). It has been suggested that one of the potential stress-related ghrelin targets is the corticotropin/releasing factor (CRF) producing neurons of the hypothalamic paraventricular nucleus, which secrete the corticotropin neuropeptide into the median eminence and activate the HPA axis (22).

Considering that disruption of circadian rhythmicity can resulting in a many number of pathologies, the aim of this study was evaluated the effect of chronic stress associated or not to obesity/hypercaloric diet intake in the temporal pattern of hormonal markers as insulin, corticosterone, and ghrelin.

2 MATERIAL AND METHODS

2.1 Animals

Animals and Reagents: The experiments were carried out with 96 male Wistar (200 - 250 g) rats with 60 days-old. Experimentally naive animals were housed in groups of five in home cages made of Polypropylene (49 x34x16cm). They were maintained under a standard LD 12:12 cycle [lights on at 07:00 h, ZeitgeberTime (ZT) 0, and lights off at 19:00 h, ZT 12], in a controlled environment (22±2°C), and water

and chow available *ad libitum* (Fed standard chow and/or hypercaloric diet). The animal's handling and experiments were performed in accordance with the international guidelines for animal welfare. The protocol of this experimental study was approved by the Ethics Committee in the Use of Animals at the Institution where the work was conducted (GPPG-HCPA: 10.0382) and is adhered to the ethical and methodological standards for medical biological rhythm research according to Portaluppi *et al.* (2008)(23).

2.2 Experimental Design

The rats were habituated to the environment for 1 week before the experiment. The animals were randomly divided into two groups: control and stress model. Each group was subdivided in more two groups: standard food (control - C, control stress - S) or hypercaloric diet (hypercaloric diet - HD and restraint stress plus hypercaloric diet –SHD), according to chronic stress exposure and type of diet used (hypercaloric diet and/or standard chow). After eighty-days of experiment, the animals were evaluated after 12 hours without food, then the rats were killed for each time ZT0 (7h), ZT12 (19h) and ZT18 (1h) and blood was collected and stored in a freezer -80C°. At night, the sacrifices were performed in the dark with a red light.

2.3 Stress procedure

The animals were subjected to restraint chronic stress model (24), it was performed using a plastic tube (25 x 7cm) adjusted to avoid discomfort, but limiting the movements of the animal, with the front open to allow breathing (25). The animals were stressed by the morning (between 9am to 12pm) 1 h/day, 5 days/week for eighty days (24). After the stress procedure, the animals were returned to the home cages. Control animals were kept in their home cages during the period of experiment. The apparatus was ventilated and did not cause physical compression, avoiding hyperthermia and sweating.

2.4 Experimental diets

The standard diet Nuvilab CR-1 (NUVITAL®) is composed of 55% carbohydrates, 22% protein, 4.5% lipids, and other constituents (fiber and vitamins), and makes a total of 2.93 kcal / g (information provided by manufacturer). A palatable hypercaloric diet (cafeteria diet) consists of about 60% carbohydrates, 20% lipids, 15% protein and 5% other constituents (sodium, calcium, vitamins, preservatives, minerals etc.) for a total of 4.186 Kcal / g and 0.42 kcal / mL (calculated based on information provided by the manufacturer on the package label). This diet was adapted to a diet known as calorie cafeteria diet or Western diet (26). As well as the standard diet, the experimental diet was replaced daily by fresh food. The exposed animals to hypercaloric diet also had access to standard food and water.

2.5 Blood Serum Collection

The animals were killed, by decapitation, and blood and tissue samples were collected 24h after the last session of restraint stress and after a 12-hour fast. Trunk blood was collected for centrifugation for 5 minutes at 5000 g at room temperature. This method was used to facilitate the collection of large volumes of blood serum for analysis. Most importantly, this model provides larger quantities of serum for determination of biochemical effects, including hormonal effects. Serum was frozen at -70 ° C for subsequent analysis.

2.6 Blood Serum Assays

The hormone levels in the blood serum samples were determined using commercial kits of Enzyme-linked immunosorbent assay (ELISA), for Insulin (Millipore, rat/mouse insulin ELISA kit, cat.#EZRMI-13K), corticosterone (IBL America, Corticosterone ELISA kit, cat.#IB79112), ghrelin (Millipore, rat/mouse ghrelin (total)

ELISA kit, cat.#EZRGRT-91K) and glucose kit PAP Liquiform (Labtest), according to the manufacturer's instructions.

2.7 Statistical Analysis

Data were expressed as the mean ± standard error of the mean (S.E.M). For verify the existence of temporal pattern of release of hormones and glucose levels it was realized comparison between ZTs of controls group by one-way ANOVA followed Student-Newman-Keuls (SNK). The baseline weight of the animals was compared between the groups using one-way ANOVA/SNK and the weekly weight were evaluated using two-way ANOVA for repeated measures followed by Bonferroni. The other data and interactions were evaluated by two-way ANOVA using time points (ZTs) as random factor (time points, obesity, chronic stress, time points x chronic stress, time points x obesity, chronic stress x obesity, and time points x obesity x chronic stress) followed by Bonferroni correction for random factor when necessary for time points and Tukey test for groups. The between-group differences were considered significant at $P<0.05$. SPSS 19.0 for Windows was used for Statistical analysis.

3. RESULTS

All the groups were analyzed in different *Zeitgeber* Time (ZT0, ZT12 and ZT 18) after 80 days of experiment.

3.1 The circadian rhythm and effect of chronic stress and obesity on corticosterone serum levels

Corroborating our previous studies, we confirm the temporal pattern of corticosterone, showing a peak in ZT12 in the control group (one-way ANOVA/SNK, $F_{(2,26)} =9.825$, $P<0.001$). In addition, the two-way ANOVA showed significant effect of chronic stress ($F_{(1,88)}=45.173$, $P=0.008$), and interaction between obesity and time points ($F_{(5,84)}=28.371$, $P=0.034$). On the other hand, the rhythmicity of this hormone not

suffers effect of stress, obesity or interaction of stress and diet, stress and ZT or stress and diet and ZT (P>0.05 for all, figure 1).

---------- Insert Figure 1 ----------

3.2 The circadian rhythm and effect of chronic stress and obesity on insulin and glucose serum levels

We found the circadian rhythm of insulin evaluating three different time points (ZTs) of control group insulin serum levels, since that was observed an increased insulin levels in ZT0 in relation to ZT12 and ZT18 (one-way ANOVA/SNK, $F_{(2, 15)}$=7.466, P=0.006). Moreover, the two-way ANOVA showed effect of obesity ($F_{(1,38)}$=8.373, P=0.044), but no effect of chronic stress or interaction between independent factors were observed (P>0.05, figure 2).

The one-way ANOVA/SNK did not showed differences in glucose levels in the control group in the three time points evaluated (one-way ANOVA/SNK, P>0.05). On the other hand, the two-way ANOVA/SNK showed effect of obesity ($F_{(1,30)}$=22.979, P=0.008). Our result also shows an interaction of obesity and chronic stress ($F_{(3,64)}$=27.388, P=0.006). There were no effects for chronic stress, time points, or interaction between obesity and time points or between obesity and chronic stress and time points (P>0.05 for all cases, figure 3).

---------- Insert Figure 2----------

---------- Insert Figure3----------

3.3 The circadian rhythm and effect of chronic stress and obesity on ghrelin serum levels

This study did not observe temporal pattern of ghrelin in three ZTs evaluated in the control group (one-way ANOVA, P>0.05). Furthermore, the levels of ghrelin did not suffer any effect of chronic stress, obesity or interaction between two factors (two-way ANOVA, P>0.05 for all, figure 4).

---------- Insert Figure 4 ----------

4. Discussion

In our study the circadian rhythmicity of corticosterone serum levels display a temporal pattern in naive rats, with a peak at ZT12. The Wistar rats are nocturnal animals, characterized by higher values of corticosterone observed during the dark phase that is the active phase of these animals (27, 28). In rodents, increased levels of corticosterone at the end of the period of inactivity serve to prepare the animal for a period of activity in which the mobilization of energetic sources and stimulating feeding behavior are needed (29). Furthermore, corticosterone levels were increased by exposure to chronic stress confirming that exposure to chronic stress activate HPA axis stimulating the production of corticosterone (30). In previous studies have been demonstrated that serum corticosterone levels in rats subjected to chronic stress do not show a significant increase in comparison to control animals; however, this increase is perceived when rats are exposed to acute stress (25, 31). Thereby, we can suggest that this increased levels of corticosterone observed in our results, could be due to the 12 h fasting period of animals before the death, since it this situation could be considered as an additional stressor resulting in higher corticosterone levels in comparison with the responses during a chronic situation. Thus, GCs mediate the

stress response, and their circadian variations are very important to allow rapid adaptation of the organism to environmental changes in order to keep homeostasis (32). In addition, we showed that the chronic stress is able to change corticosterone levels across time points evaluated, since the corticosterone serum levels fall throughout the dark phase, reaching their lowest levels at the dark–light transition (16). Moreover, our data indicated interaction of obesity and time points in the temporal pattern of corticosterone. This result agrees with others studies that showed circadian endocrine variation is associated the consumption of high-calorie foods and chronic stress (9, 33). Thus, it is reinforces the notion that chronic stress have important risk factor to the development of metabolic disorders such as obesity and type 2 diabetes (29, 34-37).

In addition, our results showed peak of insulin in the naive rats in ZT0. Rats with free access to food typically eat numerous small meals throughout the night (38). The daily activity/rest cycle of the animal also generates a feeding/fasting cycle, with most of the food ingested during the night in the Wistar rats (39, 40). Our results demonstrate the obesity is able to influences insulin levels. Moreover, the daily feeding pattern is considered the major time cue for the peripheral oscillators (41). Also, characteristic of nutrition are considered one *zeitgeber* as properties of a meal than is taste, smell and increase of ingested food (17). Circadian misalignment occurs when the internal circadian timing system is not appropriately aligned with the external environment including the dark-light cycle, sleep-wake, stress, and fasting-feeding cycle (42). On the other hand, increased levels of insulin in response to the consumption of hypercaloric diets foods with consequently obesity agree with other studies (43-46). In consequence, chronic consumption of hypercaloric diet also called “comfort food” is related of metabolic disorders like diabetes and obesity (47).

Moreover, in this study glucose serum levels did not exhibit temporal pattern in control groups in the three times evaluated. However, our results agree with another

study that indicates increased of glucose levels in the obesity (48). Also, it corroborates other studies that showed that high palatable foods intake represents an additional metabolic challenge, because high levels of glucose are associated metabolic changes, for example diabetes mellitus type 2 (11, 48-50). Several studies showed that animals exposed in chronic stress protocol, as restraint stress, lead increased intake of palatable foods (51, 52). Dallman *et al* (2003) demonstrated that the hypercaloric diet is able to reduce the effects of chronic activation of the HPA axis signs due fat deposits (7, 51, 52). On the other hand, previous studies have linked chronic stress and eating disorders (51-54).

In our study the ghrelin serum levels did not show temporal pattern considering the time points evaluated in this study. However, it is known that in humans there is a pre-prandial peak before meal times and subsequent post-meal a decrease in ghrelin concentrations (55). Contrary to another studies (56), our study showed the both factors, chronic stress and obesity were not able to modify the ghrelin levels. It is possible that the death schedules chosen did not permitting to observe the effects of chronic stress and obesity in these animals. Highlighting that circulating ghrelin may contribute to the neuroendocrine and behavioral responses along with sustaining the energetic requirement needed upon repeated exposure to stressors (22). The mechanisms by which ghrelin promotes food intake are multifaceted and include enhancing the rewarding properties of certain food (57). However, the interaction of ghrelin-engaged catecholamine neurons instead may contribute substantively to the altered, complex eating behaviors and development of obesity in humans exposed to chronic stress and in humans with major depressive disorder (22, 58).

In addition, as discussed above, disorganization in the circadian system can lead to metabolic dysfunction in response to genetic, environmental, and behavioral perturbations. Studies indicate that interruption or desynchronization of the circadian system may contribute to manifestations of metabolic syndrome and complications

occurring in obesity such as dyslipidemia, type 2 diabetes mellitus, hypertension, endothelial dysfunction and cardiovascular disease (59, 60).

In summary, taking into consideration that alteration in circadian patterns of metabolism have been associated with diseases in humans and which these circadian disturbances precede and/or follow the onset of obesity and metabolic syndrome (61), we can infer that altered feeding patterns together with stressful situations may potentially induce both central and peripheral changes in circadian rhythms. Most importantly, we demonstrated that obesity and chronic stress could influence the body's homeostasis over time.

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Figure 1. Temporal pattern of Corticosterone serum.

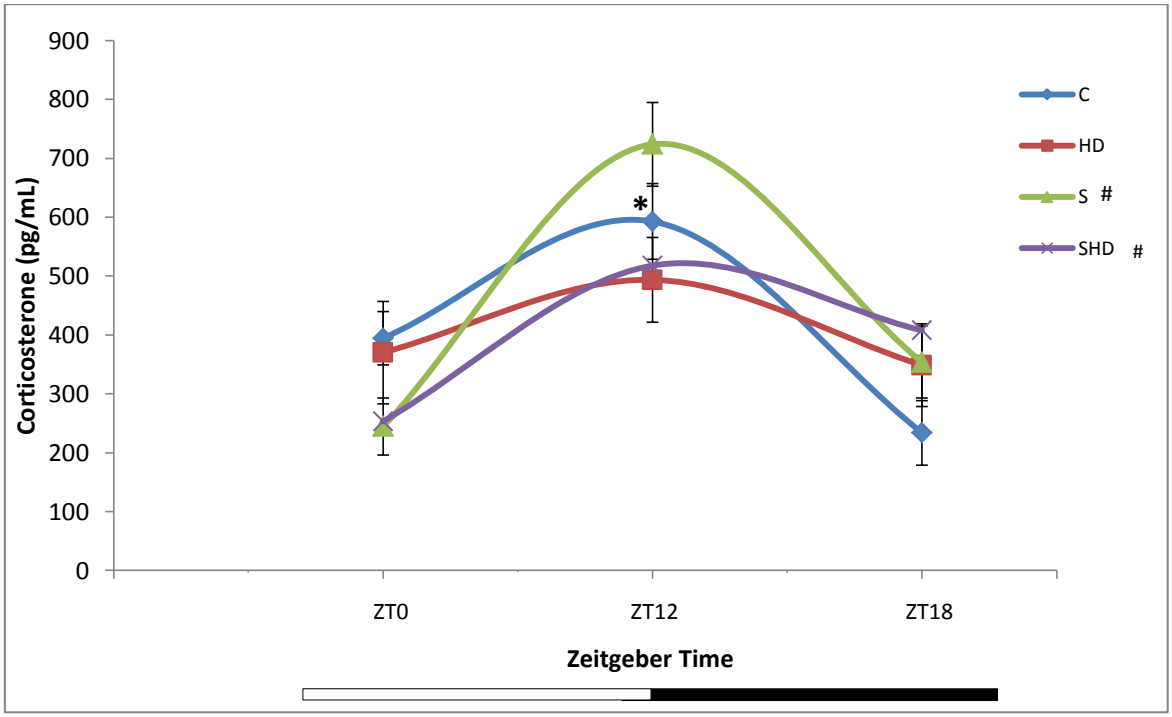

FIGURE 1: Corticosterone levels in the ZT0 (7hs), ZT12 (19hs) and ZT18 (1h) (N=8/11). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Values are expressed as mean ± S.E.M. Horizontal bar of base of graph represent day (white) and night (Black). Significant interaction of obesity and ZT (two-way ANOVA /Bonferroni, $P<0.05$).

*Indicate significant different of others ZTs (one-way ANOVA /Tukey, $P<0.05$).

Indicate significant effect of chronic stress (two-way ANOVA /Bonferroni, $P<0.05$).

Figure 2. Temporal pattern of Insulin serum.

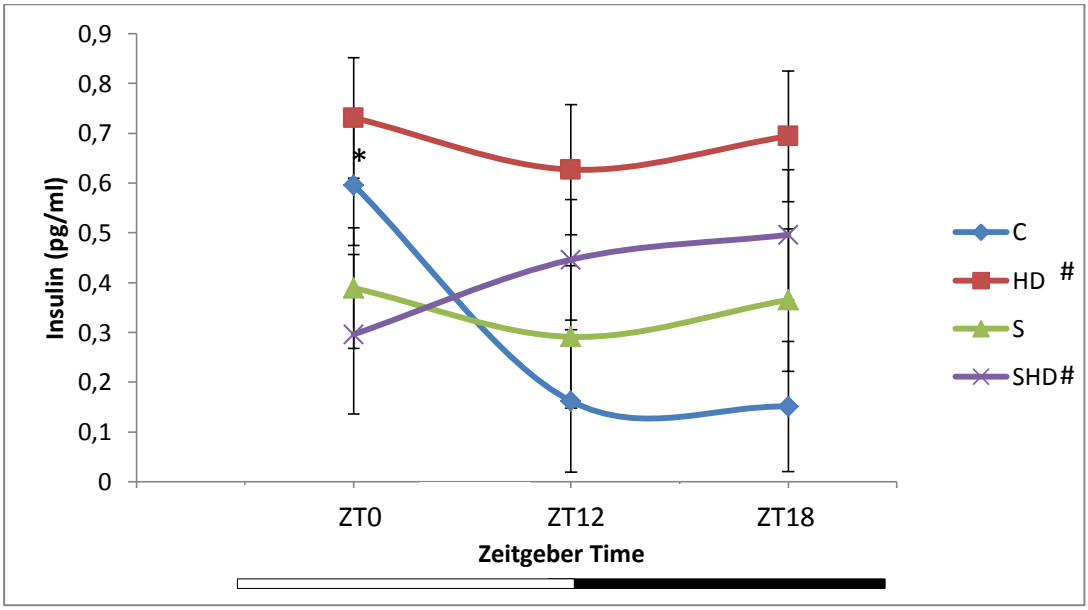

FIGURE 2: Insulin levels in the ZT0 (7hs), ZT12 (19hs) and ZT18 (1h) (N=5/8). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Values are expressed as mean ± S.E.M. Horizontal bar of base of graph represent day (white) and night (Black).

* Indicate significant different of others ZTs (one-way ANOVA /Tukey, P<0.05).

Indicate significant effect of obesity (two-way ANOVA /Bonferroni, P<0.05).

Figure 3. Temporal pattern of glucose serum.

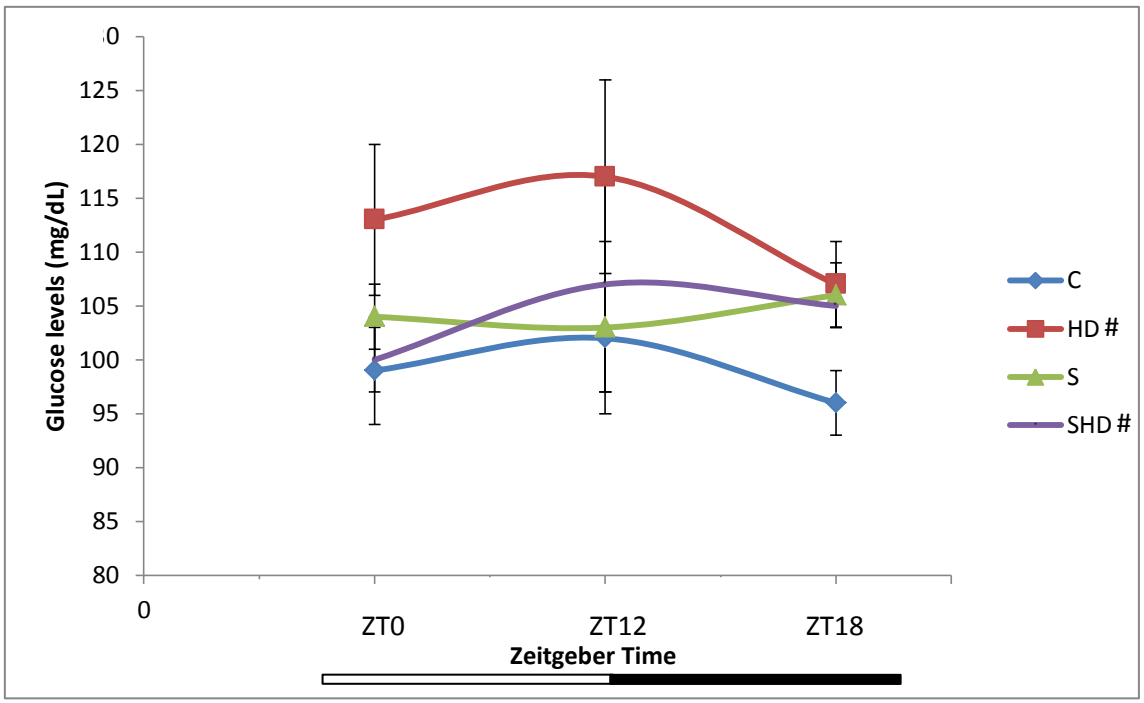

FIGURE 3: Glucose levels in ZT0 (7hs), ZT12 (19hs) and ZT18 (1h) (N=5/7). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Values are expressed as mean ± S.E.M. The control group don't showed circadian pattern (One way Anova/Tukey, P>0.05). Horizontal bar of base of graph represent day (white) and night (Black). Significant interaction of obesity and chronic stress (two-way ANOVA , P>0.05).

Indicate significant effect of obesity (two-way ANOVA, P>0.05).

Figure 4. Temporal pattern of Ghrelin serum.

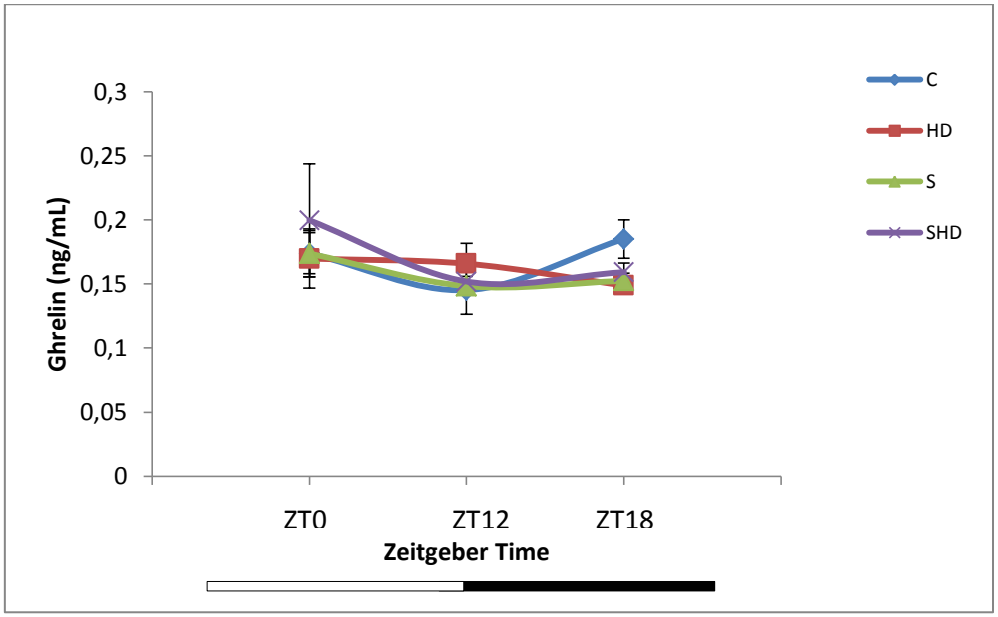

FIGURE 4: Ghrelin levels in the ZT0 (7hs), ZT12 (19hs) and ZT18 (1h) (N=5/8). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Values are expressed as mean ± S.E.M. The control group don't showed circadian pattern (one-way ANOVA/Tukey, P>0.05). Without effect of stress, diet or interaction (P>0.05). Horizontal bar of base of graph represent day (white) and night (Black).

5.2 ARTIGO II

OBESITY MODEL REVERTED STRESS INDUCED HIPERALGESIA AND INDUCED ANXIETY-LIKE BEHAVIOR

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Conflict of Interest: There were no financial interests between the authors or commercial interests in the outcome of this study.

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Running Head: Chronic stress plus hypercaloric diet.

Abstract

Studies suggest that chronic stress is associated with anxiety symptoms. These symptoms are influenced by hormonal alterations that result from hypothalamic-pituitary-adrenal (HPA) axis activation. It is also a medical condition linked to chronic stress exposure and abnormalities of food consumption. Obesity is a chronic low inflammation disease that has endocrine changes that can lead an increase or decrease in pain threshold. In the present study we induced obesity using hypercaloric diet associated with chronic stress exposure and we evaluated the nociceptive and inflammatory pain responses (tail flick and formalin tests, respectively), locomotion and like-anxiety behavior (Open field [OP] and Elevated Plus Maze tests [EPM] respectively) and evaluated stress parameter (relative adrenal weight, corticosterone serum levels) and obesity parameter (Delta weight and Lee Index). Was used 96 male Wistar rats at 60 days old were divided into 4 groups: diet standard (C), hypercaloric diet (cafeteria diet) (HD), chronic stress (chronic stress by restricting 1h/day/5 days a week between 9am and 12pm for 80 days) associated with the standard diet (S), chronic stress associated with hypercaloric diet (SHD). The groups will have their behaviors analyzed and will be dead in three hours of the day: 7h, 19h and 1h [Zeitgebers (ZT) 0: time at wich the light is turned on vivarium and ZT12, time the light is turned off vivarium, respectively and ZT18]. In the OF the stressed animals showed longer latencies to leave the first quadrant. Furthermore obese groups and showed an increase in the outer crossings: these suggest a decreased in the levels of anxiety-like behavior. We observed a significant interaction in the inner crossings, between chronic stress exposure and obesity. In the EPM there is a decrease in time spent in the open arms for stress group indicating an increased in the levels of anxiety-like behavior in these animals. And, both chronic stress and obesity influenced rearing independently, and the obesity prevents the effects of chronic stress. Also in Tail flick test stressed group showed hyperalgesia. On the other hand our results indicate that obesity

reverted stress-induced-hyperalgesia. Based on these results, it is suggested that anxiety-like behavior presented by rats exposed to chronic stress and/or hypercaloric diet (obese rats) can be due to alterations on hypothalamic-pituitary-adrenal (HPA) axis, which may in turn result in behavioral changes responses and eating disorders.

Keywords: Chronic stress, Hypercaloric diet, Hypothalamic-pituitary-adrenal axis, Nociception, Tail flick, Formalin test, Open field test, Elevated Plus Maze test, rats.

Introduction

Stress is a response by the body against insults that may disrupt homeostasis; it involves changes in physiological and neurochemical factors. It is also known that stress produces behavioral changes that enable the individual to cope with new situations (1). Eating patterns can be modified for repeated episodes of chronic stress (2); and glucocorticoids (GCs) have been associated with increased palatable food intake (3). Exposure to stressors stimulates the hypothalamic-pituitary-adrenal (HPA) axis, releases corticotropin-releasing hormone (CRH), adrenocoticotropin (ACTH), and increases circulating GCs secreted by the adrenal cortex (4). The consequences of repeated stress exposure may affect both the peripheral and central nervous systems (CNS) (5, 6). Chronic stress can promote overeating, which, in turn, can elevate cortisol, glucose, and insulin levels, and consequently weight gain (7, 8). Growing evidence suggests that abnormalities in cortisol secretion have been extensively examined in relation to human obesity (9). The exposure to stress and/or elevated stress hormone levels is also known to alter learning, memory, and emotional responses (10, 11).

Studies also show that the areas in the nervous system that are associated with cognitive and emotional aspects of eating behavior, such as the effects of reward and motivation, are activated during stress situations (12, 13). The model of reward-based eating has been suggested as a means to reduce stress response. Emotional changes can influence feeding behavior and it reflects the complex functioning of a psychobiological system organized in different levels (13, 14). In addition, palatable foods that are rich in fats and carbohydrates, known as “comfort food,” decrease stress response (15). Studies with humans show a prevalence of symptoms of anxiety in patients with eating disorders. For example, patients suffering from mood disorders presented HPA axis deregulation as the main pathophysiological alteration (16). Interestingly, altered emotional and mood states, including anxiety, affect eating

behavior and food choice (3). The consumption of comfort food evokes a psychologically comfortable and pleasurable state (14). Studies show that most subjects report a preference for sweet, high-fat foods in response to negative emotions (17); fat intake has been shown to decrease anxiety-like behavior and facilitate recovery from stress (15, 18).

In addition to these disorders the exposure to acude and chronic stress also can lead to changes in pain threshold (19). Pain is an adaptive response to aversive situations that threaten in the physical integrity of the organism, which is influenced by GCs (20). Unlike acute stress, which has been associated with a reduction in pain sensitivity (19), chronic stress has been associated with increased sensitivity to pain, producing hyperalgesia (decrease pain threshold) (21-23) and allodynia (pain induced by no noxious stimuli) (23). Several studies correlate the dysfunction of the HPA axis and multiple neurotransmitter systems including opioid, serotonergic, purinergic and noradrenergic systems, by modulating the nociceptive response (22, 24, 25).

Considering the stressful characteristics of modern lifestyle, and that exposure to stress can promote the development of physiological and behavioral alterations which, in turn, are associated with changes in eating habits that may lead to obesity, the goal of the present study was evaluated the nociceptive and nociceptive behavior of inflammatory pain responses (tail flick and formalin tests, respectively), exploratory behavior/locomotion and like-anxiety behavior (Open field and Plus maze respectively).

2. Materials and methods

2.1 Animals

The experiments were carried out on 60 day-old male Wistar rats (at the beginning of the treatment, weighting 200 - 250 g) randomized by weight and housed in Polypropylene material cages (49 x34x16cm). All animals were kept on a standard 12-hour light/dark cycle (lights on at 7.00 a.m. and lights off at 7.00 p.m.), in a temperature-controlled environment (22±2°C), and had access to water and chow *ad libitum* (hypercaloric diet and/or standard rat chow). All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. 100382) and were compliant with Brazilian guidelines involving use of animals in research (Law No. 11,794). Vigorous attempts were made to minimize animal suffering and decrease external sources of pain and discomfort, as well as to use only the number of animals that was essential to produce reliable scientific data.

2.2 Experimental Design

Rats were habituated to the maintenance room for one week before the beginning of the experiment. Animals were divided into two groups each one: control and stress model. Each group was subdivided into two additional subgroups: standard food (control - C, control stress - S) or hypercaloric diet (hypercaloric diet - HD and restraint stress plus hypercaloric food – SHD), according to chronic stress exposure and type of diet used (hypercaloric diet and/or standard chow). After 80 days of experiment, the animals were exposed in exploratory behavior, locomotion and like-anxiety behavior (open-field and plus-maze), nociceptive behavior tests (tail flick and formalin test) and after the death of the animals the adrenals were weighed and blood collected subsequent analysis.

2.3 Stress procedure

Rats were subjected to a chronic restraint stress model (2). The model was implemented using a plastic tube (25 x 7cm) adjusted to avoid discomfort but that limited the movements of the animal; the front of the tube was opened to allow breathing (26). The animals were stressed in the morning (between 9 am and 12 pm) one hour/day, five days/week for 80 days (2). After the stress procedure, animals were returned to their home cages. Control animals were kept in their home cages when experimental rats were subjected to stress. The apparatus was ventilated and did not cause physical compression in order to avoid hyperthermia and sweating.

2.4 Experimental diets

The standard diet Nuvilab CR-1 (NUVITAL ®) is composed of 55.0% carbohydrates, 22.0% protein, 4.5% lipids and other constituents (fiber and vitamins); it totals 2.93 kcal/g (information provided by manufacturer). A palatable high calorie diet (cafeteria diet) consists of about 60.0% carbohydrates, 20.0% lipids, 15.0% protein and 5.0% other constituents (sodium, calcium, vitamins, preservatives, minerals etc.) for a total of 4.186 Kcal/g and 0.42 kcal/mL (calculation based on information provided by the manufacturer on the package label). The experimental diet used was the Western diet (or palatable hyperlipidic diet) previously described by Estadela, 2004 (27). The standard diet and the experimental diet were replaced daily with fresh food. The animals administered the hypercaloric diet also had access to standard food and water.

2.5 Exploratory behavior, locomotion and like-anxiety behavior:

2.5.1 Open field test (OF)

The behavioral assessment was performed in a varnished wood cage, measuring 60 cm x 40 cm x 50 cm and with the inside lined with glass. The floor was covered with linoleum and divided up with dark lines: 12 squares of 13 cm x 13 cm each. The rats were gently placed in the left back corner and allowed to explore the surroundings for five minutes (29, 30). The number of line crossings of each animal was taken as a measure of locomotor activity (31); the latency to leave the first quadrant was taken as a measure of anxiety (32). Rearing was defined as the moment the rat rose up on its hind legs, ending when one or both front paws touched the floor again (33); and it was evaluated as exploratory activity (34). Grooming was defined as licking/washing of the head and body; it was assessed as a biological function of caring for the surface of the body (35). The start of a trial occurred immediately after the rat was placed in the environment for scoring purposes. In this test, the animal was recorded as entering a new area when all four paws crossed the boundary into a different, marked-out area. Five measures were taken during the five-min test sessions: latency to leave the first quadrant (time in seconds); number of line crossings (i.e. horizontal activity), outer and inner crossings; grooming (time in seconds); number of rearing behaviors (i.e. vertical activity); and number of fecal boluses. The box was cleaned between each trial.

2.5.2 Elevated plus-maze test (EPM)

The elevated plus-maze test was used to evaluate anxiety-like behavioral state. The maze was made of black PVC and elevated to a height of 50 cm above floor level. The apparatus included two open arms and two closed arms (50 cm x 40 cm x 10 cm), which extended from a common central platform (10 cm x 10 cm). The animal was

placed in the central area of the EPM, facing one of the open arms. Next, the following behavioral measures were recorded during the five-min test sessions: number of protected head-dipping movements (PHD); number of non-protected head-dipping movements (NPHD); number of entries in the open arms (EOA); number of entries in the closed arms (ECA); time spent on the open arms (TOA); time spent on the closed arms (TCA); time of grooming; number of rearings; and number of fecal boluses. Protected head dips involved dipping the head over the sides of the maze from within the central platform or a closed arm, whereas unprotected head dips were considered to occur when the animal dipped its head over the sides of the maze while on an open arm. In the EPM, entering a new area was recorded when all four paws crossed onto a new arm or into the central area (28). After each test, the apparatus was cleaned to remove any animal scent.

2.6 Effect of chronic stress and/or obesity in nociceptive behaviors

2.6.1 Tail flick latency (TFL)

The nociceptive response was evaluated using the tail-flick test (TFL) (29). Each animal was placed on the apparatus and its tail laid across a nichrome wire coil; the coil was heated by an electric current. The light source positioned below the tail was focused on a point 2–3 cm rostral to the tip of the tail. The equipment was calibrated to collect three consecutive baseline tail-flick latencies, between three and five seconds. If at any time the animal failed to flick its tail before the temperature reached 75 ◦C, the tail was removed from the coil to prevent damage to the skin. Three TFL baseline measures were taken at three-min intervals. Animals were familiarized with the TFL apparatus 24h before the test session; the novelty of the apparatus itself may induce antinociception (30).

2.6.2. Formalin test

The formalin test was performed as previously described (31) with minor modifications. Twenty-four hours before the test, each animal was placed in the chamber for 10 min to familiarize them with the procedure, since the novelty of the apparatus itself can induce antinociception (32). The animals were injected s.c. on the plantar surface of the left hindpaw with (0.17 ml/kg of a 2%) formalin solution (Formaldehyde P.A.®, obtained from Sigma-Aldrich, São Paulo, Brazil) diluted in 0.9% NaCl (saline). Each animal was observed in a varnished wood cage, measuring 60 × 40 × 50 cm, with the inside lined with glass, and the nociceptive response was recorded for a period of 30 min. This test produces two distinct phases of nociceptive behavior: an early, transient phase (phases I; up to 5 min after the injection) and a late, persistent phase (phase II; 15–30 min after the injection). Phase I has been considered to reflect direct stimulation of primary afferent fibers, predominantly C-fibers (neurogenic pain) (33) whereas phase II is dependent on peripheral inflammation (inflammatory pain) (34-36). The total time (seconds) spent in licking, biting, and flicking of the formalin-injected hindpaw was recorded in phases I and II. The test was performed once only in each rat.

2.7 Characterization of obesity

In order to characterize the model for obesity were analyzed weekly weight, the delta weight and Lee index. The Lee index was calculated as the cube root of body weight (g)×10/naso-anal length (cm), this index was adapted of Moura and Cols (37), and delta weight of the animals was calculated by difference of the initial weight and final weight at the experiment.

2.8 Characterization of stress

2.8.1. Biochemistry parameter: Blood Serum Assay

The rats, individually, were quickly transferred to a separate room and decapitated within 1 min; the trunk blood was drawn and blood samples were centrifuged in plastic tubes for 5 min at 5000 x g at room, serum was obtained and frozen at −80 °C until the assays perform. The levels in the corticosterone blood serum samples were determined using a commercially available Enzyme-linked immunosorbent assay (ELISA) (MP Biomedicals) (Uscnk, Life Science Inc.) according to the manufacturer's instructions. The data were expressed by pg/ml.

2.8.2. Relative weight of the adrenal

Adrenal glands were carefully dissected and weighed. The adrenals were weighed on a precision balance using a scale with a precision of 0.0001 g (38).

2.9 Statistical analysis

All results are expressed as mean ± standard error of the mean (S.E.M). Data and interactions were evaluated using two-way ANOVA (hypercaloric diet or obesity and stress); followed by Bonferroni test for multiple comparisons. Differences between groups were considered significant when $P < 0.05$.

3. Results

3.1 Effect of chronic stress and/or obesity in other behavior parameters

3.1.1 Open-field tests (Table 1)

Two-way ANOVA showed effect of chronic stress ($F_{(1,\ 48)}$=10.395, P=0.002), with a significant increase in the latency to leave the first quadrant; however, our results did not show significant effects of obesity (P>0.05) or interaction of two independents factors (P>0.05). There was a significant effect of obesity in outer

crossings ($F_{(1, 47)}$ = 25.250; *P*=0.0001), without effect of chronic stress exposure (*P*>0.05) or interaction between chronic stress and obesity ($F_{(3, 97)}$=0.303, *P*>0.05). Our results also show an interaction between chronic stress and obesity ($F_{(3, 97)}$=3.999, P<0.05) in inner crossings, but no effect of repeated stress exposure (*P*>0.05) or obesity (*P*>0.05). Total number of crossings showed a significant effect of hypercaloric diet ($F_{(1, 47)}$=24.181, P=0.001). Chronic stress exposure produced a marginally significant effect in total crossings ($F_{(1, 48)}$ = 3.335, *P*=0.07), with no interaction between chronic stress and obesity (P>0.05). There were no effects of chronic stress or obesity in rearing, grooming, and fecal boluses (*P*>0.05 for all).

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insert table 1

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3.1.2 Elevated Plus-Maze test (Table 2)

The two-way ANOVA showed effect of chronic stress in TOA ($F_{(1,49)}$=4.267, *P*<0.05), without effect of obesity or interaction between stress and obesity (*P*>0.05 for both). There were effects of chronic stress ($F_{(1,49)}$=6.212, P=0.02) and obesity ($F_{(1,48)}$= 5.714, *P*=0.02) in rearing, but no interaction between stress and obesity (*P*>0.05). There were no significant effects for chronic stress or obesity in PHD, NPHD, EOA, ECA, and TOA (*P*>0.05 for all parameters).

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insert table 2

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3.2 Effect of chronic stress and/or obesity in nociceptive behaviors

3.2.1 Tail flick test

In the tail flick test the two-way ANOVA showed effect of stress ($F_{(1,30)}$= 11.093, P=0.002) and interaction of diet and stress ($F_{(3,46)}$= 4.790, P=0.037). However, the two-way ANOVA showed no without effect of diet (P>0.05) (Fig. 1).

3.2.2 Formalin test

In the phase I of formalin test the two-way ANOVA showed without effect of stress ($F_{(1,14)}$= 0.670, P>0.05); without effect of obesity ($F_{(1,14)}$=0.001 , P>0.05) and without interaction between stress and diet factors ($F_{(1,14)}$= 1.743, P>0.05) (Fig. 2 A). In phase II the two-way ANOVA showed without effect of stress ($F_{(1,15)}$= 2.689, P>0.05); and without effect of obesity ($F_{(1,15)}$= 1.379 , P>0.05) and without interaction between stress and obesity ($F_{(1,15)}$= 0.214, P>0.05) (Fig.2 B).

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insert figure 1, 2 A and 2 B

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3.3 Characterization of obesity (weight parameters)

The weight parameters were used to confirm the presence of obesity induced by hypercaloric diet. The two-way ANOVA indicated effect of hypercaloric diet ($F_{(1,49)}$= 6.331, P=0.014) in the Lee index, but without effect of chronic stress ($F_{(1,49)}$= 0.095, P>0.05) or interaction between these independent factors ($F_{(3,99)}$= 2.920, P>0.05). The groups that consumed hypercaloric diet showed an increase in the Lee index (Fig. 3 Panel A).

For the delta weight the two-way ANOVA showed effect of chronic stress ($F_{(1,50)}$=7.107, P<0.009), and of hypercaloric diet ($F_{(1,48)}$=12.207, P<0.001) but not showed interaction between these factors ($F_{(1,99)}$= 0.436, P>0.05). The groups that consumed a hypercaloric diet obtained greater weight gain than animals that consumed standard diet. On the other hand, the stressed groups showed less weight gain (Fig. 3 Panel B).

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insert figure 3 , panel A and panel B

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3.4 Characterization of Stress

3.4.1 Corticosterone

The results of corticosterone serum levels measures are show in figure 4 Panel A. The two-way ANOVA indicated effect of stress ($F_{(1,88)}$=45.173, P=0.08) and without effect of obesity (*P*>0.05) or interaction between chronic stress and obesity (*P*>0.05).

3.4.2 Relative adrenal weight

We evaluated relative adrenal weight, expressed as adrenal weight to body weight ratio. Exposure to stress caused an increased ratio ($F_{(1,30)}$ = 5.288, P = 0.029); additionally, there was an effect of obesity ($F_{(1,30)}$ = 6.197, P = 0.019) and a significant interaction between stress and diet ($F_{(3,62)}$ = 4.182, P < 0.05), since hypercaloric diet exposure was able to prevent the increased adrenal weight/body weight ratio (Fig. 4 panel B).

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insert figure 4, panel A and panel B

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4. Discussion

The study shows behavioral alterations in male Wistar rats submitted to chronic stress associated with a hypercaloric diet. Exposure to stress induced an increase of latency to leave the first quadrant in the OF test; this result was associated with a decrease in time spent in the open arms during the EPM test. This type of behavior in the test has been associated with anxiolytic-like behaviors. Studies indicate a strong correlation between stressful events and the development of behavioral abnormalities, such as anxiety disorders (39-41); these results show that there is an association between chronic stress and anxiety-like behavior (1). Therefore corroborating other studies that show that chronic activation of the HPA axis is associated with anxiety (1, 40, 42). When facing challenging or stressful situations, rats generate both an endocrinological and a behavioral response that help to cope with the situation (4). We can suggest that our results reflect an active coping strategy in response to stress-induced activation of the HPA axis. The alterations of the HPA axis may produce changes in psychological, emotional, and behavioral responses (4, 10, 11, 18). Moreover, deregulation of the HPA axis is implicated in the pathogenesis of eating disorders (43); both deregulation and the pathogenesis of this disorders can be influenced by other factors, as hypercaloric diet (16, 40, 44). Elevated GCs levels can promote CRH release in the central nucleus of the amygdala, which is reduced by consumption of comfort foods. This behavior constitutes a negative feedback loop: stress leads to the selection and ingestion of hypercaloric foods, which in turn reduce behavioral response of the stress (3, 45).

The present study also showed that hypercaloric diet intake produced an increase in locomotion (i.e. increase in total crossings); and this effect was associated with an increase in external crossings in the OF test. Studies have suggested that rats eating a high-fat diet for two to three months have reduced sympathetic responses to stressors in comparison with animals eating high carbohydrate diets (18). Short-term exposure to a diet rich in fat also reduced the level of anxiety in the EPM test (i.e. low time in spend in closed arms (46). These studies suggest that the type of diet, for example, high-calorie diet or comfort foods, may alleviate stress response; they also show that stress-related behavior may be dependent on the type of stress, chronic or acute (47). It is our understanding that the availability of a hypercaloric diet associated with the choice between varieties of foods may have influenced the behavior of the animals.

Our results also show that there was an interaction of hypercaloric diet and stress exposure in the number inner crossings. This result indicates an effect in anxiety-like behavior when two factors were associated; it is possible that consumption of high calorie diet resulted endocrine components of the model of chronic stress used in this study. Moreover, a low level of corticosterone has been reported by studies that investigated stress and hypercaloric diets (3, 40). Female rats exposed to stress plus chocolate intake showed an increase in the number of inner crossings. This increase was associated with the fact that palatable intake counteracts the effects of stress (48). The effect of stress can be influenced by high-calorie diets (15, 48) and a hypercaloric diet may minimize the effects of stress (45). Thus, these results support the hypothesis that the stress exposure results in increased consumption of preferred diets, especially those that are high in calories. Some studies have demonstrated that the possibility of choosing the diet high in calorie can significantly dampen the HPA response to stress (45, 49).

Furthermore, our results suggested chronic ingestion and chronic stress exerts profound effects on the explorative behavior of rats. The SHD group that was exposed both the hypercaloric diet and stress increased the number of rearings, thus showing a recovery of the exploratory activity and curiosity about the environment. It has been suggested that this behavior is associated with emotional factors (50); additionally, suggested that reflect motor activity in animal models (51).

Interestingly, stress response is not only a result of GCs release, since several other systems may be involved. Additional possibilities should be taken into consideration in the complex feeding response seen after stress (52). It is well documented that the central dopaminergic and serotoninergic nervous systems are closely as associated with psychiatric disorders (53). A number of animal studies indicate that exposure to stress can alter the activity of the neuroendocrine and neurotransmitter systems that affect behavior. Exposure to stress in rodents sometimes induces emotional or psychic states including anxiety and enhanced fear (54, 55). Chronic stress results in reduced dopaminergic transmission in the prefrontal cortex (PFC), which may cause cognitive deficits (56); chronic stress also reduces serotoninergic transmission in the PFC (57). It is important to note that the chronically stressed rats showed deregulation of the HPA system (58). Others have shown decreased hippocampus, increased dopamine (amygdala and hypothalamus) and decreased serotonin (hippocampus and cerebral cortex) after exposure to chronic restraint stress (59). The results suggest that chronic restraint stress differentially affects the activity of central dopaminergic and serotonergic system; the different effects may be related to the effects observed in behavior. The serotoninergic system is involved in anxious and obsessive behaviors and impulse control (60, 61). Studies in animals and humans show that alterations of the 5-HT-1A receptor functions may play a role in anxiety (62), impulse control (63) and feeding behavior (64).

Studies in genetically modified lines of animals, specifically serotonin transporter and 5-HT-1A receptor knock-out mice, show impaired exploration; this behavior might be attributed to 5-HT-1A deregulation and increased levels of anxiety (65). Though several neurotransmitters have been associated with psychological disorders, it is well known that the dopaminergic and serotoninergic systems play an important role in disorders. Our findings may provide a better understanding of the pathogenesis of emotional and eating disorders. In addition, the central serotonergic signaling system has been shown to play an important role in appetite control and the regulation of food intake. Serotonin exerts its anorectic effects mainly through the 5-HT-2C has been shown to significantly attenuate rodent bodyweight gain. This effect is strongly associated with marked hypophagia and is probably mediated by the hypothalamic melanocortin system (66, 67).

The HPA axis activation leads to the release of adrenaline, dampening pain processing and resulting in transient analgesia (68). This initial response is followed by the release of corticosteroids in order to re-establish physiological homeostasis (69). On the other hand, the effects of chronic stress upon pain perception are not consensual as both, inhibition (analgesia) and facilitation (hyperalgesia) of nociception has been reported in different contexts (70, 71). Corroborating this data, our results showed that the stressed animals presented decrease thresholds in the tail-flick test, suggesting higher sensitivity to this type of stimulus, but no effect in the formalin test. It is possible that the different response can be related to activation of different nociceptive fibers by evaluated by these tests, since they promote different response in the assessment of nociception. The formalin test involves tonic pain, a long-term stimulus, triggering the nociceptive response, which involves stimulation of C-type fibers (72). In opposite to this, the nociception assessed by tail-flick is related to the reflex of the spinal cord (73, 74), but remains under control of supraspinal structures(75). This test involves phasic pain, the noxious stimulus is related to a short

duration and a stimulation of minimal surface areas, and the pain threshold is the measure evaluated. The test involves thermal stimulation of Aδ fibers (72). Corroborating this, it is know that the hyperalgesia induced by chronic stress is the reflex of profound changes in the neurochemistry of the spinal dorsal horn and descending pain modulatory pathways (19). The HPA axis enhanced activity could contribute to the comorbidities associated with pain due to the detrimental effects of corticosteroid hypersecretion that occur during its activation.

Indeed, our results showed interaction between chronic stress and obesity on nociception. The hypercaloric diet exposure reverted the hyperalgesia induced by chronic restraint stress in the tail-flick test. Several studies described that individuals exposed to repeated stressful conditions show a decreased nociceptive threshold, known as chronic stress-induced hyperalgesia on several nociception tests (21, 76) which may involve a different neurochemical basis related to stress model severity. Chronic stress has been shown to affect brain activity and promote long-term changes in multiple neural systems (77), like serotonergic and noradrenergic systems (78-80). However, the mechanism by which chronic stress exerts hyperalgesic effects still remains unknown.

In the obesity the pain threshold (or perception) can increase or decrease according to some factors like gender, stress, depression or individual differences(81). Also, previous studies showed that pain threshold could change in obesity (82), or with high levels of GCs (83), but its effects on pain threshold are controversial (84, 85). In this study we showed that obesity prevent the chronic restraint stress-induced-hyperalgesia in rats. This indicates a relationship between chronic restraint stress and chronic consumption of hypercaloric diet (or obesity consequently) related to mechanisms of pain modulation in CNS. The modulatory role of palatable diet such as hypercaloric diet on nociception may be an attribute of its hedonic qualities directly (86)

and/or indirectly through their action on endogenous opioid peptides in the hypothalamus (87).

In this study we observed that stressed groups had a lower weight gain and delta weight in the experiment. This result agrees with previous studies that the restraint stress protocol was able to inhibit weight gain independently of type of diet (88, 89). The chronic stress inducing weight loss is very frequent often but not always this is associated with reduction in the food intake (90, 91). This observation corroborates other studies that showed lower weight gain in animals exposed of chronic restraint stress related to GCs high levels (15). These GCs influence a variety of physiological functions responses, including food intake, body weight and energy metabolism (3). In the fact, these hormones are involved in lipid metabolism, and correlate with unpredictable life situations such as energy intake shortages (92). Food consumption was not measured in this study, but it has been suggested that increasing the activity of CRH induced anorexic effects in the CNS. Thus, we can suggest that chronic stress leading to a reduction on body weight can be involved to negative energy balance (93).

On the other hand, the hypercaloric diet used in this study was efficient in promoting obesity, since presented increased in the body weight delta and in Lee index in the animals. It has previously been reported that hypercaloric food contains a high density of calorific energy, thus the diet is the actual factor predisposing to weight gain in many animal studies (18, 89). The hypercaloric diet used in this study to promote experimental obesity has been presented as food *in natura*, better known as cafeteria diets or comfort foods (88, 94). These kinds of diet are highly energetic, tasty and contain different shapes and are closer to the food consumed in general by humans.

Moreover the hypercaloric diets are characterized by increased energy density and palatability, they frequently lead to higher energetic intake (95). It was already

reported that in chronic stressed animals the hypercaloric diet consumption did not increase body weight, but it induced an increase in abdominal fat stores (89). Consequently, our study corroborate others studies that showed stressed rats exposed to the hypercaloric diet showed lower weights regarding to animals that received only the hypercaloric diet (15, 40). According to Macedo *et al.* (2012) we can also suggest that the effect of the cafeteria diet on the establishment of obesity was higher than the weight loss imposed by stress (96). The increase of body mass index (BMI- Lee Index) is considered a predisposing factor for metabolic syndrome and propensity to obesity (97, 98).

Additionally our data showed that exposure of chronic stress led to increase the relative adrenal weight in the stressed group, showed that chronic stress exposure had an impact on the activation of the HPA axis. And, interestingly hypercaloric diet prevented this effect on the adrenals weight of stressed rats. It agrees with the hypothesis that the palatable food is able to reduce the chronic stress effects in HPA activity (15, 45, 48). For example, Fachin *et al.* (2008) showed that the chronic consumption of chocolate was able to prevent adrenal hypertrophy in chronically restraint stressed rats (89). Several studies showed that chronic GCs secretion is correlated with hypertrophy of adrenals due to chronic activation of the HPA axis (3, 89, 99). This is considered an adaptive response to physical or psychological stress. Corroborating this finding the levels of corticosterone of stressed rats presented greater than the control group. However, this effect counter previous studies have been demonstrated that serum corticosterone levels in rats subjected to chronic stress do show a significant increase in comparison to control animals; however (88, 100). Thereby, we can suggest that this increased levels of corticosterone observed in our results, could be due to the 12 h fasting period of animals before the death, since it this situation could be considered as an additional stressor resulting in higher corticosterone levels in comparison with the responses during a chronic situation.

In conclusion, our study demonstrates that ingestion of hypercaloric diet, with consequently obesity, is able to prevent effects induced by HPA axis activation as, hyperalgesia, and like-anxiety behaviors supporting to the hypothesis proposed by Dallman *et al* (2003) of that the hypercaloric diet is able to reduce the effects of chronic activation of the HPA axis signs due fat deposits (3, 45, 101). On the other hand, previous studies have linked chronic stress and eating disorders (3, 45, 88, 102). Furthermore, this study supports that chronic stress and obesity may represent risk factors to metabolic diseases and psychological disorders. Future studies should evaluate the role of limbic (emotional and cognitive) areas, like the amygdala and hypothalamus (especially, the PVN) can elucidate on the mechanisms of emotional alterations associated with chronic stress and obesity.

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LEGENDS

Table 1

Behavior/group	C	HD	S	SHD
Latency (s)	7.320±1.727	7.167±1.763	14.960±1.727 [a]	10.720±1.727[a]
Outer crossing (n)	64.160±3.904	86.042±3.985[b]	59.840±3.904	77.400±3.904[b]
Inner crossing (n)	4.360±0.652	3.500±0.665	2.040±0.652	3.800±0.652[c]
Total crossings (n)	68.520±4.081	89.542±4.165[b]	61.880±4.081	81.200±4.081[b]
Rearing (n)	28.440±1.889	32.417±1.928	27.720±1.889	29.120±1.889
Grooming (s)	9.646±2.445	8.433±2.495	10.613±2.445	15.767±2.445
Fecal boluses (n)	3.400±0.570	2.083±0.582	2.880±0.570	2.200±0.570

Effects of chronic restraint stress and/or obesity upon behavior evaluated in the open-field test (n=24-25/group). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean for each behavior.

[a] Significant effect of chronic stress (two-way ANOVA, $P<0.05$).

[b] Significant effect of obesity (two-way ANOVA, $P<0.05$).

[c] Interaction between chronic stress and obesity (two-way ANOVA, $P<0.05$).

Table 2

Behavior/Group	C	HD	S	SHD
PHD (n)	2.591±0.463	2.583±0.443	2.240±0.434	1.500±0.426
NPHD (n)	2.591±0.831	3.417±0.796	2.600±0.780	1.385±0.765
EOA (n)	1.000±0.330	1.583±0.316	1.040±0.310	0.577±0.304
	(19%)	(25%)	(21%)	(13%)
ECA (n)	4.136±0.702	4.792±0.672	3.840±0.659	3.731±0.646
	(81%)	(75%)	(79%)	(87%)
TOA (s)	24±5.29	30.033±5.072	21.093±4.970[a]	12.320±4.873[a]
	(8%)	(10%)	(7%)	(4%)
TCA (s)	276±7.683	269.262±7.356	270.752±7.207	287.680±7.067
	(92%)	(90%)	(90%)	(95%)
Grooming (s)	16.118±5.233	21.673±5.011	11.915±4.909	22.937±4.814
Rearing (s)	15.909±2.364	18.250±2.263[b]	18.480±2.217 [a]	26.923±2.174 [ab]
Fecal boluses (n)	1.364±0.438	0.875±0.419	0.960±0.411	1.077±0.403

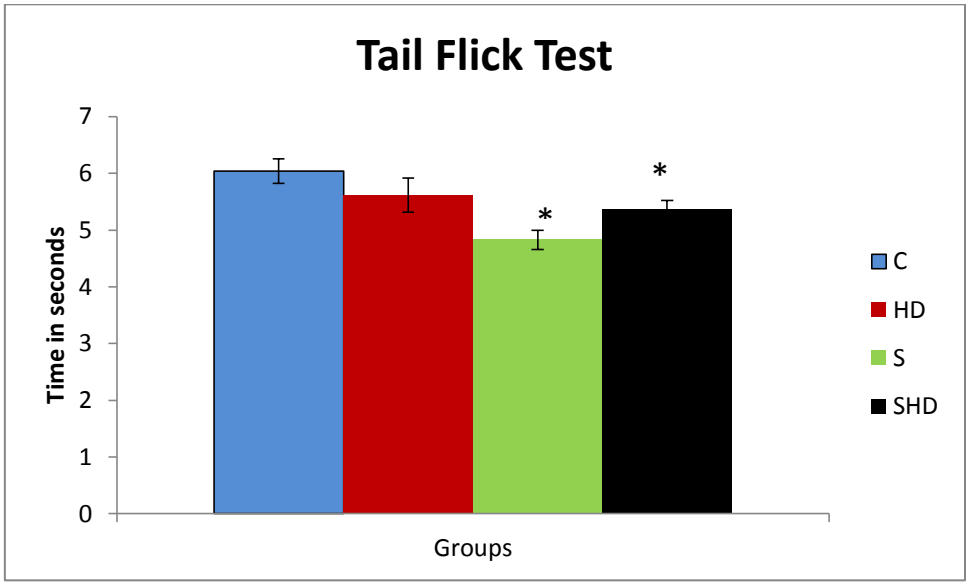
Effects of chronic restraint stress and/obesity on behavior evaluated in the elevated plus-maze test (n=22-26/Group). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Were evaluated number of protected head-dipping movements (PHD), number of non-protected head-dipping movements (NPHD), number of entries in the open arms (EOA), number of entries in the closed arms (ECA), time spent on the open arms (TOA), time spent on the closed arms (TCA), time of grooming, number of rearing and number of fecal boluses. Data are reported as mean ± std. error of mean for each behavior.

[a] Significant effect of stress variable (two-way ANOVA, $P<0.05$).

[b] Significant effect of obesity (two-way ANOVA, $P<0.05$).

Figure 1

TAIL FLICK



The Tail Flick test. Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean (N=16 for group). The two-way ANOVA showed interaction of chronic restraint stress and obesity ($P<0.05$).

* Significant effect of chronic restraint stress (two-way ANOVA, $P<0.05$).

Figure 2 A

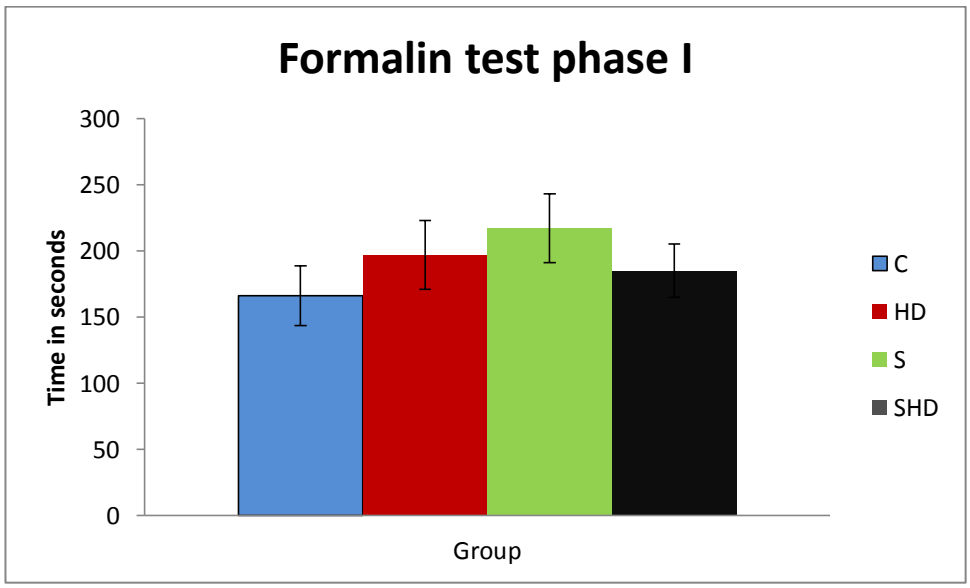


Figure 2 A. The formalin test phase I. Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean (N= 3/5 for group). The two-way ANOVA showed no difference between groups (*P*>0.05).

Figure 2 B

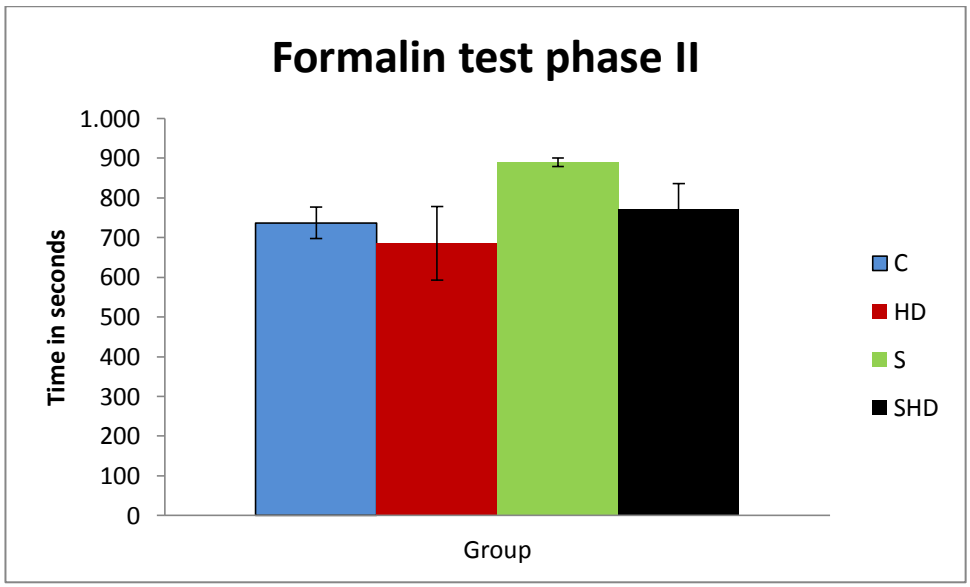
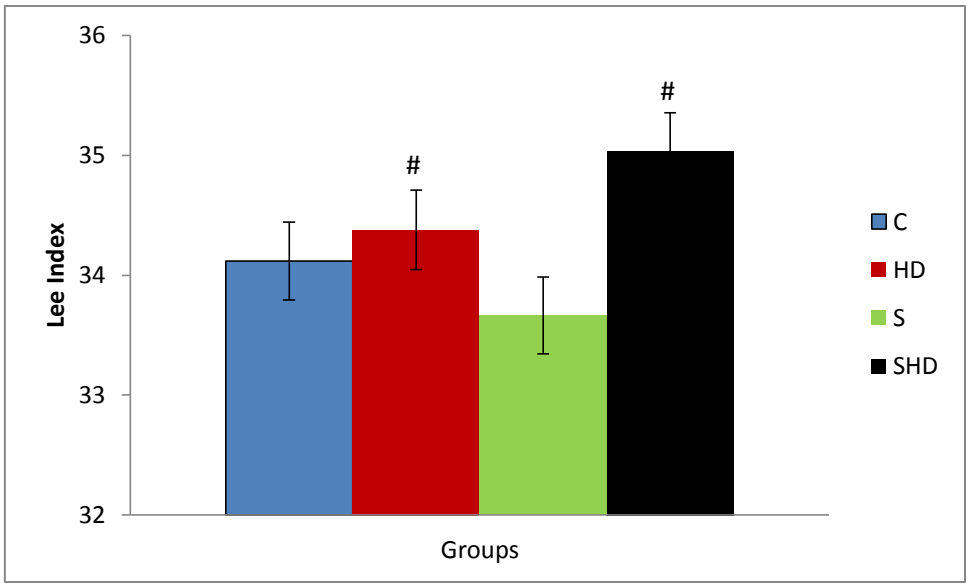


Figure 2 B. The formalin test phase II. Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean (N= 3/5 for group). The two-way ANOVA showed no difference between groups (*P*>0.05).

Figure 3 Panel A

LEE INDEX

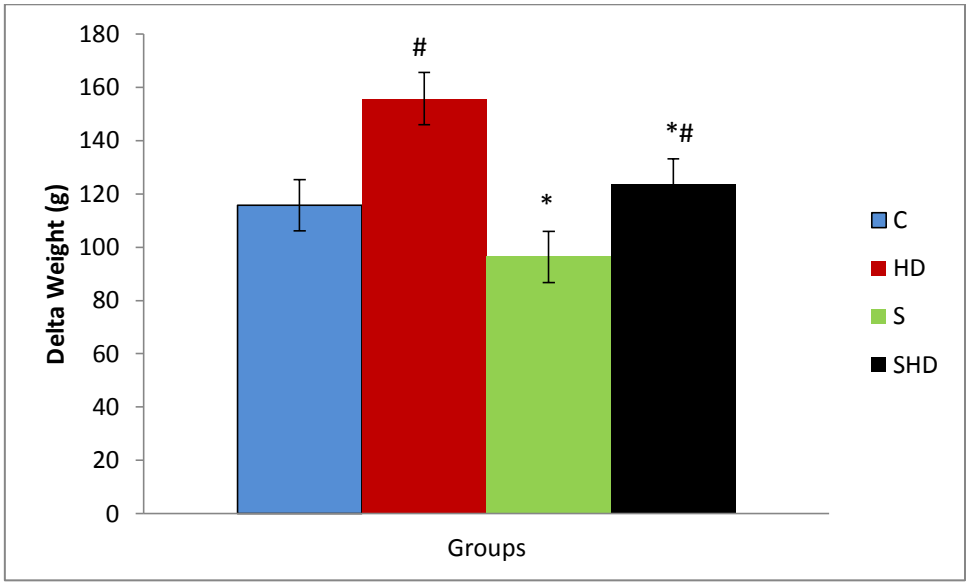


The Lee index was calculated in the end of experiment (N=24/26 for group). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean.

Significant effect of hypercaloric diet (two-way ANOVA, *P*<0.05).

Figure 3 Panel B

DELTA WEIGHT



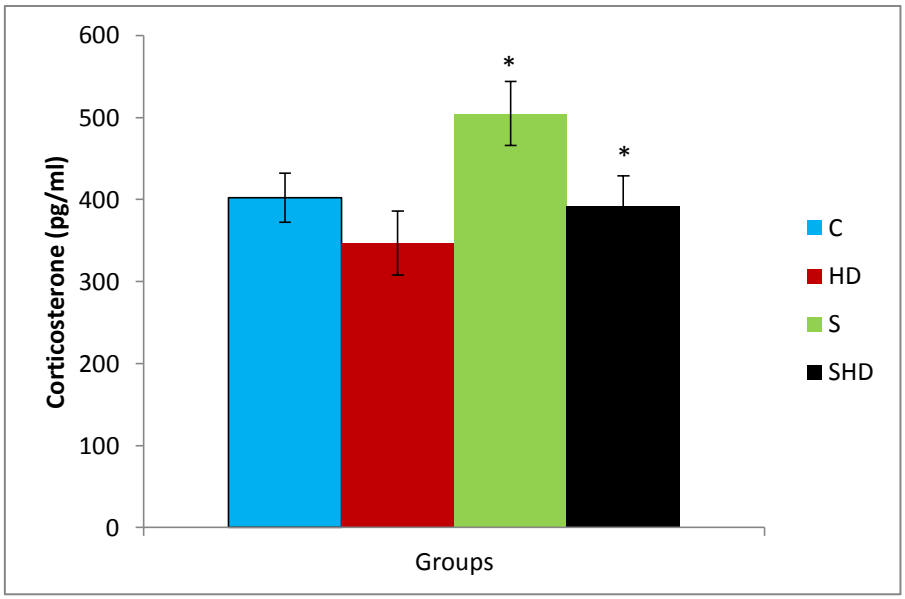
The delta weight was calculated subtracting the initial weight by the weight end of the experiment (N=25/26 for group). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean.

* Significant effect of chronic restraint stress (two-way ANOVA, $P<0.05$).

Significant effect of hypercaloric diet (two-way ANOVA, $P<0.05$).

Figure 4 A

PENEL A CORTICOSTERONE

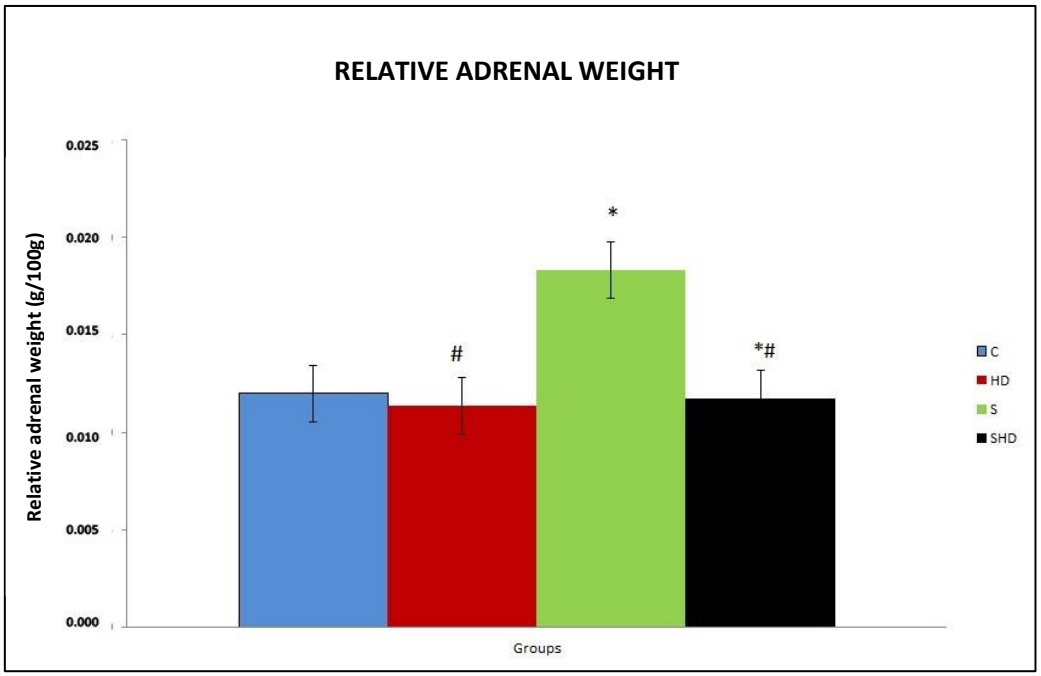


Plasma corticosterone levels after eighteen days of experiment (N=18/31 for group). Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean.

* Significant effect of chronic restraint stress (two-way ANOVA, $P<0.05$).

Figure 4 B

RELATIVE ADRENAL WEIGHT (g/100g)



The relative adrenal weight. Control group (C), hypercaloric diet group (HD), stress group (S) and stress and hypercaloric diet group (SHD). Data are reported as mean ± std. error of mean. The two-way ANOVA showed interaction between stress and obesity ($P<0.05$).

* Significant effect of chronic restraint stress (two-way ANOVA, $P<0.05$).

Significant effect of obesity (two-way ANOVA, $P<0.05$).

6 CONSIDERAÇÕES FINAIS

No presente estudo, observamos as alterações comportamentais, endócrinas e metabólicas associadas à obesidade (induzida por exposição à dieta hipercalórica) e/ou estresse crônico. Estes fatores estão presentes na vida moderna, e são relacionados a importantes alterações clínicas. Nossos resultados indicam que os animais estressados apresentam aumento de comportamento semelhante à ansiedade. Também, apresentaram alterações hipertrofia de adrenais devido à secreção crônica de glicocorticoides. Além disso, mostramos que consumo crônico de uma dieta hipercalórica induziu a obesidade e aumento na locomoção dos animais obesos indicativo de baixos níveis de ansiedade ambos os efeitos podem ser relacionados à diminuição na atividade do eixo HPA. Além disso, demonstramos que o consumo da dieta concomitante a exposição ao estresse foi capaz de prevenir a hipertrofia das adrenais e a hiperalgesia induzida por estresse crônico. Por outro lado o estresse crônico diminuiu o ganho de peso dos animais expostos à dieta hipercalórica. Adicionalmente, este estudo avaliou o padrão temporal dos hormônios corticosterona, insulina e grelina. Nossos resultados indicam padrão temporal para corticosterona e insulina, porem não foi capaz de demonstrar o padrão temporal da grelina nos horários do dia avaliados. Observamos que os níveis séricos de corticosterona sofreram efeito da exposição ao estresse crônico e interação entre dieta hipercalórica e ZTs. A obesidade alterou os níveis de insulina. Podemos concluir que, ambos os fatores, estresse crônico e obesidade exercem grande impacto no sistema endócrino estando envolvidos em alterações de comportamento.

7 Perspectivas Futuras

Como perspectivas futuras relacionadas aplicação do modelo de estresse por restrição e/ou dieta hipercalórica para à continuação deste estudo são as seguintes:

- Investigar os efeitos do estresse crônico e dieta hipercalórica sobre os sistemas neurotransmissores envolvidos na resposta nociceptiva.
- Investigar o padrão circadiano endócrino sobre o efeito da exposição do protocolo de estresse crônico e dieta hipercalórica em ZT0, ZT6, ZT12 e ZT18.
- Investigar as alterações envolvidas ao sistema opióide causadas pelo estresse crônico e dieta hipercalórica.
- Investigar as alterações e geração de radicais livres no sistema nervoso central devido à exposição ao estresse crônico e dieta hipercalórica.
- Avaliar a diferença entre os níveis de dopamina e serotonida em ratos Wistar expostos ao modelo de estresse crônico por restrição e dieta hipercalórica.

8 Divulgações e produção durante o período de mestrado

1. OLIVEIRA, C. M., Oliveira, Carla de, SCARABELOT, V. L., SOUZA, A., Macedo, I C, MEDEIROS, L. F., SILVA, F. R., Marques, P, Souza, I.C.C., Caumo, W, Torres, ILS; Avaliação do Padrão Temporal do Perfil Lipogênico e Glicemia em Ratos: Efeito da Obesidade Associada à Estrese Crônico In: XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2012, Águas de Lindóia - SP. XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE. , 2012.

2. SCARABELOT, V. L., Oliveira, Carla de, OLIVEIRA, C. M., SOUZA, A., Macedo, I C, MEDEIROS, L. F., ADACHI, L. N. S., SILVA, F. R., Marques, P, Souza, I.C.C., Torres, ILS; Chronic stress and/or hypercaloric diet: effects on behavior In: XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2012, Águas de Lindóia - SP. XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE 2012. , 2012.

3. Macedo, I C, Oliveira, Carla de, OLIVEIRA, C. M., ROZISKY, J. R., LASTE, G., MEDEIROS, L. F., SILVA, F. R., SANTOS, V. S., Torres, ILS; Chronic stress associated with hypercaloric diet on BDNF levels in hippocampus and olfatory bulb of wistar rats In: XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2012, Águas de Lindóia - SP. XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE 2012. , 2012.

4. Oliveira, Carla de, OLIVEIRA, C. M., SOUZA, A., Macedo, I C, SCARABELOT, V. L., MEDEIROS, L. F., Souza, A.C., SILVA, F. R., Marques, P, Souza, I.C.C., Caumo, W, Torres, ILS; Effect of Obesity Plus Chronic Stress on 24-Hour Pattern of Circulating Adiponectin and Leptin in Rats In: XXVII Reunião

Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2012, Águas de Lindóia - SP. XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE. , 2012.

5. CIOATO, S. G., Macedo, I C, Oliveira, Carla de, OLIVEIRA, C. M., ROZISKY, J. R., LASTE, G., Marques, P, Nonose, Y, Torres, ILS; Níveis de BDNF em hipocampo e bulbo olfatório de animais tratados com dieta de cafeteria e expostos ao estresse crônico por restrição In: 32ª Semana Científica do Hospital de clínicas de Porto Alegre, 2012, Porto Alegre. 32ª Semana Científica do Hospital de clínicas de Porto Alegre. , 2012.

6. DIEFENTHALER, F., OLIVEIRA, C. M., Oliveira, Carla de, SOUZA, A., MEDEIROS, L. F., Souza, A.C., SCARABELOT, V. L., ADACHI, L. N. S., Macedo, I C, Torres, ILS; Obesidade associada a estrese crônico: avaliação de parâmetros ponderais In: XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, 2012, Águas de Lindóia - SP. XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE 2012. , 2012.

6. Marques, P, OLIVEIRA, C. M., Oliveira, Carla de, Macedo, I C, SILVA, F. R., CIOATO, S. G., Nonose, Y, Torres, ILS; Efeitos da dieta Hipercalórica sobre o Comportamento de Ratos no Teste de Campo Aberto In: IV Semana Científica da UFCSPA e I Semana de Tecnologia e Inovação, 2011, Porto Alegre. IV Semana Científica da UFCSPA. , 2011.

9 ANEXOS

I - Aprovação do comitê de Ética

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão Científica e a Comissão de Ética no Uso de Animais (CEUA/HCPA) analisaram o projeto:

Projeto: 100382 **Versão do Projeto:** 19/11/2010

Pesquisadores:
IZABEL CRISTINA CUSTODIO DE SOUZA
LICIANE FERNANDES MEDEIROS
ISABEL CRISTINA DE MACEDO
CLEVERSON MORAES DE OLIVEIRA
WOLNEI CAUMO
CARLA DE OLIVEIRA
IRACI LUCENA DA SILVA TORRES

Título: IMPACTO DO ESTRESSE CRÔNICO ASSOCIADO A UM MODELO DE SÍNDROME METABÓLICA SOBRE O PADRÃO TEMPORAL DE MARCADORES HORMONAIS

Este projeto foi Aprovado em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais. Os membros da CEUA/HCPA não participaram do processo de avaliação de projetos onde constam como pesquisadores. Toda e qualquer alteração do Projeto deverá ser comunicada imediatamente a CEUA/HCPA.

Porto Alegre, 13 de dezembro de 2010.

Alessandro Bersch Osvaldt
Coordenador da CEUA/HCPA