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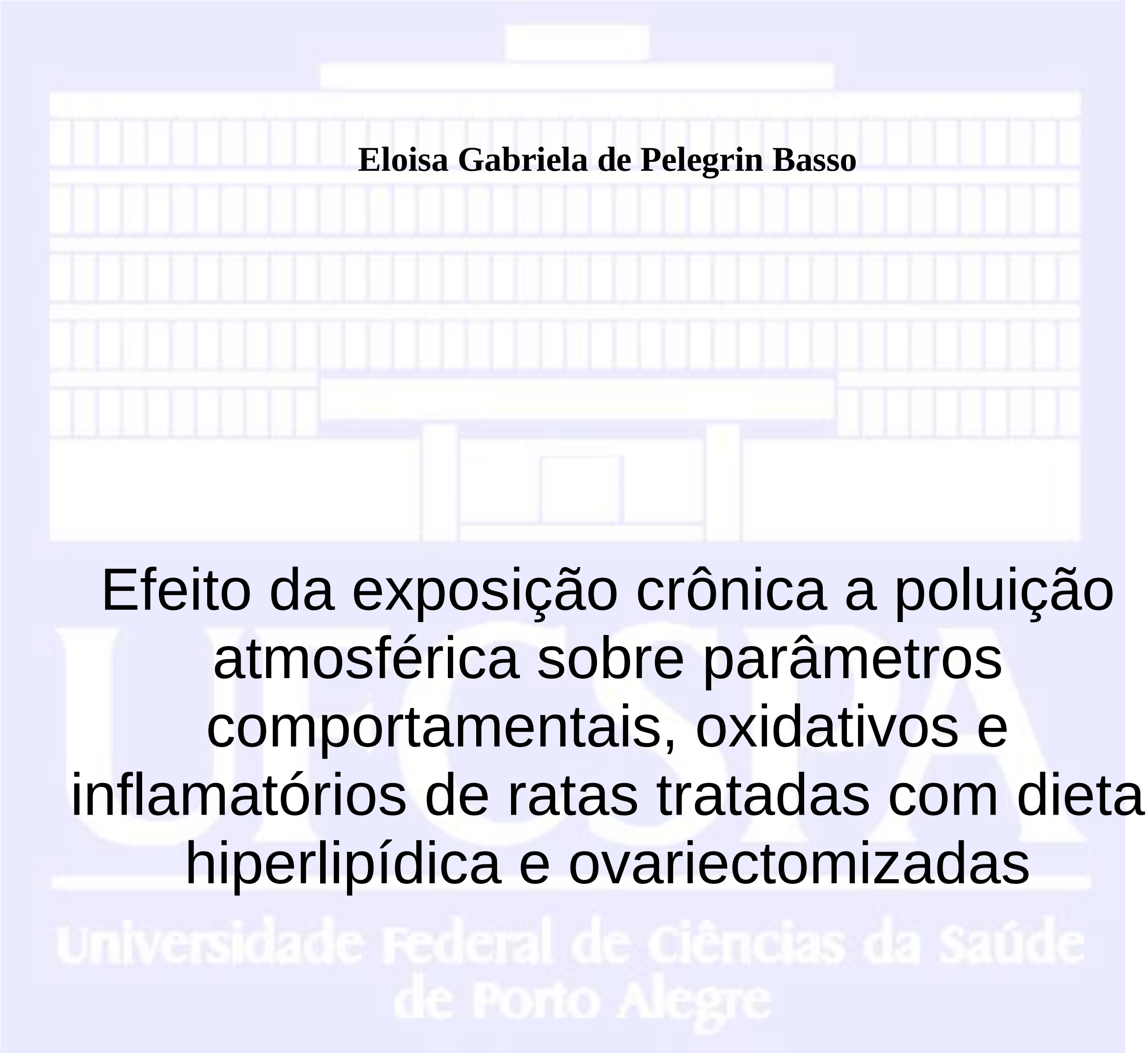

Eloisa Gabriela de Pelegrin Basso

Efeito da exposição crônica a poluição atmosférica sobre parâmetros comportamentais, oxidativos e inflamatórios de ratas tratadas com dieta hiperlipídica e ovariectomizadas

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre, como requisito parcial para a obtenção do título de Mestre em Ciências da Saúde.

Orientadora: Cláudia Ramos Rhoden
Co-orientador: Thiago Gomes Heck

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"Só espero que nunca percamos de vista uma coisa: tudo começou com um rato".

Walt Disney, 1954

RESUMO

A poluição atmosférica está associada a 7 milhões de morte anualmente. Num quadro pós-menopáusico, as mulheres quando expostas a poluição atmosférica, possuem maior incidência de doenças cardiovasculares e alterações comportamentais. Além disso, a obesidade também é um fator que interfere na qualidade de vida das mulheres e na pós menopausa, os efeitos da obesidade podem ser potencializados, causando alterações comportamentais e celulares, como o estresse oxidativo. Este estudo teve como objetivo avaliar os efeitos da exposição crônica a poluição atmosférica sobre parâmetros comportamentais, oxidativos e inflamatórios de ratas tratadas com dieta hiperlipídica e ovariectomizadas. Para isto foram utilizados 30 ratas Wistar fêmeas, alimentadas com DHL e distribuídos aleatoriamente em dois grupos que receberam instilação intranasal de 50 µL de solução salina diariamente por 12 semanas (representando um ambiente limpo) ou partículas totais em suspensão (PTS) em solução salina (250 µg, representando um ambiente poluído). Na 12° semana, as ratas foram ovariectomizadas (OVX) ou submetidas a cirurgia falsa (Sham) e continuaram a receber solução salina ou partículas por mais 12 semanas, formando assim os quatro grupos experimentais: DHL (n = 6), DHL+OVX (n = 9), DHL+Poluído (n = 6) e DHL+Poluído+OVX (n = 9). Na 24ª semana, foram realizados os testes comportamentais de Campo Aberto, Labirinto em Cruz Elevado e Nado Forçado e os animais foram eutanasiados. Os níveis séricos de citocinas (IL-6, IL-10) e proteínas de choque térmico de 70 kDa (eHSP70) foram mensurados (ELISA), e foram avaliados marcadores de estresse oxidativo (TBARS e SOD) no hipotálamo, hipocampo, córtex cerebral e cerebelo. O grupo DHL+Poluído+OVX apresentou maior ganho de massa corporal pós a ovariectomia, e também aumento na adiposidade (P = 0,003) e o comportamento tipo depressivo (P = 0,002) e diminuiu os níveis séricos de IL-6 (P = 0,038). A exposição crônica a poluição atmosférica promoveu comportamento depressivo em ratas fêmeas ovariectomizadas e obesas, independente de marcadores de estresse oxidativo e ou de inflamação.

Palavras-chave: Poluição do Ar. Dieta rica em gordura. Comportamento animal. Estresse oxidativo. Perfil inflamatório. Ansiedade. Depressão.

ABSTRACT

Air pollution is associated with 7 million deaths annually. In a postmenopausal picture, women exposed to air pollution have a higher incidence of cardiovascular disease and behavioral changes. In addition, obesity is also a factor that interferes with women's quality of life and menopause, the effects of obesity can be potentiated, causing behavioral and cellular changes such as oxidative stress. The aim of this study was to evaluate the effects of chronic exposure to air pollution on behavioral, oxidative and inflammatory parameters of rats treated with high-fat diet and ovariectomized. Thirty DHL-fed female Wistar rats were randomly assigned to two groups that received intranasal instillation of 50 µL of saline daily for 12 weeks (representing a clean environment) or total suspended particles (TSP) in saline (250 µg, representing a polluted environment). At week 12, the rats were ovariectomized (OVX) or sham surgery (Sham) and continued to receive saline or particles for an additional 12 weeks, thus forming the four experimental groups: DHL (n = 6), DHL+OVX (n = 9), DHL+Polluted (n = 6) and DHL+Polluted + OVX (n = 9). At week 24, the Open Field, High Cross Maze and Forced Swimming behavioral tests were performed and the animals were euthanized. Serum cytokine (IL-6, IL-10) and 70 kDa heat shock protein (eHSP70) levels were measured (ELISA), and oxidative stress markers (TBARS and SOD) were evaluated in the hypothalamus, hippocampus, cerebral cortex. and cerebellum. DHL + Polluted + OVX group showed higher body mass gain after ovariectomy, increased adiposity (P = 0.003) and depressive behavior (P = 0.002) and decreased serum IL-6 levels (P = 0.038). Chronic exposure to atmospheric pollution promoted depressive behavior in obese and ovariectomized female rats, independent of oxidative stress and / or inflammation markers.

Keywords: Air Pollution. High-fat diet. Animal behavior. Oxidative stress. Inflammatory profile. Anxiety. Depression

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

CO: Monóxido de Carbono
CONAMA: Conselho Nacional do Meio Ambiente
DNA: Ácido desoxirribonucleico
E1: estrona
E2: estradiol
E3: estriol
eHSP70: Proteína de Choque Térmico extracelular
EO: Estressse oxidativo
ERO: Espécie reativa de oxigênio
FAO: *Food and Agriculture Organization of the United Nations* – Organização das Nações Unidas para Alimentação e Agricultura
HPA: Hidrocarbonetos Policíclicos Aromáticos
HSPs: *Heat Shock Proteins* - Proteína de Choque Térmico
HSP70: Proteína de Choque térmico de 70 KDa
HSP72: Proteína de Choque térmico de 72 KDa
iHSP70: Proteína de Choque Térmico intracelular
MP: Material Particulado
MP_{10}: Material Particulado Grosso $< 10\ \mu M$
$MP_{2,5}$: Material Particulado Fino $< 2,5 \mu M$
$MP_{0,1}$: Material Particulado Ultrafino $< 0,1 \mu M$
NO_2: Dióxido de Nitrogênio
O_3: Ozônio
WHO: World Health Organization
ONU: Organização das Nações Unidas
OPAS: Organização Pan-americana de Saúde
PIs: Partículas Inaláveis
PTS: Partículas Totais em Suspensão
RE: Receptores estrogênicos
SNC: Sistema Nervoso Central
SO_2: Dióxido de Enxofre

SUMÁRIO

1 INTRODUÇÃO

1.1 Poluição atmosférica

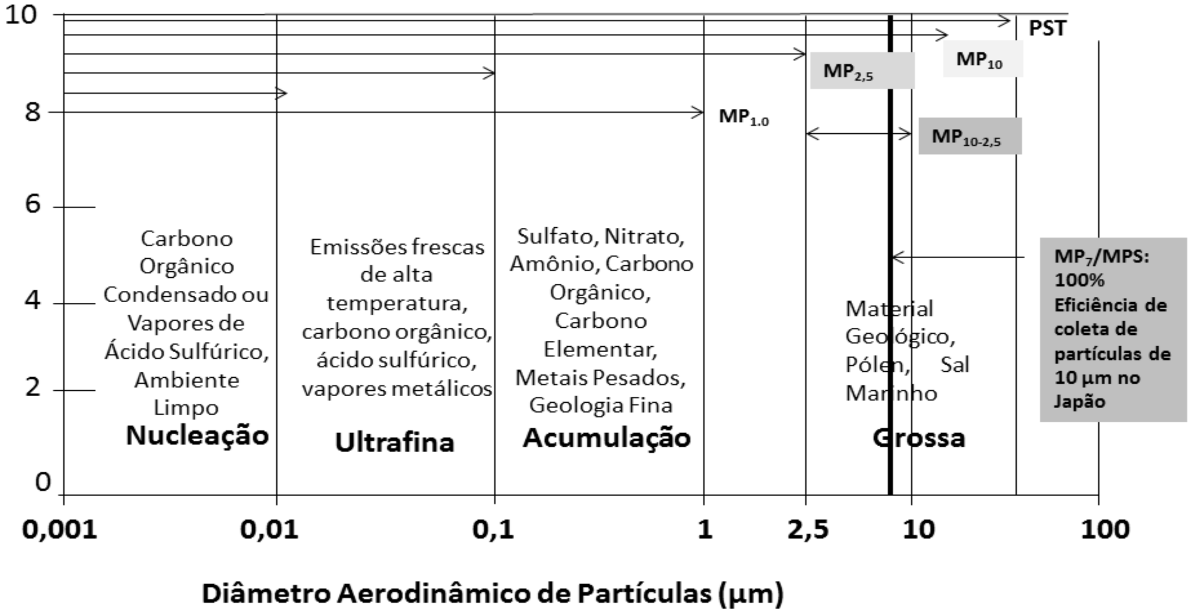
A poluição atmosférica está associada a 7 milhões de morte anualmente e 90% da população mundial está exposta ao ar poluído, conforme demonstra a Organização Mundial da Saúde (2018). Segundo a Resolução Nº 491/2018 do CONAMA, poluente atmosférico refere-se a qualquer forma de matéria em quantidade, concentração, tempo ou outras características, que tornem ou possam tornar o ar impróprio ou nocivo à saúde, inconveniente ao bem-estar público, danoso aos materiais, à fauna e flora ou prejudicial à segurança, ao uso e gozo da propriedade ou às atividades normais da comunidade (CONAMA, 2018).

Os poluentes do ar são classificados de acordo com sua fonte de emissão para a atmosfera. Existem dois tipos de fontes poluidoras, as fontes biogênicas e as fontes antropogênicas. As fontes biogênicas, ou naturais, são decorrentes dos eventos naturais do ambiente, como a atividade vulcânica, erosões, matéria orgânica em decomposição, queima da biomassa, desastres naturais, porém na maior parte das áreas urbanas as atividades humanas são as principais fontes emissoras de poluentes (fontes antropogênicas). Estas são oriundas da queima de combustíveis, processos industriais, minerações, meios de transporte e evaporação de combustíveis (CETESB, 2018).

Os principais poluentes encontrados na atmosfera são o material particulado (MP), o dióxido de nitrogênio (NO_2), o dióxido de enxofre (SO_2), os hidrocarbonetos policíclicos aromáticos (HPA), o monóxido de carbono (CO) e o ozônio troposférico (O_3). Os quatro primeiros (MP, NO_2, SO_2 e CO) são poluentes primários, pois são emitidos para a atmosfera diretamente de sua fonte de emissão. Entretanto o O_3 é um poluente secundário, que resulta da reação química entre poluentes primários e componentes naturais da atmosfera (CETESB, 2018). Dentre os poluentes, destacamos o Material Particulado (MP), pois ele tem recebido maior ênfase em pesquisas cientificas, tanto epidemiológicas, como experimentais (Goettems-Fiorin et al, 2016; Mai et al, 2017). O MP é caracterizado como uma mistura complexa de partículas sólidas e líquidas de substâncias orgânicas e inorgânicas suspensas no ar (OMS, 2018). Ele é classificado de acordo com o diâmetro das partículas, devido à relação existente entre o diâmetro e seu potencial causador de efeito adverso à saúde, sendo que quanto menor a partícula, maiores são os efeitos no organismo (Xu et al, 2011).

Analisando os efeitos do acúmulo de partículas do MP no organismo, a OMS possui uma classificação referente ao tamanho aerodinâmico das partículas. Classificam-se em Partículas Totais em Suspensão (PTS), representadas pelas partículas cujo diâmetro é menor que 50 µm e Partículas Inaláveis (PIs), cujo diâmetro é menor que 10 µm. Entre as PIs, há uma subdivisão em partículas grossas (MP_{10}), partículas finas ($MP_{2,5}$) e partículas ultrafinas ($MP_{0,1}$). O tamanho das partículas são relevantes para a dinâmica do MP na atmosfera e absorção no corpo humano e refletem as faixas usadas em estudos em saúde (OMS, 2016) (Figura 1).

Figura 1 - Principais características da distribuição de massa de partículas atmosféricas.



Fonte: Adaptado de WHO (2016) *Outdoor Air Pollution*. MP_{10}: partículas com diâmetro aerodinâmico < 10 µm; $MP_{2,5}$: material particulado com partículas de diâmetro aerodinâmico < 2,5 µm; MP_7/MPS: material particulado em suspensão; PST: partículas suspensas totais; PU: partículas ultrafinas.

Com a exposição constante a poluentes atmosféricos, a OMS estabelece valores de referência de máximo de emissão de poluentes visando amenizar os efeitos nocivos à saúde, pois mesmo em baixas dosagens, a poluição pode afetar a saúde. Os valores estabelecidos pelas “Diretrizes de qualidade do ar da OMS” (2005) preconiza que o ar, deve ter até no máximo de material particulado fino ($MP_{2.5}$) 10 µg / m^3 de média anual e 25 µg / m^3 de média diária. No entanto, 92% da população mundial já vivem em locais onde os níveis de qualidade do ar excedem a esses parâmetros (CONAMA, 2018).

A exposição à poluição atmosférica desencadeia diversos problemas, devido a interação das partículas nos pulmões com o sistema circulatório e que pode causar interferências no funcionamento fisiológico normal do indivíduo (Kinney 2008), nos sistemas cardiovascular (Shah et al. 2013; Dominici et al. 2006), nervoso (Zanchi et al, 2010;

Calderón-Garcidueñas et al. 2008) e reprodutivo (Veras, 2009), e também pode estar associada a desfechos neurológicos adversos. Fonken et al (2011) observaram, que mostrou que a exposição ao MP leva à depressão e a deficiência cognitiva, e promovendo uma supra regulação de marcadores inflamatórios e alterações nas estruturas do hipocampo. A exposição a poluição do ar induz a obesidade através nos sistemas hormonais no controle do peso, podendo alterar também a atividade nos sistema simpático, sistema inflamatório (através dos adipócitos), desregulação de lipídeos e glicose e estresse oxidativo, além de promover resistência à insulina (Sneha et al., 2014).

Estudos anteriores do nosso grupo demonstram que o contato direto com o MP pode afetar o cérebro de ratos e que esse comprometimento é dependente da estrutura, onde o hipocampo e o cerebelo são as mais afetadas pelo estresse oxidativo provocado pela exposição ao MP (Fagundes et al, 2015). Em um estudo experimental que expôs ratos a poluição do ar durante o período pré e pós natal concluiu que essa exposição prejudica a memória discriminativa de curto prazo e sugere que os períodos gestacionais e a primeira infância são a chave para desencadear esses efeitos. Além disso, eles salientam que o estresse oxidativo cortical pode ter um papel importante na medicação do comprometimento da memória de curto prazo induzida por poluentes urbana (Zanchi et al, 2010).

A presença de metais de transição na poluição do ar, pode levar à formação de espécies reativas de oxigênio (EROs), que podem causar danos oxidativos ao DNA na reação de Fenton (OMS 2016). O estresse oxidativo (EO) é uma conseqüência da produção excessiva de radicais livres e também devido à falha do mecanismo de defesa antioxidante que protege as células removendo os radicais livres (McCord 1993). Quando um excesso de radicais livres é gerado e / ou excede o mecanismo de defesa antioxidante, isso causa importantes danos às células através da alteração do DNA, proteínas e lipídios e, obviamente, isso causa um consumo de antioxidantes e o estresse oxidativo aparece (McCord 1993; Gutteridge 1995).

1.1.1 Poluição em mulheres

A exposição ao $MP_{10,}$ em mulheres no período pós-menopausa (idade acima de 60 anos), aumenta em cinco vezes o risco de mortalidade por doença cardíaca, comparado com as mulheres no período pré-menopausa (antes dos 45 anos) expostas aos mesmos poluentes (Miller et al 2007). Esses pesquisadores concluíram que a exposição prolongada ao material particulado fino atmosférico está associada a uma maior incidência de doença cardiovascular e morte em mulheres pós-menopáusicas (Miller et al. 2007). Outro estudo (Honda et al 2018) mostrou que a exposição a longo prazo de PM2,5 e PM10, foram associadas ao aumento do risco de hipertensão incidente em uma coorte nacional de mulheres na pós menopausa.

Estudos recentes do nosso grupo (Goettems-Fiorin, et al 2019) mostram grupos expostos ao ROFA e submetidos a ovariectomia apresentam um aumento na taxa de neutrófilos e neutrófilos / linfócitos, diminuição da defesa antioxidante (atividade da SOD) e aumento nos níveis de HSP70 intracelular no fígado, sugerindo que alterações no sistema reprodutivo, como a diminuição dos níveis de estrogênio, predispõem os organismos femininos a efeitos de poluição do ar particulado, afetando a expressão metabólica, oxidativa, pró-inflamatória e de choque térmico (Goettems-Fiorin et al 2019).

1.2 Pós menopausa

Os processos naturais de envelhecimento resultam em alterações fisiológicas no cérebro e no corpo de todos os organismos. A senescência reprodutiva ocorre por volta da quinta década de vida, quando o conjunto de folículos ovarianos é esgotado através da morte celular programada, chamada atresia (Koebele, 2016). O climatério é caracterizado pela perda da função ovariana (Dalal; Argawal, 2015) e as alterações hormonais resultantes, sobretudo os baixos níveis de estrogênio estão associados ao desenvolvimento de doenças cardiovasculares e metabólicas, estresse oxidativo, obesidade e podem agravar o processo de senescência oriundo da inflamação generalizada (Benedusi et al 2014).

O estrogênio é um hormônio esteroide sexual feminino produzido nos ovários e pelas glândulas supra-renais, responsável pela regulação e coordenação de múltiplas funções em órgãos, células e genes por meio de receptores e utiliza vias de sinalização para ativar respostas moleculares e genômicas para sobrevivência celular e corporal (Rettberg et al, 2014). Existem três formas de estrogênio no corpo feminino: Estrona (E1), Estradiol ou 17 β-estradiol (E2) e o Estriol (E3). O E1 e o E3 estão presentes no organismo em níveis baixos e apresentam menor atividade sobre os receptores estrogênicos (RE) (Gruber et al, 2002, Morissete et al, 2008). Já o E2, é o mais abundante e potente estrógeno humano, é produzido principalmente pelos ovários, regulado pelo eixo-hipotálamo-hipófise.

A vida pós-reprodutiva surge com uma gama de mudanças fisiológicas, comportamentais e cerebrais e podem afetar a qualidade de vida (Al-Safi; Santoro 2014; Jenabi et al, 2015). A pós menopausa está associada à disfunção metabólica (Della Torre et al 2014) e patologias que envolvem inflamação (por exemplo, osteoporose e distúrbios metabólicos, incluindo diabetes, aterosclerose, doenças articulares e até neurodegeneração) (Hotamisligil et al 2006; Komm 2008). Estudos recentes mostraram que a ovariectomia resultou em alterações metabólicas e variação da massa gorda corporal, apresentou perda óssea, um declínio na função muscular, além de aumento do estresse oxidativo e da inflamação (Noh et al., 2018; Tagliaferri et al., 2015).

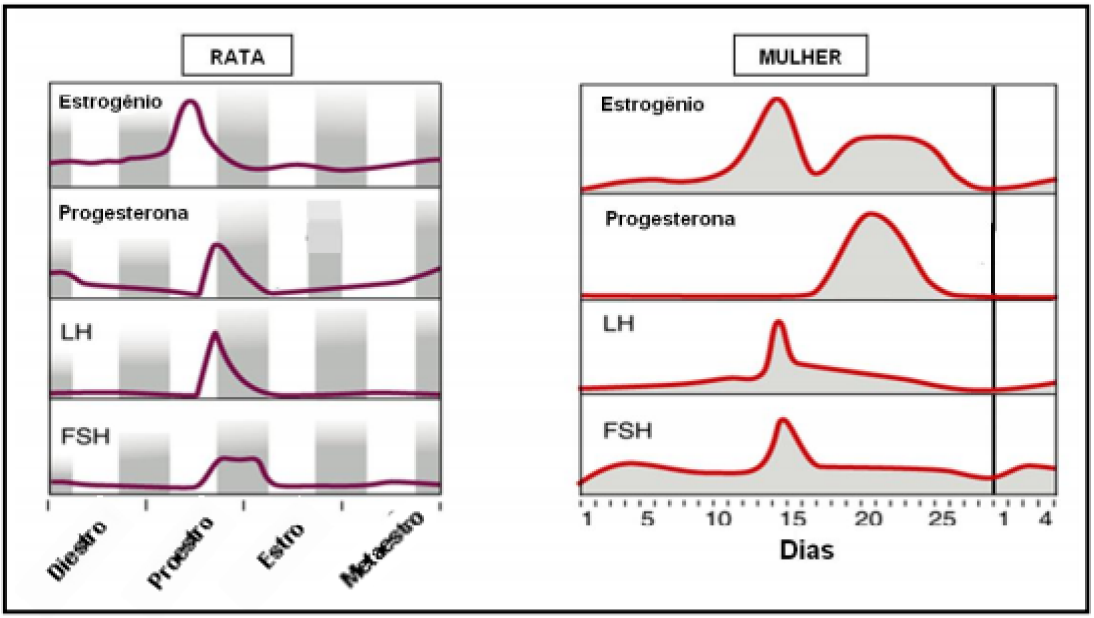
O processo inflamatório gera EROs, e essas possuem duas funções distintas no organismo. São capazes de prevenir a doenças, por auxiliar o sistema imunitário, mediando a sinalização celular e desempenhando papel essencial na apoptose (Celep; Marotta 2014; Seifried et al., 2007), e também podem oxidar lipoproteínas e outras importantes estruturas celulares, causar danos ao ácido desoxirribonucleico (DNA) e às proteínas, podendo, levar ao processo de estresse oxidativo, contribuindo para o desenvolvimento e complicações de doenças autoimunes, infecciosas, metabólicas, cardiovasculares, neurodegenerativas, dentre outras (Celep; Marotta, 2014; Giacco; Brownlee, 2010).

A utilização de animais experimentais para o estudo do ciclo reprodutivo feminino é cientificamente aceito. A ovariectomia cirúrgica em modelos animais é considerada o modelo que melhor reproduz as mudanças pós-menopáusicas (Savonenko; Markowska 2003). Como ratas possuem ciclos reprodutivos curtos, são animais ideais para verificar alterações que ocorrem durante o ciclo reprodutivo (Spornitz et al, 1999; Marcondes et al, 2002). Além disso, 60-70% dos roedores envelhecidos passam espontaneamente para um estado anovulatório policolicular de estro constante, caracterizado por níveis sustentados de 17 β-estradiol plasmático (Brinton, 2014).

A ovariectomia em modelos animais é utilizada para estudar muitas doenças relacionadas à menopausa, como doenças cardiovasculares e osteoporose (Oliveira, 2014; Moreau 2012). Esse procedimento mimetiza mulheres na pós menopausa, aumentando a suscetibilidade à obesidade e suas comorbidades associadas, como a inflamação (Hong et al 2009; Viera et al 2007), como já demonstrado em um estudo de Min (2018), que observou um aumento na massa corporal e na adiposidade, sugerindo que o declínio nos níveis de estrogênio, devido à ovariectomia, influenciam a distribuição corporal e diferenciação de adipócitos (Min 2018).

As alterações hormonais que ocorrem tanto no ciclo estral (ratas) como no ciclo menstrual (mulheres) são muito semelhantes (Figura 2).

Figura 2 - Comparação entre as alterações hormonais durante o ciclo estral de ratas e o ciclo menstrual de mulheres.



Fonte: Silva, 2011.

A ausência dos estrogênios, através da menopausa em mulheres e ovariectomia em modelos experimentais, estão associados ao aumento de peso, diminuição da massa magra (Douchi et al 2002), acúmulo de gordura visceral (Tchernof et al 2004) e aumento de lipídeos no fígado. Os estrógenos são moléculas anti-inflamatórias (Ghisletti et al 2005; Saijo et al 2011; Vegeto et al 2001), assim considerando que a inflamação generalizada de baixo grau é um fator importante na determinação da senescência (Hotamislgil 2006) a perda de estrogênio ovariano em mulheres idosas pode agravar o processo de senescência por meio de um aumento da inflamação generalizada.

Os estrogênios têm papeis bem estabelecido na função reprodutiva, mas seus efeitos em outros sistemas têm sido trazidos à luz, a saber, nos tecidos adiposo, nervoso, cardiovascular, muscular e ósseo, implicando esses hormônios em diversos papéis fisiológicos (Monteiro, 2014). Em um estudo de base populacional, o *The French Three-City Study,* tanto a menopausa cirúrgica quanto a menopausa natural precoce foram associadas com efeitos negativos, em longo prazo, sobre a função cognitiva, que não foram inteiramente compensados por terapia de reposição estrogênica (Ryan et al 2014).

Mulheres são mais afetadas com a produção EROs durante o envelhecimento, pois a redução acentuada de E2 também tem sido associada ao aumento dos níveis de estresse oxidativo. Em um ciclo normal, as mulheres possuem altas concentrações de E2, o que promove um efeito antioxidante benéfico no organismo. A perda de estrogênio na menopausa está associada ao aumento de doenças metabólicas (Lizcano 2014) por mecanismos relacionados ao aumento do estrese oxidativo (Oliveira 2018) inflamação e equilíbrio de HSP70 (Crist et al 2009; Heck et al 2017).

O estado pós-menopausal e a privação de estrógenos causam impactos em múltiplos sistemas fisiológicos, incluindo o ósseo, cardiovascular, imune e o sistema nervoso central (SNC) (Wise 2001). Estudos nos mostram que, uma dieta rica em gordura saturada diminui a função cerebral e as habilidades cognitivas em roedores e humanos (Molteni et al, 2004; Wu et al, 2004).

Dados na literatura sugerem que a depressão é duas vezes mais comum em mulheres do que em homens e as diferenças sexuais estão presentes na resposta antidepressiva (Gorman, 2006; Marcus et al., 2005; Sloan e Kornstein, 2003; Thiels et al., 2005). A diferença entre os gêneros pode ser relacionada à flutuação hormonal durante o ciclo reprodutivo, principalmente o estrógeno (Pigott 1999). Nesse contexto, mudanças na secreção de estrógeno durante a pré-menopausa e a menopausa podem ser relevantes para o aumento do risco de ansiedade nestes períodos (Hill et al, 2007).

Estudos que utilizam a técnica de ovariectomia analisaram os efeitos da privação do estrogênio sobre o comportamento, e observaram que a retirada dos ovários aumenta o comportamento típico de ansiedade em ratas e a administração subcutânea de estradiol é capaz de reverter esses efeitos em vários testes (Diaz-Velliz et al, 1997; Bowman et al, 2002; Frye; Walf 2004; Walf; Frye 2006).

1.3 Obesidade

A obesidade é definida como uma condição inflamatórias crônica que envolve deposição excessiva de tecido adiposo, níveis aumentados de citocinas pró-inflamatórias, quimiocinas, proteases e fatores de crescimento que vem sendo associados a patologias relacionadas à idade como as doenças neurodegenerativas (Spielman 2014).

O desenvolvimento da obesidade pode ser decorrente de diversos fatores, dentre os quais se destacam os ambientais, como o consumo de dietas inadequadas e o sedentarismo (Rosini et al, 2012). Diante disso, considera-se a obesidade como uma epidemia mundial, e projeta-se que em 2025, cerca de 2,3 bilhões de adultos estejam com sobrepeso, e mais de 700 milhões, obesos (OMS, 2018). Em relação ao Brasil, segundo o relatório da Organização das Nações Unidas (ONU) para Alimentação e Agricultura (FAO) e da Organização Pan-

Americana de Saúde (OPAS) “Panorama da Segurança Alimentar e Nutricional na América Latina e Caribe” (2016) observa-se que mais da metade da população está com sobrepeso e a obesidade já atinge a 20% das pessoas adultas, sendo que as mulheres possuem maior prevalência, correspondendo 22,7%. Diante desse quadro, observa-se que aspectos ambientais e comportamentais possuem grande interferência no desenvolvimento da obesidade (Jequier; Bray 2002), pois se associam a outras doenças, como diabetes e transtornos psiquiátricos, como depressão ou ansiedade (Stunkard et al 2003; Philips and Perry, 2015).

Sivanathan et al (2015) mostraram que a alimentação rica em gordura aumenta o comportamento de ansiedade crônica das ratas adultas as tarefas do teste de transição claro/escuro e do campo aberto, em comparação com ratos alimentos com dieta contendo baixo teor de gordura.

Existem mecanismos celulares que auxiliam no combate ao estresse oxidativo e à inflamação, as Proteínas de Choque Térmico (*Heat Shock Proteins* – HSP), especialmente a HSP70, são exemplos desses mecanismos. A HSP70 é mais conservada e tem demonstrado modular a inflamação em alguns estudos (Feder 1999; Van Molle et al, 2002; Borges et al, 2012). Assim, Uchida et al (2012) propôs que, sob condições estressantes, as chaperonas moleculares como a HSP70, são liberadas como um sinal de perigo para as células imunes, para promover respostas inflamatórias e proteger as células.

A HSP70 no ambiente intracelular (iHSP70) é essencial para a sobrevivência das células, desempenhando funções chaperona, anti-inflamatória e como proteção complementar contra o estresse oxidativo celular. Por outro lado, sob condições de estresse, estas proteínas, principalmente a HSP72, podem ser exportadas da célula e detectadas no ambiente extracelular (eHSP70), sendo capazes de se ligar à superfície celular, modular células imunes (pró-inflamatória) e atuar como “sinal de perigo” (Calderwood et al, 2007; De Maio 2011).

2 JUSTIFICATIVA

Neste contexto, destaca-se que os elementos descritos acima, como o consumo de DHL, a exposição às partículas urbanas e a privação de E2, promovem efeitos análogos quando se avaliam parâmetros metabólicos, oxidativos, inflamatórios e comportamentais. Ainda assim, poucos estudos tem relatado o efeito da associação destas condições no processo de alterações comportamentais. Portanto, neste estudo propomos investigar os efeitos da exposição crônica da poluição atmosférica sobre parâmetros comportamentais, oxidativos e inflamatórios de ratas tratadas com dieta hiperlipídica e ovariectomizadas.

3 OBJETIVOS

3.1 Objetivo Geral

Avaliar os efeitos da exposição crônica da poluição atmosférica sobre parâmetros comportamentais, oxidativos e inflamatórios de ratas tratadas com dieta hiperlipídica e ovariectomizadas.

3.2 Objetivos específicos

- Avaliar o efeito da exposição crônica da poluição atmosférica sobre a massa corporal, ganho de massa corporal (pré e pós ovariectomia) e adiposidade de ratas tratadas com DHL e ovariectomizadas;
- Observar o efeito da exposição crônica da poluição atmosférica sobre o comportamento exploratório (Teste do Campo Aberto), na ansiedade (Labirinto de Cruz Elevado) e no comportamento do tipo depressivo (Nado Forçado) de ratas tratadas com DHL e ovariectomizadas;
- Investigar o efeito da exposição crônica da poluição atmosférica sobre parâmetros de estresse oxidativo (TBA e SOD) do hipotálamo, hipocampo, córtex cerebral e cerebelo de ratas tratadas com DHL e ovariectomizadas;
- Avaliar o efeito da exposição crônica da poluição atmosférica sobre marcadores inflamatórios (IL-6, IL-10 e HSP70 extracelular) de ratas tratadas com DHL e ovariectomizadas.

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5 ARTIGO CIENTÍFICO

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Chronic exposure to air pollution promotes depression-like behavior in rats treated with high-fat diet and ovariectomized independent of oxidative stress

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Chronic exposure to atmospheric pollution promotes depression-like behavior in rats treated with high fat and ovariectomized diet independent of oxidative stress

Abstract

This study aimed to evaluate the effects of exposure to atmospheric pollution on behavioral, oxidative and inflammatory parameters of rats treated with high fat and ovariectomized diet. First, female rats (n = 30) were fed HDF and randomly assigned to two groups receiving an intranasal instillation of 50 µL of saline daily for 12 weeks (representing a clean environment) or total suspended particles in saline (250 µg, representing a polluted environment). The animals then received ovariectomy (OVX) or false surgery (Sham) and continued to receive saline or particles for an additional 12 weeks, comprising four groups: HFD (n = 6), HFD + OVX (n = 9), HFD + Polluted (n = 6) and HFD + Polluted + OVX (n = 9). At week 24, open field testing, elevated cross maze and forced swimming were performed. One day after the last behavioral test, the animals were euthanized, and serum estradiol, cytokine (IL-6, IL-10) and 70 kDa heat shock protein (eHSP70) levels were evaluated (ELISA). Oxidative stress markers (TBARS and SOD activity) of brain structures were also evaluated. The association HFD + Polluted + OVX increased adiposity (P = 0.003) and depressive behavior (P = 0.002) of rats, and decreased serum IL-6 levels (P = 0.038). Chronic TSP exposure promotes depressive behavior in ovariectomized obese rats, independent of oxidative stress.

Keywords: Air Pollution; High-Fat diet; Animal Behavior; Oxidative stress; Inflammatory profile; Anxiety; Depression;

Introduction

Air pollution is associated with 7 million deaths annually, and 90% of the world's population is exposed to polluted air (WHO, 2018), characterized as one of the major public health problems worldwide. Studies show effects of exposure to air pollution on the respiratory system (Kinney 2008), cardiovascular (Shah et al. 2013; Dominici et al. 2006), reproductive (Veras, 2008) and central nervous system (CNS) (Calderón-Garcidueñas et al. 2008; Zanchi et al., 2010). CNS changes can trigger behavioral changes, as demonstrated by an animal model study that showed the association between pollution exposure and anxiety (Salvi et al., 2017). These authors showed that the emission of vehicular pollutants led to alterations in the open field and elevated cross maze tests, accompanied by the depressive behavior observed in the forced swimming test (Salvi et al., 2017). The effects of air pollution on behavior are associated with oxidative stress in different brain structures (Zanchi et al. 2008) and altered cytokine levels (Bolton et al. 2012; Bolton et al. 2014).

Another important public health factor that may influence behavioral changes is obesity. Approximately 13% of the world's adult population (11% of men and 15% of women) are obese (WHO, 2018), and one consequence is the development of psychological problems, including anxiety (Vasques et al., 2004; Gariepy et al., 2010). Studies in experimental models have shown that young male Sprague Dawley rats exposed to a high-fat diet for 10 weeks and had more entries and time spent in the center of the Open Field (Marwitz et al 2015), while Sivanathan (2015) found females. Long Evans fed high-fat diets may develop anxiety, as well as Bilbo (2010) and Sasaki (2013), who have shown that consumption of a high-fat diet (HFD) and consequent obesity influence brain behavior and function in different stages of the life cycle, as demonstrated by anxiety behavior in labyrinthine tasks and the open field test (Bilbo et al., 2010; Sasaki et al., 2013). In addition, HFD consumption impairs rat cognition, as demonstrated by the Morris Aquatic Maze test (Pintana et al., 2012; Pipatpiboon et al., 2013).

Obesity triggers an increase in free radical production and may induce an oxidative stress (OS) profile in human and animal model studies (Pratchayasakul et al. 2015). In addition, obesity increases the production of proinflammatory cytokines such as adipokines, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) by adipose tissue (Teixeira 2012). On the other hand, obesity decreases the expression of 70 kDa intracellular heat shock proteins (HSP70), and these changes interfere with cell cytoprotection (Chung et al., 2008, Kido et al., 2011). Although HSP70 can be released into the bloodstream (called eHSP70 due to its extracellular location), thus eHSP70 can modulate behavior by acting as chaperokine signaling (Heck et al., 2011; Bobkova et al., 2015), the relationship between eHSP70, obesity and behavior is not yet established.

Postmenopausal women represent the group most susceptible to the development of obesity (Brown and Clegg, 2010; Kim, 2012) and hypoestrogenism-related nervous disorders. Estrogen is a neuromodulatory and neuroprotective hormone (Craig and Murphy 2007; Spencer et al., 2008), which reduces anxiety-related behavior and produces antidepressant effects (Chaves et al., 2009). The decrease in estrogen levels and the metabolic changes triggered by its decline make the female organism predispose to a higher production of reactive oxygen and OS species (Crist et al., 2009). The OS profile may be associated with the activation of transcription factors, such as nuclear factor kappa B (NF-κB), responsible for increased production and release of proinflammatory cytokines (Newsholme and De Bittencourt 2014). In addition, HSP70 expression (which has anti-inflammatory properties inhibiting NF-KB activation) is sensitive to OS (Ahn and Thiele, 2003) and estrogen (Hamilton et al., 2004) levels. When estrogen deficiency occurs, as in menopause, HSP70 concentrations in intracellular and extracellular environments may be altered (Voss et al., 2003; Hou et al., 2010).

Although air pollution, obesity and decreased estrogen levels are independently associated with behavioral changes and are also independently associated with changes in OE, inflammation and HSP70 levels, the effects of the combination of these factors (air pollution, obesity and (estrogen levels) still need to be elucidated. Thus, our study aims to investigate the effects of chronic exposure to air pollution on behavioral, oxidative and inflammatory parameters of rats treated with a high-fat diet and ovariectomized.

Method

Animals

Female Wistar rats (*Rattus norvegicus albinus*) (n = 30) (eight weeks old) from the Regional University of the Northwest of the State of Rio Grande do Sul (UNIJUÍ) were kept in the plastic cages (47 cm X 34 cm X 18 cm) under controlled temperature conditions (22 ± 2ºC), light-dark cycles (light from 7:00 am to 7:00 pm). The animals received water and high-fat diet *ad libitum*. The management of the animals was in compliance with Law No. 11,794 of 08/10/2008, Law No. 6,899 of July 15, 2009, and Resolution No. 879 of February 15, 2008 (CFMV), as well as resolutions of the National Animal Experimentation Council and all procedures, were in accordance with the National Institute of Health's (NIH-USA) Guidelines for the Care and Use of Laboratory Animals. This study was approved by the UNIJUÍ Ethical Committee on the Use of Animals (CEUA 076 / 15).

Experimental Design

Animals (eight weeks old) were submitted to the High-Fat Diet (HFD - described below) and exposed to air pollution (Total Suspended Particles - TSP see below) for 12 weeks. After this period, the rats underwent ovariectomy, remaining with the previous treatments for another 12 weeks. Thus, four experimental groups (Figure 1) were randomly formed: HFD (n = 6), HFD+OVX (n = 9), HFD+Polluted (n = 6) and HFD+Polluted+OVX (n = 9). At the 24th week of the experiment, all animals were submitted to Open Field, Elevated Plus Maze and Forced Swimming, with a one-day interval between tests. One day after the last test (Forced Swimming), the animals were euthanized through the guillotine, without anesthesia. The whole blood was collected in eppendorfs with (for analysis of interleukin 6 and 10 and extracellular HSP70 levels) or without EDTA (for analysis of estradiol 17β). We also collected hypothalamus, hippocampus, cerebral cortex and cerebellum that were dissected and frozen in liquid nitrogen and stored for later homogenization.

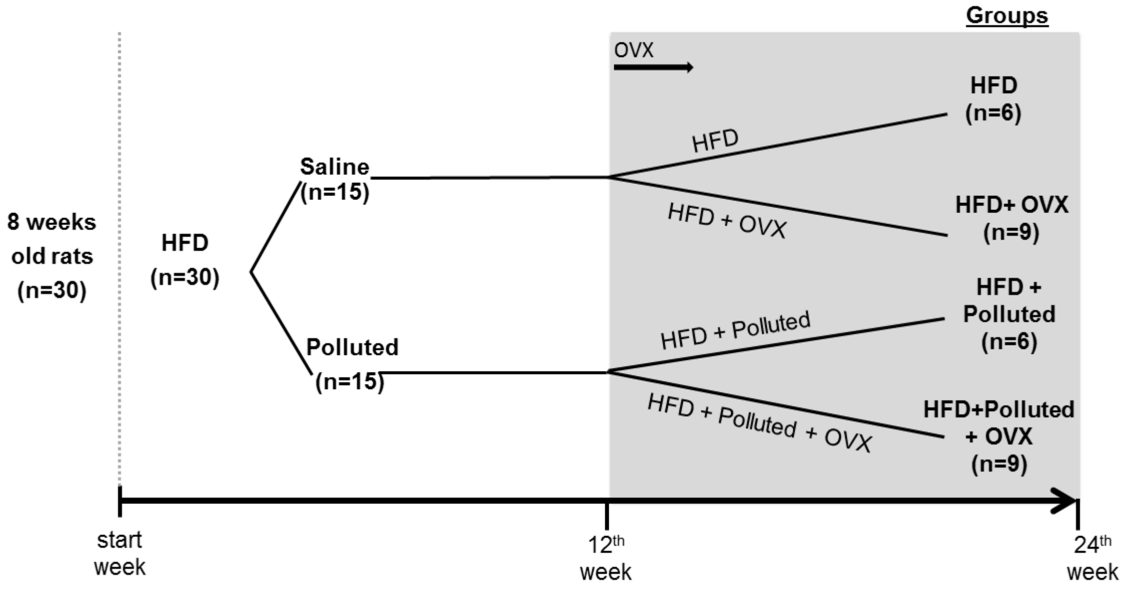

Figure 1. Experimental design.
Eight-week-old Wistar rats were submitted to a high-fat diet (HFD) and exposed to air pollution (Polluted) for 12 weeks. After this period, the rats underwent ovariectomy, remaining with previous treatments for another 12 weeks. Groups: HFD (n = 6), HFD + OVX (n = 9), HFD + Polluted (n = 6) and HFD + Polluted + OVX (n = 9).

High-Fat Diet

The high-fat diet (HFD) consisted of 58.3 % of fats and was produced in Biological Laboratory/UNIJUÍ (LeBIO/UNIJUÍ) and stored at 2-8 ºC. The ingredients (except starch and lard) were adjusted to be present in the same amount as in the standard diet for rodents. To meet this standard, 13.7 % egg albumin, 7.4 % amino acid supplement (Aminomix™) and 1.1 % oyster and dry bone meal were used to prepare the experimental diet weekly (Winzell e Ahren 2004; Goettems-Fiorin et al. 2016).

Exposure to air pollution

Total suspended particles (TSP) were collected from an electrostatic precipitator from a steel plant (Carvalho-Oliveira et al 2015). The particles were suspended in saline at a concentration of 250 µg / 50 µL and administered in the Polluted groups, the other groups received 50 µL of saline. The intranasal instillation procedure was performed once a day, 5 days a week, for 24 weeks with an automatic pipette. We pipetted the pollutant into the animal's nostril, which inhaled it through an apnea reflex (Medeiros et al. 2004).

The TSP exhibited iron predominance, which represented 64% of the total mass of the analyzed elements. No organic compounds were detected by gas chromatography / mass spectrometry in the TSP sample. (Carvalho-Oliveira 2015). The composition of the total suspended particles is detailed in the table below (Table 1).

Table 1: Composition of Total Suspended Particles (TSP)

Metal	µg / g (mean ± SD)
Al	243.4 ± 86.79
Ca	17326.07 ± 1029.77
Cu	47.14 ± 11.23
Fe	82507.29 ± 6371.3
K	12160.76 ± 851.3
Na	1646.66 ± 253.39
Ni	ND
Pb	1590.87 ± 146.16
S	7666.44 ± 474.09
Si	5922.42 ± 308.92
Ti	181.05 ± 24.7
V	ND
Zn	111.67 ± 11,69

Al: aluminum; Ca: calcium; Cu: copper; Fe: Iron; K: potassium; Na: sodium; Ni: nickel; Pb: lead; S: sulfur; Si: silicon; Ti: titanium; V: vanadium; Zn: Zinc.

Ovariectomy

The animals were submitted to the following surgical protocol (Zou, 2011): Pre-anesthetic medication: 5 mg / kg morphine intraperitoneal (i.p.); induction of anesthesia: 4 % of isoflurane (BioChimico®); maintenance of anesthesia: 2 % isoflurane (BioChimico®). The ovariectomy (OVX) corresponded to the bilateral removal of the ovaries achieved by the longitudinal incision of the paralimbic region. The non-ovariectomized animals were submitted to false operation (Sham) where their ovaries were identified, surgically exposed and repositioned for posterior suturing of the musculature and skin. After the surgical procedures, all animals (Sham e OVX) received appropriate postoperative treatment, composed of body temperature and pain signals monitoring, and meloxicam (Ouro Fino®) 0.2 % (2 mg / kg, subcutaneously) administration after 24 hours (single dose).

Biometric Profile

Body mass (g) of the animals was checked at the beginning (T = 0), at the 12th week and the end of the 24 weeks of the experiment. For body mass measurement, three weightings of each animal were performed on a semi-analytical balance (Marte®) and the mean of these three values was used as the final results. The body mass gain of these animals was calculated, being Delta 1 (pre-ovariectomy) (T = 12 – T = 0) and Delta 2 (post-ovariectomy) (T = 24 – T = 12). At the end of 24 weeks, we verified the visceral white adipose tissue mass of the animals and calculated the percentage of adiposity by the equation: adipose tissue mass multiplied by 100 / final body mass.

The percentage of adiposity at the end of the experiment with body mass gain (delta 2) and interleukin levels 6 were also correlated.

Measurement of 17β-estradiol (E2)

At the end of 24 weeks, the animals were euthanized and whole blood was collected, without anticoagulant, to obtain serum used determine the 17 β estradiol (E2) dosing. Determination of 17 β-estradiol (E2), a serum sample and a quantitative dosage were used using the automated system ADVIA Centaur XP (Siemens Healthcare Diagnosis) by chemiluminescence methodology with sensitivity and *in vitro* limits between 20 and 3000 ρg / ml. The ovariectomized rats presented lower levels of estradiol than the sham-operated rats (Sham = 29.9 ± 10.8 vs Ovariectomized = 23.3 ± 5.1 pg / ml, P = 0.041).

Behavior

Open Field Test (OFT)

The OFT assessed behavioral responses such as motor activity and exploratory behavior. It is also used to measure anxiety (Hall 1934). This test was conducted in the white square arena (60 x 60 x 40 cm) divided into 16 quadrants. The animals were placed individually in the center of the arena and recorded for five minutes. The sessions were video-recorded and analyzed by a blind experimenter to treatment groups. We measured and analyzed for each rat: exploratory behavior (adding of the number of squares crossed and frequency of rearing), the frequency of grooming and the amount of fecal bolus (Walsh e Cummins 1976). The apparatus was cleaned with alcohol (70% v / v) to eliminate the olfactory information of territoriality represented by urine and feces and reduce the influences of other animals on the behavior.

Elevated Plus Maze Test (EPM)

The EPM test assessed the anxiety-like behavior. The EPM was originated from the work of Montgomery (1955), who used a high Y-maze. However, in the current version, EPM consists of a maze made of dark wood, shaped like a symmetrical cross with two open arms (without lateral walls of 50 x 10 cm) and two closed (50 x 10 x 40 cm side walls) arranged perpendicularly forming a central area and the labyrinth was raised 50 cm above the ground. The animals were placed individually in the central area and recorded for five minutes. The sessions were video-recorded and analyzed by an experimenter blind to treatment groups. As parameters of anxiety-like behavior were considered: the time spent in the open and closed arms, the transition to each arm (Pellow et al. 1985). After each animal session, the floor of the appliance was cleaned with 70 % alcohol, to minimize the interference of odors left by the animal, and allowing to dry and circulate some air.

Forced Swimming Test (FST)

FST originally developed by Porsolt (1979) for antidepressant drug screening is also used as a model for a rodent depression-like phenotype. Each rat was placed in a plastic cylinder (25 cm in diameter and 51 cm high) filled with water (23 ± 2 ° C) to a depth of 30 cm so that the animal could not touch the bottom with its tail or feet for 5 minutes. The behavior of the rat was observed. As a rule, the animal is actively fighting early on, trying to escape. After a while, the rat acquires an immobile posture with the minimum movements necessary to keep it floating. This immobility is considered a state of negative mood or behavioral despair that is considered relevant to human depression (Bogdanova et al. 2013; Carter & Shieh 2015; Hoffman 2016). No pretesting was performed.

All behavioral experiments were performed between 2:00 pm and 4:00 pm and carried out in a temperature- and noise- controlled room.

Evaluation of Oxidative Stress and Inflammatory Profile

Tissue Preparation

At the end of 24 weeks of intervention, one day after the behavioral tests, the animals were euthanized through the guillotine without anesthesia. Whole blood was collected in eppendorf with EDTA and centrifuged at 3,000 rpm for 5 min. Plasma was used for cytokine analysis (IL-6, IL-10 and eHSP70).

We also collected the cerebral cortex, cerebellum, hippocampus and hypothalamus that were dissected, weighed, frozen in liquid nitrogen and stored for later homogenization. In these tissues, we analyzed lipoperoxidation through the Thiobarbituric Acid Reactive Substances Test (TBARS) and the activity of the enzyme Superoxide dismutase (SOD). For this purpose, a portion of the tissues was homogenized in potassium phosphate buffer (KPi, pH 7.4) containing PMSF. Homogenization was performed in a tissue homogenizer (CT-136.1, Cientec®).

Determination of Protein Concentration

The basis for characterization of the components was determined by the Bradford method (1976) at 578 nm by the microplate reader spectrophotometric technique using bovine serum albumin (0.04-3.0 mg / mL points) as standard.

Determination of Lipid Peroxidation

Lipid peroxidation concentrations in brain structures were analyzed by the thiobarbituric acid reactive substances method (TBARS; Buege and Aust, 1978). The homogenates were precipitated with 10 % trichloroacetic acid, centrifuged and incubated with thiobarbituric acid for 15 min at 100° C. Then, the absorbance was measured at 505 nm by the microplate reader spectrophotometric technique. The MDA standard was prepared from 1.1.3.3 Tetramethoxypropane (concentrations between 0.0005 and 0.016 mg / mL). The results were expressed as µmol MDA / mg protein.

Determination of SOD Activity

The activity of brain structures of SOD was performed by the inhibition of pyrogallol auto-oxidation (Marklund e Marklund 1974). Briefly, into a cuvette, 944 µl of Tris-EDTA buffer (TRIS 2-amino-2-hydroxymethylpropane-1,3-diol, 50 nM, EDTA ethylenediaminetetraacetic acid, 1 mM, pH 8.2) was added 4 µl of catalase (CAT; 30 µM), 20 µl of homogenate and 32 µl of pyrogallol (24 mM in 10 mM HCl) and mixed and the SOD activity was determined in a spectrophotometer (420 nm) for 120 seconds. The results were expressed in the Unit of SOD / mg protein.

Determination of IL-6, IL-19 and eHSP70 Expression

Interleukins 6 and 10 were analyzed by enzyme-linked immunosorbent assay by the RayBio specific kit for each interleukin. Both techniques use specific antibodies to detect interleukin, measured at 450 nm. The IL-6 kit has a sensitivity of 10 ρg / mL, and does not show cross-reactivity with any of the cytokines tested CINC-2 of rat, CINC-3, IL-1 alpha, IL-1 beta, IL-4, IL-6, GM-CSF, IFN-gamma, Leptin, Lix, MCP-1, MIP-3 alpha, beta-NGF, TIMP-1, TNF-alpha, VEGF. The IL-10 kit has a sensitivity of 30 ρg / mL and does not cross-react with any of the cytokines tested: CINC-2 of rat CINC-3, Fractalkine, IL-1 alpha, IL-1 beta, IL-4, IL-10, GM-CSF, IFN-gamma, Leptin, Lix, MCP-1, MIP-3 alpha, beta-NGF, TIMP-1, TNF-alpha. All cytokines were reported as picogram per milliliter, according to the instructions manual of the respective kits.

The eHSP70 levels were analyzed by the ENZO Life Science kit also by immunoenzymatic assay and detected at 450 nm. The kit has a detection limit of the assay is 0.007 ng / mL (7 ρg / mL). The sensitivity was determined by interpolation at 2 standard deviations above the mean signal of the zero replicate patterns (n = 22). Cross-reactivity to a number of related compounds was determined by diluting the cross-reactants in the assay buffer at various concentrations. These samples were then measured in the assay. The cross-reactivity of HSP70 in rats is 117.6%, according to the instructions manual of the kit.

Statistical Analysis

Shapiro Wilk normality test was performed for all variables. For results without normal distribution, the Kruskal-Wallis test was performed followed by Dunn's post hoc test. For variables with normal distribution, a one-way analysis of variance (ANOVA) was performed followed by a Tukey post hoc. For the analysis of estradiol levels, the Mann Whitney test was performed.

For the correlation between the adiposity percentage and body mass gain (Delta 2) Pearson correlation was performed and for the correlation between the adiposity percentage and IL-6 levels the Spearman correlation was performed.

All statistical analyzes were performed using Graph Pad 6.0 software. The significance level was set at 5%. Results were expressed as mean ± standard deviation or median ± interquartile range.

Results

In order to verify the efficacy of the consumption of the high-fat diet, a control group (n = 6) was created that received a standard diet, however, we chose to work only with the rats that consumed the high-fat diet.

The body mass of rats was verified at baseline (T= 0), at week 12 (before ovariectomy) and at the end of the study (after ovariectomy T= 24). There were no changes in

T = 0 (P = 0.547) or T = 12 (P = 0.541) regarding body mass, however at week 24 (Figure 2A) the HFD + OVX group had higher final body mass than the groups. C, HFD and HFD + Polluted (P = 0.0005).

When analyzing the pre-ovariectomy body mass gain, (Delta 1) no changes were observed at this time of the experiment (P = 0.582), but in the second half of the experimental period, which refers to post ovariecomy (Delta 2), we observed the effect of ovariectomy (HFD+OVX and HFD+OVX+Polluted) on the body mass gain of the animals compared to group C, in addition, the HFD+OVX group showed higher body mass gain than the HFD+Polluted group (P = 0.0008; Figure 2B). The percentage of adiposity was influenced by ovariectomy as observed in Figure 2C, where the ovariectomized groups (HFD+OVX and HFD+Polluted+OVX) had a higher percentage of adiposity than group C (P = 0.0003; Figure 2C).

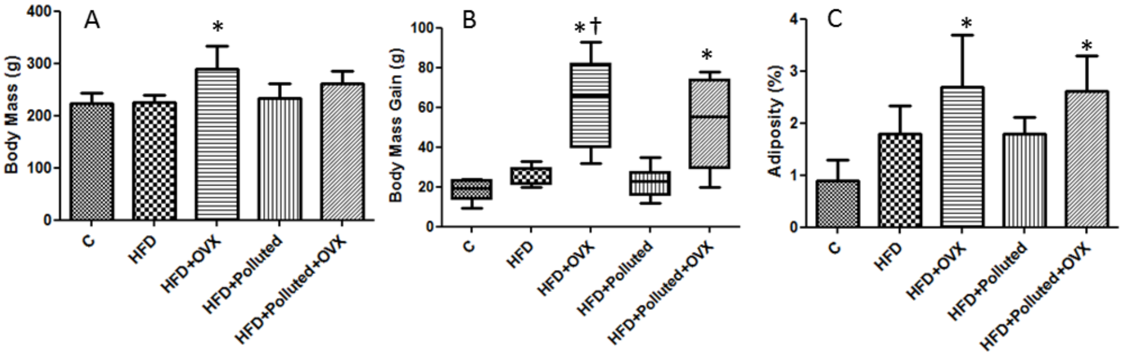

Figure 2: Effects of exposure to air pollution on body mass (g) at T = 24 (A), pre-ovariectomy body mass gain (g) (B) and adiposity percentage (C) of rats treated with a high-fat diet and ovariectomized. (A) - body mass at T = 24, *vs C, HFD and HFD+Polluted, P = 0.0005; One-way ANOVA followed by Tukey test. (B) Delta 2 (T = 24 - T = 12) *vs C; †vs HFD + Polluted, P = 0.0000, Kurskal Wallis Test followed by Dunn's. (C) - Percent adiposity, *vs C, Kurskal Wallis test followed by Dunn's. (P = 0.003). Data are expressed as mean ± SD or median and interquartile range.

Figure 3 shows that there is a positive correlation (r = 0.808) between body mass gain after ovariectomy (Delta 2) and adiposity percentage, indicating that the higher the body mass gain the higher the percentage of adipose tissue (P = 0.0001). We also observed that the groups that presented adiposity percentage above 2.5% and body mass gain after ovariectomy above 50 g were the ovariectomized groups (HFD+OVX and HFD+Polluted+OVX), suggesting that the body mass gain It was based on the accumulation of adipose tissue due to ovariectomy.

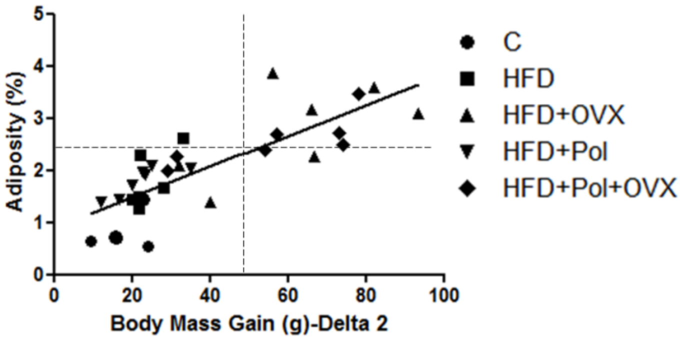

Figure 3: Correlation between body mass gain after ovariectomy (Delta 2) and percentage of adiposity in rats chronically exposed to air pollution treated with a hyperlipid and ovariectomized diet. Pearson correlation (P = 0.0001; r = 0.808).

In the Open Field Test (figure 4) there were no differences between groups in terms of exploration (P = 0.388, Figure 4A), grooming (P = 0.394, Figure 4B) and number of fecal cakes (P = 0.616; Figure 4C).

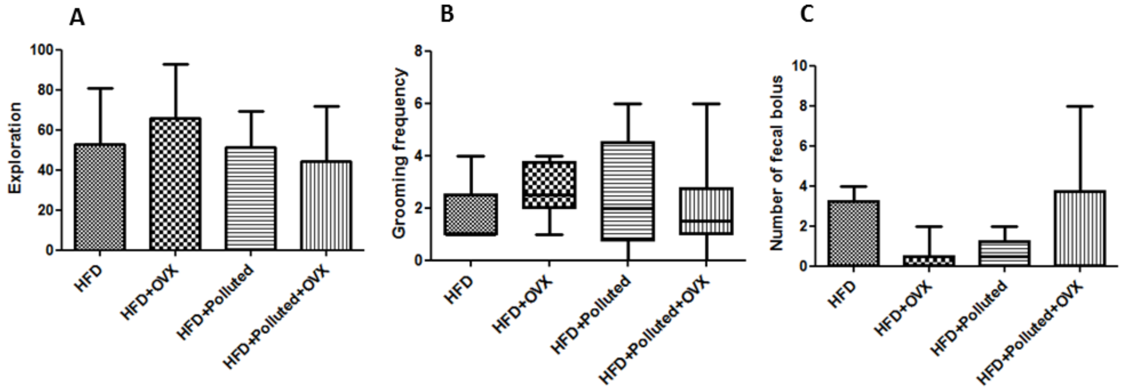

Figure 4: Effect of exposure to atmospheric pollution on exploratory behavior in rats treated with high fat and ovariectomized diet. A - Exploration behavior (sum of number of crossed squares and frequency of creation); P = 0.388; One way ANOVA; B - frequency of preparation; P = 0.394; Kruskal Wallis. C - Number of fecal cakes; P = 0.616 Kruskal-Wallis test. Data are expressed as mean ± SD or median and interquartile range.

In addition, there were no differences in length of stay (P = 0.398, Figure 5A) and frequency of entry into the closed arms (P = 0.446, Figure 4B) in EPM. In addition, there was no difference in length of stay in the open arm (P = 0.053; Figure 5C). However, the HFD+OVX group showed an anxious behavior, evidenced by the decrease in EPM open arms inputs (P = 0.007; Figure 5D) compared to the HFD group.

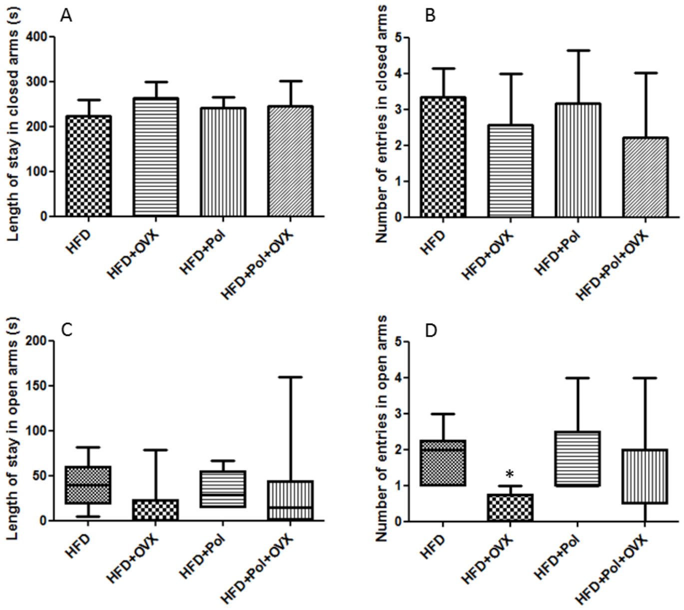

Figure 5: Effect of exposure to air pollution on anxiety behavior in rats treated with hyperlipidic diet and ovariectomized. (A) - Length of stay in the closed arm (s); P = 0.397; One way ANOVA; (B) - Frequency of entrances in closed arms; P = 0.282; Kruskal Wallis; (C) - Length of stay in the open arm (s); P = 0.053; Kruskal-Wallis test. (D) - Frequency of open arm entrances. *vs HFD; P = 0.007. Kruskal-Wallis test, Dunn's post hoc. Data expressed as mean ± SD or median and interquartile range.

In terms of depressive behavior, the polluted groups (HFD+Polluted and HFD+Polluted+OVX) showed greater immobility compared to the HFD group in the forced swim test (Figure 6), and the HFD+Polluted+OVX group showed greater immobility compared for the ovariectomized group (HFD+OVX) (P = 0.002, Figure 6).

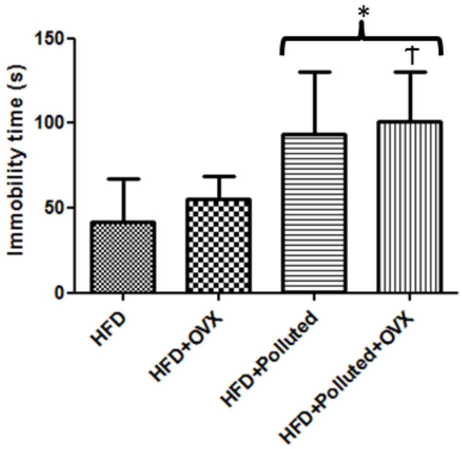

Figure 6: Effect of exposure to air pollution on depressive-type behavior in rats treated with high fat diet and ovariectomized. Immobility (s) *vs HFD and †vs HFD+OVX; P = 0.002; One-way ANOVA, followed by Tukey's test. Data expressed as mean ± SD.

Table 2 presents the results of the oxidative stress markers in the following brain structures: hypothalamus, hippocampus, cortex and cerebellum. We observed no difference between the groups in lipid peroxidation levels and SOD antioxidant activity levels in the hypothalamus (P = 0.453 and P = 0.116), hippocampus (P = 0.530 and P = 0.586), cortex (P = 0.831 and P = 0.202) and cerebellum (P = 0.847 and P = 0.204).

When compared to historical control data, we found no significant differences in the above parameters (data not shown).

Table 2. Effect of exposure to atmospheric pollution on oxidative stress parameters of rats treated with high-fat diet and ovariectomized.

	HFD (n=6)	**HFD +OVX (n=9)**	**HFD +Polluted (n=6)**	**HFD +Polluted +OVX (n=9)**	**P value**
Hypoyhalamus					
TBA (µmolMDA/mgProtein)	0.18 ± 0.24	0.33 ± 0.21	0.17 ± 0.11	0.29 ± 0.25	0.351
SOD (USOD/mgProtein)	45.8 ± 12.1	33.7 ± 18,7	32.6 ± 12.7	49.8 ± 13.8	0.126
Hippocammpus					
TBA (µmolMDA/mgProtein)	0.02 ± 0.02	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.539
SOD (USOD/mgProtein)	44.4 ± 10.9	36.6 ± 11.0	41.2 ± 9.4	40.0 ± 12.2	0.599
Cortex					
TBA (µmolMDA/mgProtein)	0.04 ± 0.03	0.04 ± 0.02	0.04 ± 0.03	0.03 ± 0.03	0.831
SOD (USOD/mgProtein)	32.8 ± 19.5	48.5 ± 10.2	34.3 ± 16.5	37.5 ± 16.4	0.202
Cerebellum					
TBA (µmolMDA/mgProtein)	0.35 ± 0.25	0.32 ± 0.26	0.45 ± 0.29	0.38 ± 0.31	0.830
SOD (USOD/mgProtein)	35.4 ± 18.5	39.9 ± 9.51	46.8 ± 13.2	32.2 ± 11.8	0.260

MDA: Malondialdehyde; SOD: superoxide dismutase. Data expressed in mean ± SD; One Way ANOVA.

When analyzing the inflammatory profile (Table 3) we observed that the OVX groups (HFD+OVX and HFD+Polluted+OVX) showed a decrease in IL-6 levels compared to the HFD group (P = 0.038) whereas IL-10 e eHSP70 levels were not different between groups (P = 0.300 and P = 0.073, respectively).

Table 3. Effect of exposure to air pollution on inflammatory markers of rats treated with high-fat diet and ovariectomized.

	HFD (n=6)	**HFD +OVX (n=9)**	**HFD +Polluted (n=6)**	**HFD+ Polluted +OVX (n=9)**	**P value**
IL-6 **(ρg/mL)**	10,212 (691.4-15,295)	404.4 (17.4-5,252)*	5,305 (679.4-10,472)	324.4 (19.4-9,152)*	0.038
IL-10 **(ρg/mL)**	964 (727-2,296)	939 (632-3,330)	798 (426-1,421)	1,009 (604-2,784)	0.300
eHSP70 **(ρg/mL)**	600 (90-3,190)	880 (360-2,350)	690 (520-3,97)	350 (140-690)	0.073

Data expressed as median and interquartile range. Kruskal Wallis test followed by Dunn's test. *Significant difference to HFD (P=0.038).

As the ovariectomized groups (HFD+OVX and HFD+Polluted+OVX) had lower plasma levels of IL-6, and had higher accumulation of adiposity, we correlated these two variables and found a negative correlation between them as observed in Figure 7 (r = -0.438; P = 0.022).

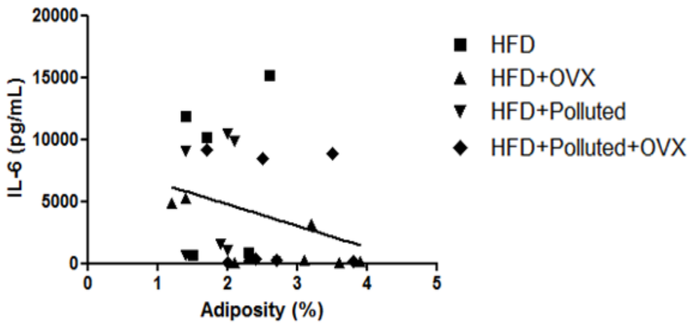

Figure 7: Correlation between adiposity percentage and interleukin 6 levels of rats chronically exposed to air pollution treated with a high-fat diet and ovariectomized. Spearman correlation (P = 0.022; r = -0.438).

Discussion

In our study, we investigated the effects of chronic exposure to air pollution on behavioral changes, oxidative stress, and inflammatory markers in rats treated with a hyperlipidic and ovariectomized diet. We found that exposure of environmental particles promoted depressive-type behavior without impact of low estrogen levels and changes in redox state.

Studies show that high-fat diet-induced obesity in animal models better mimics the physiological functions of an obese human body (Wang et al 2017). Thus, we used a 58.3% fat diet, a well-known protocol for inducing metabolic diseases in rats and mice, such as obesity and type 2 diabetes (Goettems-Fiorin et al. 2016; Kostrycki et al. 2016). Chronic DHL consumption may increase body adiposity, triglycerides and plasma glucose levels (Banin et al, 2014). Regarding behavioral effects, 20 weeks of DH intake (49% fat) (since weaning) in male rats induces anxious behavior observed by the EPM test (André et al. 2014), and adult rats who consumed a DHL (60 Fat%) for 10 weeks showed an increase in anxiety behavior in the light / dark transition and open field test tasks compared to the group receiving a low fat diet (Sivanathan et al 2015). Our study also treated adult rats with DHL (58.3%), however we did not observe the effect of eating this diet, but the decrease in estrogen levels through ovariectomy promoted anxiety behavior in EPM, and exposure to pollution in the FST. Our data suggest that hormonal changes and pollution impact the behavior of rats treated with HFD.

In inflammatory markers, we found a decrease in IL-6 plasma levels in the HFD+OVX and HFD+Polluted+OVX groups, and no change in IL-10 levels. As these groups presented higher adiposity and lower IL-6 levels, our data show a negative correlation between these two parameters. Although IL-6 can be considered an adipocyte cytokine (adipokine), it is well known that plasma levels of this cytokine are considered muscle derived signaling (myocin), with many metabolic benefits against obesity and diabetes, including inflammatory pathways (Pedersen et al. al. 2008). Thus, the decrease in IL-6 levels, demonstrated by ovariectomized groups, may represent a loss of systemic signaling function of cytokines in rats treated with HD, promoting behavioral changes found in our study. Corroborating these observations Baier et al. (2009) showed that rats with low IL-6 levels had impaired memory tasks and reduced exploratory behavior.

Another factor that should be taken into account is the content of dietary lipids, since the type of fatty acids induces a differentiated response to storage and the consequent relation with changes in lipid metabolism, being that saturated fatty acids are considered more adipogenic and fatty acids interfering with normal levels of lipoproteins, triglycerides and markers of oxidative stress and inflammation (Ohashi et al., 2014; Lorente-Cebrían et al., 2013; Micha; Mozaffarian, 2010).

Freeman (2014) shows that IL-1, IL-6 and TNF - α are examples of proinflammatory cytokines orchestrating the inflammatory response to many stimuli, both systemically and in the brain. IL-6, in particular, is an immune-mediated inflammatory signaling molecule and is associated with immune central nervous system communication (McAfoose and Baune, 2009; Yirmiya and Goshen, 2011; Spooren et al., 2011; Gruol, 2015). In addition, IL-6 has functional properties during neurological development and brain function (Stolp et al. 2013), suggesting that decreased IL-6 plasma levels may induce behavioral changes.

White adipose tissue (WAT) has receptors that respond to signals from the central nervous system and other hormonal systems. Here we highlight the receptors for insulin, glucagon, IL-6 and TNF-α (Kershaw and Flier 2004). Adipose tissue resident immune cells in an obesity setting have emerged as the primary source of IL-6 (Hotamisligil 2008), indicating that IL-6 is related to the onset of insulin resistance. However, it should be mentioned that cytokines collectively regulate adipose tissue metabolism. In addition to IL-6, adipocytes can synthesize and release tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) (Coppack 2001; Kern et al. 2001; Wu et al. 2007). The balance between pro and anti-inflammatory cytokines is the determinant of homeostasis (You 2011). In the depressive and anxious state this balance is broken, with higher proinflammatory cytokine (IL-1, IL-6, IL-8 and TNF-α) and lower levels of anti-inflammatory cytokines (IL-10 and TGF-β) (O'Brien et al. 2004; O'Brien et al. 2007; Dhabhar 2009). Thus, no change in plasma levels of IL-10, an anti-inflammatory cytokine, may be the result of complex cytokine network signaling in HD-treated ovariectomized rats. Sinvanathan (2015) has shown that chronic DHL consumption can alter anxiety-like behavior, at least in part, through changes in glucocorticoid signaling mechanisms in the limbic brain regions.

Menopause is a physiological condition that affects women between 45 and 55 years old, characterized by cessation of ovarian function (Dalal and Agarwal 2015). The resulting hormonal changes are associated with cardiovascular and metabolic diseases, with various impacts on women's quality of life (Al-Safi and Santoro 2014; Jenabi et al. 2015). In postmenopausal women, metabolic diseases are related mainly due to decreased estrogen levels (Martins et al., 2018), as shown in our HFD+OVX and HFD+Polluted+OVX groups. Estrogen is a signaling molecule that regulates and coordinates multiple functions in organs, cells and genes (Retteberg et al. 2014) and plays an important role in homeostatic control of food intake and body weight (Eckel, 2011; Asarian and Geary, 2013).

Estrogen has relevant roles in cognition and neuroprotection (Gibbs and Aggarwal 1998) and decreased levels are associated with neurodegenerative diseases (Shepherd 2001). Thus, in OVX rats, after 12 weeks of hypoestrogenism, animals exhibited greater anxious behavior compared to themselves three weeks earlier (Picazo 2006). In our study, HFD+OVX showed a decrease in open arm entries in the EPM test, indicating an anxiety behavior, which was already observed by Moreira et al (2015). Although hypoestrogenism is related to increased ROS production and decreased antioxidant defense (Crist et al, 2009), in our study we found no changes in brain structures in relation to oxidative stress biomarkers. Since all animals were on HFD treatment, the effects of low estrogen levels in the redox state may be obscured by the effects of obesity on oxidative biomarkers. Obesity induces a decrease in enzymatic and non-enzymatic antioxidant defenses (CAT, SOD and glutathione), and an increase in lipid peroxidation in the hippocampus (Freeman et al. 2014) and in the prefrontal cortex (Sousa et al. 2007). Thus, exposure to particulate air pollution in our study daily had no effect on oxidative stress or inflammatory markers in DHA-treated rats, but induced depressive behavior in these animals. Environmental pollution can affect the central nervous system (Calderön-Garcidueñas et al. 2007; Suglia et al. 2008), since subchronic (30 days) exposure to ROFA (20 µg) reduced peripheral and general exploration in observed rats. in the open field test (Zanchi et al. 2008).

Studies show that climacteric, represented by decreased estrogen levels, promotes changes in inflammatory markers and oxidative stress (Machi et al, 2016) and led to the development of insulin resistance and cognitive impairment associated with obesity (Evsen et al., 2013 ; Henderson, 2008; Matsuzawa-Nagata et al., 2008; Pintana et al., 2012).

We also measured eHSP70 levels in our study. Under stress conditions such as oxidative stress and inflammation (Geiger and Gupte 2011), these proteins are overexpressed in various tissues, and their plasma levels are associated with observed obesity damage (Krause et al. 2015), particulate air pollution (Baldissera et al. 2018) and the combination of these factors (Goettems-Fiorin et al. 2016). Since oxidative stress is a critical factor in altering eHSP70 levels, the absence of difference in oxidative stress markers between experimental groups is in agreement with no change in eHSP70 levels (Malyshev et al. 2000). Moreover, as decreased HSP70 expression and release may represent an aging effect with impaired cellular homeostasis (Balch et al. 2008; Swindell et al. 2009), ovariectomy alone may be insufficient to alter the response to thermal shock of rats (Miragem et al. 2015).

Conclusion

Chronic exposure to air pollution promotes depression-like behavior in rats treated with a high-fat and ovariectomized diet, independent of oxidative stress in brain structures. However, we observed that ovariectomy, without influence of exposure to air pollution, increased body mass gain and adiposity, promoted anxiety behavior and decreased interleukin 6 levels, even without changes in oxidative stress parameters. Our study reinforces that these factors can act independently in inducing behavioral changes.

Acknowledgments

Declaration of interest**:** The authors report no conflicts of interest.

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6 CONCLUSÃO

A realização do presente estudo mostrou que a exposição a poluição atmosférica, associada ao consumo de uma dieta hiperlipídica em ratas ovariectomizadas foi capaz de desencadear um comportamento do tipo depressivo. Também constatamos que o efeito da ovariectomia no ganho de massa corporal e adiposidade, e promoveu um comportamento de ansiedade.

Nos parâmetros de estresse oxidativos avaliados, TBA e SOD, as intervenções não foram capazes que alterar o estado redox do hipotálamo, hipocampo, córtex cerebral e cerebelo, bem como não alteraram os níveis de interleucina-10 e a expressão de HSP70 extracelular, no entanto a ovariectomia foi capaz de diminuir os níveis de interleucina 6. Entretanto ainda há necessidade de mais estudos para investigar que outras modificações estão acontecendo, em nível celular, e até mesmo analisar outras estruturas cerebrais que possam estar envolvidas nas modificações comportamentais.

7 ANEXOS

7.1 ANEXO A - Parecer da Comissão de Ética no Uso de Animais (CEUA)

UNIJUÍ UNIVERSIDADE REGIONAL

CEUA Comissão de Ética no Uso de Animais da UNIJUÍ

PARECER CONSUBSTANCIADO CEUA - 076/2015

Comissão de Ética no Uso de Animais da UNIJUÍ

PARA USO EXCLUSIVO CEUA	
PROTOCOLO Nº 044/15	ESPÉCIE ANIMAL: Ratos
DATA DE PROTOCOLO: 30/10/2015	QUANTIDADE: 64
DATA DA REUNIÃO: 27/11/2015	SEXO: Fêmeas
DURAÇÃO: Início: 01/02/2016 Término: 30/03/2018	IDADE E PESO (aproximado): 21 dias - 30 – 50g

I – IDENTIFICAÇÃO

(X) PROTOCOLO DE PESQUISA

II – PESQUISADOR RESPONSÁVEL

Pauline Brendler Goettems Fiorin

III – INSTITUIÇÃO/DEPARTAMENTO

DCVIDA – UNIJUÍ

IV – TÍTULO DO PROTOCOLO

COMBINAÇÃO ENTRE DIETA HIPERLIPÍDICA E EXPOSIÇÃO CRÔNICA À MP 2,5 EM RATAS OVARECTOMIZADAS: EFEITOS EM PARÂMETROS METABÓLICOS, OXIDATIVOS E INFLAMATÓRIOS NO DESENVOLVIMENTO DO DIABETES.

V – CONSIDERAÇÕES DO PARECERISTA

O protocolo apresenta a sua aplicabilidade e conformidade com os requisitos éticos, sendo de parecer favorável à realização deste.

VI – SITUAÇÃO

(x) APROVADO

De acordo,

Ijuí, 10 de dezembro de 2015.

Prof. Dr. Fernando Silvério Ferreira da Cruz
Coordenador da CEUA UNIJUÍ

Comissão de Ética no Uso de Animais - CEUA/UNIJUÍ
Telefone: (55) 3332-0301
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Rua do Comércio, 3000, Bairro Universitário, Ijuí/RS – Brasil CEP 98700-000.

7.2 ANEXO B - Normas de Publicação da Revista “Inhalation Toxicology”

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