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**Efeitos da Exposição Crônica ao ROFA em
Parâmetros Metabólicos, Oxidativos e
Inflamatórios de Ratas Obesas Ovariectomizadas**

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Coorientador: Prof. Dr. Thiago Gomes Heck

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“Não se pode criar experiência. É preciso passar por ela.”
Albert Camus

1. RESUMO

INTRODUÇÃO: Estudos epidemiológicos e experimentais têm demonstrado forte associação entre obesidade (induzida por dieta hiperlipídica - DHL) e exposição à poluição atmosférica (Material Particulado Fino – MP_{2,5}) no desenvolvimento de doenças crônicas. Além disso, a redução nos níveis de estrogênio no período pós-menopausa também promove efeitos diretos no metabolismo, que podem ser agravados por outros fatores de susceptibilidade, como a combinação entre DHL e exposição ao MP_{2,5}. Os três fatores implicados na presente proposta deste estudo (MP_{2,5}, DHL e hipoestrogenismo) caracterizam-se individualmente pelo desenvolvimento de um quadro pró-inflamatório e pró-oxidativo que predispõe as mulheres ao desenvolvimento de doenças cardio-metabólicas. Contudo, poucos estudos têm documentado o efeito sinérgico destes fatores. Nesse sentido, se propôs investigar os efeitos metabólicos, oxidativos e inflamatórios da exposição ao MP_{2,5} em ratas obesas ovariectomizadas. **METODOLOGIA:** Para realização integral deste estudo foram utilizadas 54 ratas Wistar com 8 semanas, subdivididas inicialmente em 4 grupos (CTRL; ROFA; DHL; DHL+ROFA), que receberam como intervenções: exposição à solução salina ou ao poluente ROFA (250µg) via instilação intranasal (5 vezes/semana); dieta padrão (11,4% de gordura) ou dieta hiperlipídica (DHL, 58,3% de gordura). Na 12ª semana de estudo metade das ratas cada grupo foram submetidas à cirurgia bilateral de retirada de ovários (OVX) e as demais restantes, submetidas à falsa operação (*Sham*). Desta forma, a partir da 12ª semana de estudo os animais constituíram 8 grupos diferentes, os quais continuaram seus tratamentos experimentais até completarem 24 semanas de estudo, constituindo ao final do estudo, os seguintes grupos (n=6-9/grupo): CTRL; ROFA; DHL; DHL+ROFA; OVX; ROFA +OVX; DHL+OVX; DHL+ROFA +OVX. Ao final do estudo foram realizadas análises biométricas, parâmetros hematológicos e bioquímicos, e parâmetros de estresse oxidativo (TBARS, CAT e SOD) e inflamatório (IL-10, IL-6, HSP70). Os dados foram analisados estatisticamente por ANOVA de uma via (post hoc Tukey) no programa GraphPad 5.0 (p<0,05). **RESULTADOS:** A redução nos níveis de estrogênio (OVX) promoveu aumento do peso corpóreo e de adiposidade, desenvolvimento de edema hepático, hipercolesterolemia e

intolerância à glicose, bem como perfil pró inflamatório (redução de IL-10 e aumento na razão IL-6 / IL-10). O grupo ROFA+OVX apresentou aumento de neutrófilos e da razão Neutrófilo/Linfócito, redução de defesa antioxidante (SOD) e aumento nos níveis de HSP70 hepática, indicando perfil pró-oxidativo, pró-inflamatório e resposta ao estresse celular. O consumo de DHL de forma isolada promoveu aumento dos níveis de colesterol hepático, e quando associado a OVX promoveu aumento de triglicerídeo hepático e formação de edema no fígado, no entanto, quando associados à exposição à poluição atmosférica (ROFA+DHL+OVX) não houve efeitos dessa associação. CONCLUSÃO: Em condição de declínio nos níveis de estrogênio, na pós-menopausa, ocorre um maior risco para desenvolvimento de doenças metabólicas, quando associado a fatores de risco isolados, como poluição atmosférica ou consumo de dieta hiperlipídica.

PALAVRAS-CHAVE: ovariectomia; estrogênio; metabolismo hepático; dieta hiperlipídica; material particulado; ROFA; poluição atmosférica.

2. ABSTRACT

INTRODUCTION: Epidemiological and experimental studies have shown a strong association between obesity (induced by high-fat diet - HFD) and exposure to air pollution (Fine Particulate Material - PM_{2.5}) in the development of chronic diseases. In addition, the reduction of estrogen levels in postmenopausal also promotes direct effects on metabolism, which may be aggravated by other susceptibility factors, such as the combination of HFD and PM_{2.5} exposure. These three factors implicated in the present proposal of this study (PM_{2.5}, HFD and hypoestrogenism) are individually characterized by the development of a pro-inflammatory and pro-oxidative condition, that predispose women to the development of cardio-metabolic diseases. However, few studies have documented the synergistic effect of these factors. In this sense, it was proposed to investigate the metabolic, oxidative and inflammatory effects of PM_{2.5} exposure in ovariectomized obese rats. **METHODS:** A total of 54 8-week-old Wistar rats were initially divided into 4 groups (CTRL; ROFA; DHL; DHL + ROFA). The following interventions were used: exposure to saline solution or ROFA pollutant (250µg). intranasal instillation (5 times / week); standard diet (11.4% fat) or high fat diet (HFD, 58.3% fat). In the 12th week of the study, half of the rats in each group underwent bilateral ovarian removal surgery (OVX) and the remaining underwent to false surgery (Sham). Thus, from the 12th week of study the animals constituted 8 different groups, which continued their experimental treatments until completing 24 weeks of study, constituting the following groups (n = 6-9 / group): CTRL; ROFA; HFD; HFD+ROFA; OVX; ROFA+OVX; HFD+OVX; HFD+ROFA+OVX. At the end of the study, biometric, hematological and biochemical parameters, was analyzed and oxidative stress (TBARS, CAT and SOD) and inflammatory (IL-10, IL-6, HSP70) parameters were performed. Data were statistically analyzed by one-way ANOVA (post hoc Tukey) in GraphPad 5.0 software (p <0.05). **RESULTS:** Reduction in estrogen levels (OVX) promoted increased body weight and adiposity, development of hepatic edema, hypercholesterolemia and glucose intolerance, as well as pro-inflammatory profile (reduction of IL-10 and increase in IL-6 / IL-10). ROFA + OVX group showed increased neutrophils and Neutrophil / Lymphocyte ratio, reduced antioxidant defense (SOD) and increased hepatic HSP70 levels,

indicating pro-oxidative, pro-inflammatory profile and cell stress response. HFD consumption alone increased liver cholesterol levels, and when associated with OVX promoted hepatic triglyceride and liver edema formation, however, when associated with exposure to air pollution (HFD+ROFA+OVX) no effects were observed. **CONCLUSION:** In a condition of reduction of estrogen levels, in post-menopause, there is a higher risk for the development of metabolic diseases, when associated with isolated risk factors, such as air pollution exposure or high-fat diet consumption.

KEYWORDS: ovariectomy; estrogen; liver metabolism; high-fat diet; particulate matter; ROFA; atmospheric pollution.

3. LISTA DE ABREVIATURAS

ANOVA: Análise de Variância

CAT: Catalase

CO: Monóxido de carbono

CONAMA: Conselho Nacional do Meio Ambiente

DHL: Dieta Hiperlipídica

DM: Diabetes mellitus

DM2: Diabetes mellitus tipo 2

DNA: Ácido Desoxirribonucleico

EDTA: *do inglês Ethylenediaminetetraacetic Acid*

eHSP70: Proteína de Choque térmico extracelular

ELISA: *do inglês Enzyme-Linked Immunosorbent Assay* (Ensaio de Imunoadsorção Enzimática)

EO: Estresse oxidativo

EPA: *do inglês Environmental Protection Agency* (EUA) (Agência de Proteção Ambiental)

ERO: Espécies Reativas de Oxigênio

GLUT-4: transportador de glicose 4

GPx: Glutathione Peroxidase

GTT: *do inglês Glucose Tolerance Test* (Teste de Tolerância à Glicose)

H₂O: Água

H₂O₂: Peróxido de Hidrogênio

HCl: Ácido Clorídrico

HDL: *do inglês High Density Lipoprotein* (lipoproteína de alta densidade)

HFD: *do inglês High Fat Diet* (Dieta Hiperlipídica)

HOMA-IR: *do inglês Homeostatic Model Assessment* (Avaliação do Modelo Homeostático)

HSP70: Proteína de Choque térmico de 70 KDa

HSPs: *do inglês Heat Shock Proteins* (Proteínas de Choque térmico)

i.n.: Intranasal

i.p.: Intraperitoneal

IAUC: *do inglês Incremental Area Under the Curve* (Área incremental sob a Curva)

iHSP70: Proteína de Choque térmico intracelular

IL-1: Interleucina 1

IL-10: Interleucina 10

IL-6: Interleucina 6

IMC: Índice de Massa Corporal

IR: *do inglês Insulin Resistance* (Resistência à Insulina)

KPi: *do inglês Potassium Phosphate Buffer* (Tampão Fosfato de Potássio)

LPO: Lipoperoxidação

MDA: Malondialdeído

MP: Material Particulado

MP₁₀: Material Particulado Grosso <10µM
MP_{2,5}: Material Particulado Fino <2,5µM
NO₂: Dióxido de Nitrogênio
O₂: Oxigênio
O₂^{•-}: Radical Superóxido
O₃: Ozônio
OH[•]: Radical Hidroxila
OMS: Organização Mundial da Saúde
OVX: Ovariectomia
PCR: Proteína C reativa
PM: *do inglês Particulate Matter* (Material Particulado)
PM_{2.5}: *do inglês Fine Particulate Matter* (Material Particulado Fino <2,5µM)
PMSF: *do inglês Phenylmethanesulfonyl Fluoride* (Fluoreto de Fenilmetanossulfonilo)
ROFA: *do inglês Residual Oil Fly Ash* (Resíduo da queima de óleo diesel)
SD: *do inglês Standard Deviation* (Desvio Padrão)
SDS: *do inglês Sodium Dodecyl Sulfate* (Dodecilsulfato de sódio)
SEM: *do inglês Standard Error of Mean* (Erro Padrão Médio)
SO₂: Dióxido de Enxofre
SOD: Superóxido Dismutase
TBARS: *do inglês Thiobarbituric Acid Reactive Substances* (Teste de Substâncias Reativas ao Ácido Tiobarbitúrico)
TNF-α: *do inglês Alpha Tumor Necrosis Fator* (Fator de Necrose Tumoral Alfa)
TRIS: 2-amino-2-hydroxymethyl-propane-1,3-diol
VCD: 4-vinylcyclohexene diepoxide

4. REFERENCIAL TEÓRICO

4.1. OBESIDADE

Condições de sobrepeso e obesidade são estabelecidas como principais fatores de risco para uma série de doenças crônicas, incluindo diabetes mellitus (DM), doenças cardiovasculares e câncer. Globalmente, a etiologia para o aumento das taxas de obesidade se devem à ingestão de alimentos altamente energéticos que são ricos em gordura, e um aumento na inatividade física devido à natureza cada vez mais sedentária do estilo de vida (WHO, 2013).

A obesidade é definida simplesmente como uma condição de acúmulo anormal ou excessivo de gordura no tecido adiposo, que pode ser prejudicial à saúde. São considerados indivíduos sobrepesos, aqueles que apresentem Índice de Massa Corporal (IMC) igual ou superior a 25 kg/m^2 , e obesos, aqueles que apresentam IMC igual ou superior a 30 kg/m^2 , calculado a partir do peso em quilos dividido pelo quadrado da altura em metros (WHO, 2000).

A Organização Mundial de Saúde aponta a obesidade como um dos maiores problemas de saúde pública no mundo. A projeção é que, em 2025, cerca de 2,3 bilhões de adultos estejam com sobrepeso; e mais de 700 milhões de obesos. No Brasil, a obesidade vem crescendo cada vez mais. Alguns levantamentos apontam que mais de 50% da população está acima do peso, ou seja, na faixa de sobrepeso e obesidade (ABESO, 2019).

Em Porto Alegre, o índice de obesidade (IMC) em adultos é de 19,9%, sendo 19,4% para homens e 20,3% para mulheres. No conjunto da população adulta, o grau de obesidade é ainda maior para indivíduos entre 55 e 64 anos (BRASIL, 2017).

No Brasil, o crescimento da população idosa tem ocorrido de forma acelerada e a expectativa de vida da população feminina é maior em relação à masculina. Como resultado do aumento da expectativa de vida nos países desenvolvidos, um maior número de mulheres vai experimentar mudanças relacionadas ao climatério, e passarão a segunda metade de suas vidas em um estado de deficiência de estrogênio (GONÇALVES et al., 2016; LIZCANO; GUZMÁN, 2014).

Na faixa etária entre 55 e 64 anos, dados da população de Porto Alegre indicam que as mulheres possuem um percentual de 25% de obesidade, ou seja, um quarto das mulheres nesta faixa etária apresenta-se em situação de obesidade, período no qual as mulheres vivenciam a pós-menopausa (BRASIL, 2017).

As razões para o aumento da obesidade em mulheres na menopausa não são claras, alguns estudos sugerem que a ausência de estrogênios pode ser um importante fator desencadeante da obesidade (LIZCANO; GUZMÁN, 2014). O ganho de peso entre mulheres de meia idade tem sido frequentemente relatado, mas as inter-relações entre obesidade, ganho de peso e a transição da menopausa permanecem incompletamente compreendidas (AL-SAFI; POLOTSKY, 2015).

Muitas mulheres relatam ganhar peso à medida que passam pela menopausa. Para a maioria, o ganho de peso é modesto e pode ser reduzido com um esforço consciente para limitar o consumo de energia e aumentar o gasto de energia. No entanto, muitas mulheres que já estão acima do peso e obesas ganham mais peso à medida que se aproximam da menopausa.

Estudo conduzido em São Paulo avaliou 930 mulheres com idade média de $56,15 \pm 6,26$ anos, que atingiram a menopausa na idade média de $47,15 \pm 5,80$ anos, e constatou o número de 294 obesas (31,61% da amostra total), sendo o IMC médio de $33,9 \pm 3,2$ kg/m². Ou seja, um terço do grupo de menopausadas apresenta-se em condição de obesidade, sendo importante ainda considerar que 39,14% apresentavam sobrepeso (LIMA, 2015), ou seja, mais de 70% destas mulheres apresentam aumento de acúmulo de gordura corporal. As autoras consideram que o hipoestrogenismo estaria basicamente implicado na modificação da distribuição da gordura corporal, o que favorece o desenvolvimento de adiposidade visceral e consequente obesidade. Além disso, esse tipo de distribuição de gordura, está associado à hipertensão, resistência à insulina, hipertrigliceridemia, e diminuição das concentrações séricas de HDL, desencadeando síndrome metabólica e aumentando o risco de doenças crônicas como diabetes tipo 2 e doenças cardiovasculares (LIMA, 2015).

Modelos animais têm sido utilizados para elucidar os mecanismos celulares através dos quais a obesidade é desenvolvida. O modelo que mais se

assemelha ao desenvolvimento da obesidade humana é o modelo de obesidade exógena, caracterizado pelo consumo de dietas com maior aporte calórico, através de uma sobrecarga de carboidratos ou de gordura, mimetizando uma condição sócio comportamental. Neste modelo experimental, acrescenta-se, ou associa-se à ração padrão substâncias altamente calóricas (Luís & Cesaretti, 2006).

Dietas hiperlipídicas (DHL), contendo mais de 40% de energia com base em gordura animal, têm apresentado maiores efeitos sobre o ganho de peso quando comparado a outros tipos de dieta (BUETTNER; SCHÖLMERICH; BOLLHEIMER, 2007). Desta forma, enfatizamos em nossa pesquisa os efeitos do consumo de dieta hiperlipídica contendo mais de 58% de gordura animal, especificamente banha de porco misturada à ração padrão, associado a outros fatores de risco, como exposição à poluição atmosférica e o hipoestrogenismo.

4.2. POLUIÇÃO ATMOSFÉRICA

O termo poluente diz respeito a substâncias químicas, partículas ou gases tóxicos, introduzidas no meio ambiente por diversas fontes e que causam efeitos adversos aos seres vivos e/ou no ecossistema, comprometendo o solo, a água e o ar atmosférico (EPA, 2010). A poluição atmosférica, por sua vez, pode ser definida como 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)

As principais fontes de poluentes atmosféricos são as antropogênicas, diferenciadas em fontes fixas e fontes móveis. As mesmas são classificadas como fontes móveis, que incluem a maioria das formas de transporte, tais como automóveis, caminhões e aviões, ou fontes fixas, que incluem instalações industriais e habitacionais.

Os poluentes emitidos diretamente pelas fontes poluidoras são chamados de poluentes primários, como o Material Particulado (MP), Dióxido de Enxofre (SO₂), Dióxido de Nitrogênio (NO₂), Monóxido de carbono (CO), enquanto os que resultam de interações com o meio ambiente são poluentes secundários.

O Material Particulado (MP) é o que, dentre os diversos poluentes, tem recebido maior ênfase em pesquisas científicas epidemiológicas e experimentais. O termo Material Particulado se refere à uma mistura de sólidos e gotículas de líquido suspensas no ar (EPA, 2019a). São partículas compostas por um núcleo de carbono sobre o qual os componentes químicos orgânicos de alto peso molecular e metais pesados se depositam (KELLY; FUSSELL, 2012).

O material particulado pode ser classificado em: Partículas Totais em Suspensão, que são aquelas cujo diâmetro é menor que 50 μm , ou Partículas Inaláveis, são definidas como aquelas cujo diâmetro é menor que 10 μm , que são ainda classificadas em partículas grossas – MP_{10} (2,5 a 10 μm), partículas finas – $\text{MP}_{2,5}$ (<2,5 μm) ou ultrafinas – $\text{MP}_{0,1}$ (<0,1 μm) (CETESB, 2019).

O tamanho das partículas está diretamente ligado ao seu potencial para causar problemas de saúde. Pequenas partículas com menos de 10 μm de diâmetro representam os maiores problemas, porque elas podem penetrar profundamente nos pulmões e algumas podem atingir a corrente sanguínea. A exposição a essas partículas pode afetar tanto os pulmões quanto o coração, e outros sistemas (EPA, 2019b).

Em 2005, o padrão estabelecido pela Organização Mundial de Saúde para concentrações de $\text{MP}_{2,5}$ passou de 65 $\mu\text{g}/\text{m}^3$ para 25 $\mu\text{g}/\text{m}^3$, durante o período de 24 horas e de 15 $\mu\text{g}/\text{m}^3$ para 10 $\mu\text{g}/\text{m}^3$, durante o período de um ano (WHO, 2005). Em publicação em Maio de 2018, a OMS segue considerando os mesmos padrões, 25 $\mu\text{g}/\text{m}^3$ para o período de 24 horas, e 10 $\mu\text{g}/\text{m}^3$ para a média anual (WHO, 2018).

No Brasil, os padrões de qualidade do ar final foram estabelecidos pela resolução do CONAMA nº 03/90, atualizados em Resolução Nº 491, de 19 de novembro de 2018, que dispõe sobre padrões de qualidade do ar. Considerando os níveis de material particulado fino ($\text{MP}_{2,5}$) de 25 $\mu\text{g}/\text{m}^3$ para o período de 24 horas, e 10 $\mu\text{g}/\text{m}^3$ para a média anual, os mesmos indicados pela OMS (CONAMA, 2018). No entanto, não há um monitoramento efetivo da qualidade do ar em nosso país. Conforme dados apresentados pelo Ministério do Meio Ambiente, o último levantamento nacional foi realizado em 2012, por meio do 1º Diagnóstico da Rede de Monitoramento da Qualidade do Ar no Brasil, publicado em 2014 (IEMA, 2014). Este levantamento indica os níveis de

poluição em algumas cidades do nosso país, principalmente da região centro-oeste, evidenciando médias anuais de $MP_{2,5}$ entre 19 e 34 $\mu g/m^3$ no ano de 2012, todas acima do preconizado pela OMS. Em relação ao padrão de poluição atmosférica de nosso estado (RS), o site da Fundação Estadual de Proteção Ambiental Henrique Luiz Roessler - RS (FEPAM) não apresenta registro dos níveis de poluentes atmosféricos (FEPAM, 2019). Ainda importante considerar que, apesar da Secretaria Municipal do Meio Ambiente (SMAM) de Porto Alegre possuir três estações para monitoramento da qualidade do ar na cidade (Estação Humaitá, Centro e Azenha), desde 2014 não são realizados registros (SMAM, 2019).

A poluição do ar pode afetar nossa saúde de várias maneiras, dependendo do tempo de exposição (curto ou longo prazo), do tipo de poluente, da concentração dos agentes poluidores, da faixa etária, da condição prévia de saúde, entre outros.

Estudos científicos descrevem a exposição à poluição por partículas a uma variedade de problemas, incluindo: morte prematura em pessoas com doença cardíaca ou pulmonar; ataques cardíacos não fatais; arritmia cardíaca; asma; função pulmonar diminuída; aumento dos sintomas respiratórios, como irritação das vias aéreas, tosse ou dificuldade para respirar. Pessoas com doenças cardíacas ou pulmonares, crianças e adultos mais velhos são os mais propensos a serem afetados pela exposição à poluição por partículas (EPA, 2019b).

Grupos sensíveis (também chamados de populações em risco) - Um termo usado para uma categoria de pessoas com maior risco de sofrer efeitos adversos à saúde relacionados às exposições à poluição do ar. Esses grupos podem apresentar risco aumentado devido a fatores intrínsecos (biológicos), fatores extrínsecos (externos, não biológicos), maior tempo de exposição e / ou aumento da dose em determinada concentração. A gravidade dos efeitos na saúde que esses grupos experimentam pode ser muito maior do que na população em geral (EPA, 2019).

Estima-se que a poluição do ar causou 4,2 milhões de mortes prematuras em todo o mundo em 2016, que é atribuída principalmente à exposição ao

material particulado fino (MP_{2,5}), por desenvolvimento de doenças cardiovasculares, respiratórias e cânceres (WHO, 2018).

Portanto, o estabelecimento de padrões de qualidade de ar em grandes cidades tem contribuído para realização de estimativas que auxiliem na administração de problemas ambientais e da saúde da comunidade. Bem como, a realização de pesquisas que enfatizem os mecanismos pelos quais a exposição à poluição agrava à saúde, traz um alerta sobre a necessidade do desenvolvimento de políticas de saúde pública, e de padrões de qualidade do ar mais rígidos.

Estudos de exposição à poluentes em modelos animais, que mimetizam a exposição à poluição atmosférica na população humana viabilizam o estudo dos mecanismos fisiopatológicos pelos quais o organismo desenvolve inúmeras doenças relacionadas à poluição atmosférica. Desta forma, muitos estudos com modelos de exposição *in vivo*, em experimentação animal, vêm sendo realizados. Tais estudos utilizam modelos variados de exposição à poluição atmosférica, como exposição em câmara, onde o ar é propulsionado do ambiente para a área interna da câmara, sendo filtrado (com série de filtros para impedir entrada de poluição) e não filtrado, onde os animais ficam expostos à poluição local (CHUANG et al., 2017; KAMPFRATH et al., 2011; SUN et al., 2009), concentrador de partículas de poluição atmosférica (RHODEN et al., 2004), os quais são utilizados para exposição à concentração real de partículas no ambiente, durante um período de tempo. E estudos que utilizam métodos de instilação intratraqueal e intranasal (GOETTEMES-FIORIN et al., 2016, 2019; YAN et al., 2011; ZANCHI et al., 2008). No modelo de instilação intranasal, conforme utilizado em nosso estudo, o animal é contido pelo manipulador (suspensão pela região cervical, sustentado com a mão sobre o dorso), e então é realizada administração via intranasal da suspensão em líquido (com concentração conhecida de poluente) diretamente na narina do animal que por reflexo de apnéia inala a suspensão (MEDEIROS et al., 2004).

Em nosso estudo utilizamos o modelo de exposição por instilação intranasal de ROFA. O ROFA, ou *residual oil fly ash* (resíduo da queima de óleo diesel), é o termo utilizado para se referir aos resíduos principalmente inorgânicos que permanecem após a oxidação incompleta de compostos de

carbono (GHIO et al., 2002). Geralmente as partículas de ROFA possuem tamanho menor do que 2,5 µm, e são quimicamente consideradas complexas quando comparadas às demais partículas de poluição atmosférica, por apresentar sulfatos, silicatos, carbono e nitrogênio. Além dos elementos citados, também apresenta uma grande quantidade de metais que estão naturalmente presentes em combustíveis (petróleo, parafina, e óleo diesel) e permanecem quando a fração volátil é destilada (DOMENICO, 2015). Este poluente tem sido usado em estudos experimentais como uma partícula substituta para investigar os mecanismos das respostas à inalação de MP em animais (ARANTES-COSTA et al., 2008; BISELLI et al., 2011).

Uma vez que os principais efeitos que se pretende analisar em nosso estudo, estão relacionados ao estresse oxidativo, o ROFA pode ser considerado um poluente aplicável, pois, por ser constituído principalmente por diversos metais, promove reações de Fenton, produzindo espécies reativas de oxigênio (GHIO et al., 2002)

4.3. A COMBINAÇÃO ENTRE OBESIDADE E EXPOSIÇÃO À POLUIÇÃO ATMOSFÉRICA: IMPLICAÇÕES METABÓLICAS, NO PERFIL REDOX E INFLAMATÓRIAS

O comportamento alimentar, estilo de vida e fatores ambientais têm afetado a saúde da população, resultando em altas taxas de morbimortalidade por doenças crônicas, como Diabetes tipo 2 (DM2) (WHO, 2013). A obesidade é sabidamente um fator de risco para o desenvolvimento de DM2 e segundo os dados da *World Health Organization* (2015) existem atualmente 600 milhões de pessoas obesas no mundo. Além disso, estatísticas mundiais destacam que 3,7 milhões de mortes são atribuídas a exposição à poluição atmosférica ambiental, dados principalmente gerados pela inalação de material particulado (MP) (EPA, 2012). Portanto, destaca-se que, de maneira paralela, tanto a obesidade e quanto a exposição à poluição representam riscos à saúde da população.

As mulheres configuram o gênero mais susceptível ao desenvolvimento de doenças relacionadas ao metabolismo e esta característica está associada às mudanças hormonais do climatério. Mulheres com idade acima de 40 anos apresentam maior prevalência DM2 quando comparada aos homens (TANG et

al., 2003). Assim, pode-se destacar um terceiro elemento (além da obesidade e da exposição ao MP) no desenvolvimento do DM2 em mulheres: a redução dos níveis do hormônio estrogênio no período pós-menopausa (KIM, 2012).

Na última década, estudos epidemiológicos têm demonstrado a existência de forte associação entre obesidade, exposição crônica ao MP e o desenvolvimento à DM2. Em estudos experimentais, a combinação entre a obesidade, induzida pelo consumo de dietas hiperlipídicas (DHL), e a inalação de MP tem evidenciado um desenvolvimento precoce do quadro pró-inflamatório e pró-oxidativo, característico do processo fisiopatológico subclínico do DM2 (SCHUSTER, 2010; SUN et al., 2009). Tendo em vista que a privação de estrogênio nas mulheres também está associada a um prejuízo nas defesas antioxidantes e no perfil inflamatório, neste estudo experimental buscamos mimetizar a vida urbana, onde mulheres estão expostas ao longo da vida a fatores dietéticos (DHL) e ambientais (inalação de MP) que predispõem ao desenvolvimento de doenças metabólicas, e ao atingirem a menopausa, perdem os efeitos protetores do estrogênio o que pode agravar/acelerar o desenvolvimento de doenças crônicas, como DM2.

A obesidade, principalmente originada pelo aumento da adiposidade abdominal, é caracterizada por inflamação subclínica crônica sistêmica tendo como tecido chave, o tecido adiposo, que produz e secreta uma série de fatores pró-inflamatórios, como a interleucina 1 (IL-1), a interleucina 6 (IL-6) e fator α de necrose tumoral (TNF- α), associados ao desenvolvimento de doenças cardiovasculares (DCV), dislipidemias e DM2 (NEHETE et al., 2014). O aumento da adiposidade também predispõe ao desenvolvimento de perfil pró-oxidativo, considerando o aumento da produção de espécies reativas de oxigênio (ERO) e a diminuição da capacidade antioxidante (SHARMA; BERNATCHEZ; DE HAAN, 2012). Assim, a obesidade se configura como um fator predisponente aos efeitos nocivos de fatores ambientais como a exposição a poluentes atmosféricos (GOLDBERG et al., 2006).

Dentre os poluentes do ar, o que tem recebido maior ênfase em pesquisas atuais é o material particulado (MP), constituído de poluentes formados a partir dos processos de combustão (EPA, 2011). O MP_{2,5} é capaz de invadir o trato respiratório e o sistema vascular (PEARSON et al., 2010) e, assim, causar efeito sistêmico, atuando como potente indutor da produção de

ERO e da liberação de citocinas pró-inflamatórias (TNF- α , IL-6, IL-8) para a circulação (BRAZ NOGUEIRA, 2009; LODOVICI; BIGAGLI, 2011; XU et al., 2011).

Os efeitos prejudiciais do MP_{2,5} à saúde dependem do tempo de exposição e da concentração deste no ambiente. Estudos mostram que exposições repetidas, em doses elevadas de MP_{2,5}, à longo prazo, parecem causar efeitos cumulativos maiores e mais persistentes (POPEIII; DOCKERY, 2006). Recentemente, nosso grupo identificou que exposição sub-crônica (12 semanas) a baixas doses de MP_{2,5} (5 μ g/10 μ L intranasal diariamente) potencializou a disfunção metabólica em camundongos alimentados com dieta hiperlipídica (DHL), promovendo intolerância à glicose, aumento dos níveis de glicemia e triglicerídeos (GOETTEMES-FIORIN, 2014).

Rajagopalan & Brook (2012) demonstraram, em investigação experimental, a relação entre DHL, exposição à MP_{2,5} e DM2, cujos resultados mostram aumento de glicemia de jejum, glicemia pós-prandial, insulina e de HOMA-IR (método utilizado para quantificar resistência à insulina). Outros estudos com modelos experimentais animais expostos à MP_{2,5} revelam aumento de macrófagos no tecido adiposo, com mudança para fenótipo pró-inflamatório, caracterizado por aumento de TNF- α e IL-6 e diminuição na produção de IL-10. Destaca-se, portanto, o potencial nocivo da associação entre consumo de DHL e exposição ao MP_{2,5} no desenvolvimento de distúrbios metabólicos e doenças crônicas. E ainda pode-se destacar um terceiro elemento (além da obesidade e da exposição ao MP) no desenvolvimento de doenças crônicas: a ausência do hormônio estrogênio no período pós-menopausa.

4.4. ESTROGÊNIO: IMPLICAÇÕES DE SUA FALTA NA REGULAÇÃO DO METABOLISMO

Mulheres com idade acima de 40 anos apresentam maior prevalência DM2 quando comparada aos homens (TANG et al., 2003), em decorrência de alterações hormonais relacionadas ao climatério.

O climatério é um processo típico e gradual que acontece em mulheres de meia idade e ocorre devido ao declínio da produção hormonal de estrogênio resultante da perda da função ovariana (MIQUEL et al., 2006; TE VELDE et al.,

1998). O estrogênio regula e coordena múltiplas funções em órgãos, células e genes, por meio da interação com receptores, utilizando vias de sinalização para ativar respostas moleculares e genômicas. Além do seu papel regulador da função sexual e reprodutiva (RETTBERG; YAO; BRINTON, 2014), atua no metabolismo da glicose e de lipídeos, na manutenção do remodelamento ósseo e de funções neurológicas (CAMPOREZ et al., 2013). A identificação de que o estrogênio modula a disponibilidade de glicose, principalmente, por meio de suas ações em proteínas da via de sinalização da insulina e sobre a expressão e translocação de GLUT-4 criaram novas possibilidades de investigação com potencial para contribuir no desenvolvimento de terapias coadjuvantes para prevenção e tratamento de DM2 em mulheres (BARROS; GUSTAFSSON, 2011).

A redução acentuada em estrogênio também tem sido associada ao aumento dos níveis de estresse oxidativo. Em altas concentrações, o estrogênio tende a ter um efeito antioxidante benéfico, no entanto, em baixas concentrações, parece predominar um estado pró-oxidante no organismo (DOSHI; AGARWAL, 2013). Em mulheres saudáveis na pré-menopausa geralmente há um equilíbrio adequado entre EROs e os mecanismos antioxidantes (DOSHI; AGARWAL, 2013). Entretanto, mulheres na pós-menopausa apresentam uma menor capacidade antioxidante total e concentrações plasmáticas maiores de lipoperóxidos, sugerindo que a falta de estrogênio causa um desequilíbrio no estado redox (LEAL et al., 2000).

Estudos experimentais, com ovariectomia em roedores, também mostraram que a privação de estrogênio pode causar aumento de peso corpóreo, com aumento de gordura intra-abdominal (BARROS; GUSTAFSSON, 2011), fator que predispõe ao desenvolvimento de perfil pró-oxidativo e pró-inflamatório, prejudicando a sinalização da insulina (SHEN; KUMAR; SHI, 2014). O hipoestrogenismo imposto pela ovariectomia (OVX), quando associado ao consumo de DHL, promove aumento de adipócitos e de marcadores inflamatórios, como, por exemplo, IL-6, reforçando a ideia de que o comportamento alimentar agrava as complicações da inflamação associadas ao hipoestrogenismo. Análises bioquímicas e moleculares também evidenciaram que a combinação entre OVX e DHL causa distúrbios no perfil metabólico, com aumento dos níveis de colesterol total, triglicerídeos, e no

perfil oxidativo, com aumento de lipoperoxidação hepática e redução do nível de GSH e atividade de catalase no fígado (LUDGERO-CORREIA et al., 2012; VUKOVIĆ et al., 2014).

Neste contexto, destaca-se que os elementos descritos acima, como o consumo de DHL, a exposição à MP e a privação de estrogênio, promovem efeitos análogos quando se avaliam parâmetros metabólicos, oxidativos e inflamatórios. Contudo, poucos estudos têm documentado o efeito sinérgico destas condições no processo fisiopatológico de doenças relacionadas ao metabolismo.

Para avaliar a condição do declínio nos níveis de estrogênio, em função da perda ovariana, diversos modelos experimentais vêm sendo utilizados. Em mulheres, a senescência reprodutiva ocorre tipicamente em torno da quinta década de vida, quando seu conjunto finito de folículos ovarianos imaturos é exaurido por meio de uma combinação de ciclos ovulatórios e apoptose, condição denominada de atresia (KOEBELE; BIMONTE-NELSON, 2017).

São descritos basicamente três modelos experimentais de menopausa em roedores – o modelo ovário-intacto, OVX e VCD (4-vinilciclohexano diepóxido) – que auxiliam na compreensão do sistema reprodutivo feminino, bem como mudanças cerebrais, comportamentais e metabólicas que ocorrem com perturbações e alterações neste sistema (KOEBELE; BIMONTE-NELSON, 2017).

No modelo de ovário intacto, pesquisadores utilizam o rato fêmea com ovário intacto para avaliar as mudanças nas interações entre cérebro, pituitária e ovário conforme a idade dos animais (KOEBELE; BIMONTE-NELSON, 2017). Outro modelo é realizado a partir do uso de ovotoxinas, como o VCD (4-vinilciclohexano diepóxido), pois acredita-se que o mesmo atue acelerando a atresia nas células ovarianas (KOEBELE; BIMONTE-NELSON, 2017; RETTBERG; YAO; BRINTON, 2014).

O uso de ovariectomia, remoção de ambos os ovários, como um modelo para indução das condições de pós-menopausa, é muito difundido para pesquisa translacional. Neste modelo cirúrgico da menopausa, a remoção bilateral dos ovários ocorre em animais jovens, saudáveis, reprodutivamente competentes. As intervenções experimentais ocorrem no momento da ovariectomia ou começam, uma vez que o 17β -estradiol atingiu um nível baixo

a não detectável no plasma, o que tipicamente ocorre dentro de 1 a 2 semanas. Este modelo continua sendo amplamente utilizado para mimetizar a menopausa humana (BRINTON, 2012).

A partir de modelo de menopausa em roedores, por ovariectomia, propomos investigar as implicações do hipoestrogenismo em ratas que realizaram consumo de DHL e foram expostas cronicamente ao MP_{2,5}. Para tal, utilizamos importantes biomarcadores de parâmetros metabólicos, oxidativos e inflamatórios, conforme descrito abaixo.

4.5. BIOMARCADORES DO PERFIL REDOX E INFLAMATÓRIO

Estresse oxidativo, citocinas e HSP70

Em nosso estudo foram utilizados alguns biomarcadores para avaliar o estresse oxidativo, como lipoperoxidação e atividade de enzimas antioxidantes e parâmetros inflamatórios, por meio da avaliação da HSP70 e citocinas inflamatórias.

O estresse oxidativo é um desequilíbrio bioquímico, que se apresenta quando a produção de pró-oxidantes, como radicais livres ou espécies reativas de oxigênio excede a capacidade antioxidante natural do organismo, resultando em dano (HALLIWELL, B.; GUTTERIDGE, 2007; SIES, 1993) e consequentemente levando ao desenvolvimento de um amplo espectro de doenças humanas (Halliwell, 1994).

Os principais agentes pró-oxidantes são as espécies reativas de oxigênio (ERO), como as radicalares, radicais superóxido ($O_2^{\cdot-}$) e hidroxila ($OH^{\cdot}$), e também algumas espécies não-radicalares derivadas do O_2 , como por exemplo, o peróxido de hidrogênio (H_2O_2) (HALLIWELL, B.; GUTTERIDGE, 2007).

Os antioxidantes, podem ser classificados como exógenos, obtidos principalmente por meio da dieta, ou endógenos, sendo produzidos pelo nosso organismo a fim de evitar o estresse oxidativo e a consequente destruição tecidual. Os sistemas de defesa antioxidantes, tem a capacidade de inibir a oxidação de outras moléculas, transferindo elétrons de uma substância para um agente oxidante (ERO), atuando em duas linhas, divididas em defesas em enzimáticas [superóxido dismutase (SOD), catalase (CAT) e glutathione

peroxidase (GPx)] e não-enzimáticas (vitaminas A, C e E; glutatona, ácido α -lipóico; carotenoides, ubiquinona ou coenzima Q10).

O desequilíbrio bioquímico, entre fatores pró e antioxidantes, que desencadeia o estresse oxidativo, pode ocorrer em condições de sobrepeso e obesidade (Furukawa et al., 2004), exposição a alguns fatores de risco, como poluentes do ar (Delfino et al., 2011) ou em condições de hipoestrogenismo, como no caso da pós-menopausa (DOSHI; AGARWAL, 2013).

Sob condições fisiológicas normais as células sincronizam sua atividade metabólica, expressão gênica, e outros processos celulares básicos, como a própria capacidade pró-oxidante/antioxidante, para manutenção da homeostase. Quando estas células são expostas a estresse, elas se comunicam para ajustar seu metabolismo para uma nova condição (ASEA; DE MAIO, 2007). O estresse oxidativo, assim como, o estresse térmico, induzem a expressão de HSP70. Altos níveis plasmáticos desta proteína estão correlacionados, simultaneamente, com o prejuízo no balanço energético, com alteração no status pró/anti-inflamatório e com o desequilíbrio dos sistemas pró/antioxidante do organismo.

As proteínas de choque térmico (*Heat Shock Proteins* - HSPs) são proteínas altamente conservadas durante a evolução das espécies e são encontradas em organismos eucarióticos e procarióticos. As HSPs podem ser agrupadas de acordo com suas massas moleculares em famílias (HSP110, HSP100, HSP90, HSP70, HSP60 e HSP30). Dentre estas, especificamente, as pertencentes à família de HSPs de 70 kDa (HSP70) são proteínas muito conservadas (SMOCK; BLACKBURN; GIERASCH, 2011) que possuem um potencial fator citoprotetor, anti-apoptótico e anti-inflamatório, em diversas condições estressantes (HECK; SCHÖLER; DE BITTENCOURT, 2011).

O aumento na síntese de HSPs, pode apresentar um aumento na tolerância ao estresse, prevenindo, danos proteicos relacionados ao estresse oxidativo (Kalmar & Greensmith, 2009). Obesidade, sedentarismo e consumo de dietas ricas em gordura podem produzir efeitos sobre a expressão de HSP70. Essas condições produzem um ciclo vicioso de inflamação e estresse oxidativo, que leva a perda do equilíbrio homeostático e podem ser indicativo

de possível dano tecidual e desenvolvimento de patologias (CHUNG et al., 2008; HOOPER; HOOPER, 2005).

As HSP70 têm a capacidade de proteger as células de dano oxidativo induzido por diferentes desafios. Estudo realizado por Kido et al. (2011) sugere que a exposição a inalação de poluição do ar induz a expressão HSP70 sistêmica, podendo ser um importante mediador imunológico que contribui para outros agravantes (disfunção vascular e eventos cardiovasculares). A exposição à suspensão de ROFA por três dias consecutivos (750 µg) promoveram aumento nos níveis plasmáticos de eHSP70 (HSP70 extracelular) e alterações no estresse oxidativo plasmático, evidenciando que a HSP70 representa um potencial biomarcador inflamatório e oxidativo (BALDISSERA et al., 2018). Além disso, estudo conduzido por Goettems-Fiorin et al. (2016) destaca que a exposição ao poluente atmosférico MP_{2.5} potencializa a disfunção metabólica em camundongos tratados com DHL, por meio de alterações na resposta ao estresse celular, avaliada pela razão [eHSP72] / [iHSP70], biomarcador do estado inflamatório crônico de baixo grau, elevando o risco de desenvolver diabetes tipo 2.

Desta forma, as HSP70, têm sido usadas, recentemente, como biomarcadores para avaliações precoces dos efeitos nocivos à saúde, como os causados por exposição à poluição atmosférica (Baldissera et al., 2018; Goettems-Fiorin et al., 2016; Mukhopadhyay et al., 2003) ou obesidade (CANGERI DI NASO et al., 2015; CHUNG et al., 2008; KRAUSE et al., 2015). Além disso, as HSP70 são potenciais biomarcadores na condição de menopausa, e também das disfunções associadas à esta condição, uma vez que o estrogênio é um indutor fisiológico da resposta ao choque térmico (heat shock response) (MIRAGEM et al., 2015).

As citocinas são proteínas secretadas pelas células imunológicas, em resposta a microrganismos e outros antígenos, e podem apresentar efeitos pró-inflamatórios e anti-inflamatórios. Neste sentido, o tecido adiposo é uma importante fonte de citocinas, e a adiposidade contribui para o ambiente pró-inflamatório, sendo que os níveis séricos de citocinas apresentam-se elevados tanto em humanos e animais com excesso de adiposidade (PARK; PARK; YU, 2005), desta forma, a obesidade verificada pelo IMC apresenta correlação com

os níveis de proteína C reativa (PCR), enquanto a adiposidade visceral apresenta-se significativamente associada à concentração de interleucina 6 (IL-6). A obesidade é bem conhecida como um estado inflamatório de baixo grau e a resposta inflamatória em decorrência do consumo de DHL é caracterizada pela ativação de NF- κ B e produção de citocinas pró-inflamatórias, como TNF- α , IL-1 e IL-6 (KHALIL; OMRAN; ALSHEIKH, 2018).

A exposição à poluentes atmosféricos também promove respostas inflamatórias por meio de citocinas. A exposição ao MP_{2,5} é positivamente associada à expressão da IL1, IL6 e TNF- α (CHEN et al., 2018). Estudo realizado por Chao et al. (2018), com motoristas, evidenciaram um aumento na atividade pró-inflamatória, com aumento nos níveis plasmáticos de TNF- α .

Estudos recentes em menopausa, indicam que esta condição também está associado a um estado inflamatório sistêmico, com aumento dos níveis séricos das principais citocinas pró-inflamatórias, como IL-1, IL-6 ou e também o TNF- α (MALUTAN et al., 2014). Níveis aumentados de citocinas pró-inflamatórias e alterações nos níveis séricos de citocinas anti-inflamatórias após a menopausa podem estar associadas à função de monócitos e macrófagos, que é prejudicada devido à deficiência de estrogênio. As implicações dessas citocinas no desenvolvimento e progressão de doenças importantes, como osteoporose, doenças cardiovasculares, afrontamentos ou síndrome depressiva, têm sido extensivamente estudadas na pós-menopausa, mas ainda existem muitas lacunas (MALUTAN et al., 2014). A condição pró-inflamatória pode estar associada ao aumento do peso corporal e a adiposidade, que em decorrência da ovariectomia, também promovem redução nos níveis de IL-10 e aumento nos níveis da razão IL-6/IL-10 (GOETTEMES-FIORIN et al., 2019).

Parâmetros de estresse oxidativo, bem como, de inflamação e estresse celular, como, avaliação de citocinas, e das proteínas de choque térmico (HSP70), são potenciais biomarcadores para avaliar a condição de obesidade, desenvolvida por consumo de dieta hiperlipídica, os efeitos da exposição crônica à poluentes atmosféricos, como ao MP_{2,5} e também à condição de hipoestrogenismo, induzido por ovariectomia. Desta forma, utilizaremos em nosso estudo os referidos biomarcadores para avaliar as implicações das três

condições propostas neste estudo: consumo de DHL, exposição à $MP_{2,5}$ e declínio do estrogênio induzido por ovariectomia em modelo experimental.

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6. OBJETIVOS

6.1. OBJETIVO GERAL

Avaliar se privação de estrogênio, induzida por ovariectomia, altera o perfil metabólico, inflamatório, estado redox e estresse celular hepático em ratas alimentadas com dieta hiperlipídica e expostas cronicamente ao MP_{2,5}.

6.2. OBJETIVOS ESPECÍFICOS

Avaliar o efeito da privação de estrogênio em animais submetidos à DHL e MP_{2,5} quanto a (ao):

- ✓ Perfil biométrico (pelo peso corpóreo e percentual de peso dos tecidos relacionados ao metabolismo);
- ✓ Parâmetros hematológicos;
- ✓ Perfil metabólico (pelos níveis plasmáticos de glicose, triglicerídeos, colesterol HDL e LDL, pela resposta aos testes de tolerância à glicose, e níveis de triglicerídeo e colesterol hepático);
- ✓ Perfil redox (pelos níveis hepáticos de lipoperoxidação, e pela atividade das enzimas antioxidantes CAT e SOD).
- ✓ Perfil inflamatório sistêmico e tecidual (pelos níveis plasmáticos e hepáticos de IL-6 e IL-10, e níveis plasmáticos e hepáticos de HSP70);

7. ARTIGOS

ARTIGO 1

PUBLICADO

Título: Ovariectomy predisposes female rats to fine particulate matter exposure's effects by altering metabolic, oxidative, pro-inflammatory, and heat-shock protein levels

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Objetivo: Avaliar se a redução nos níveis de estrogênio afeta o tecido hepático e altera o metabolismo da glicose em ratas expostas à poluição por material particulado fino.

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Ovariectomy predisposes female rats to fine particulate matter exposure's effects by altering metabolic, oxidative, pro-inflammatory, and heat-shock protein levels

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Abstract

The reduction of estrogen levels, as a result of menopause, is associated with the development of metabolic diseases caused by alterations in oxidative stress (OS), inflammatory biomarkers, and 70-kDa heat-shock protein (HSP70) expression. Additionally, exposure to fine particulate matter air pollution modifies liver OS levels and predisposes organisms to metabolic diseases, such as type 2 diabetes (T2DM). We investigated whether ovariectomy affects hepatic tissue and alters glucose metabolism in female rats exposed to particulate air pollution. First, 24 female Wistar rats received an intranasal instillation of saline or particles suspended in saline 5 times per week for 12 weeks. The animals then received either bilateral ovariectomy (OVX) or false surgery (sham) and continued to receive saline or particles for 12 additional weeks, comprising four groups: CTRL, Polluted, OVX, and Polluted+OVX. Ovariectomy increased body weight and adiposity and promoted edema in hepatic tissue, hypercholesterolemia, glucose intolerance, and a pro-inflammatory profile (reduced IL-10 levels and increased IL-6/IL-10 ratio levels), independent of particle exposure. The Polluted+OVX group showed an increase in neutrophils and neutrophil/lymphocyte ratios, decreased antioxidant defense (SOD activity), and increased liver iHSP70 levels. In conclusion, alterations in the reproductive system predispose female organisms to particulate matter air pollution effects by affecting metabolic, oxidative, pro-inflammatory, and heat-shock protein expression.

Keywords Air pollution · Menopause · Estrogen · Metabolic diseases · Heat shock proteins · Oxidative stress · Inflammation

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Introduction

Women have an estimated life expectancy of 72–82 years, and there were at least 1.1 billion postmenopausal women globally as recently as 2011 (North American Menopause Society 2010). Since most postmenopausal women live in the cities of developing countries with higher levels of air pollution emission (Yu et al. 2018), many postmenopausal women experience chronic air pollution exposure. The loss of ovarian function and, subsequently, low estrogen levels in menopause is associated with the development of cardiovascular and metabolic diseases, such as type 2 diabetes mellitus (T2DM), and may aggravate the senescence process via an increase in generalized inflammation (Benedusi et al. 2014). Since estrogen regulates multiple functions in various organs, cells, and genes (Rettberg

et al. 2014), a decline in circulating estrogen levels may increase cardiovascular risk by effects related to increased adiposity and impaired lipid metabolism (Schneider et al. 2006). Hence, almost two-thirds of menopausal women are overweight or obese and have vasomotor problems, with a high prevalence of hypertension and T2DM (Santos et al. 2012). Experimental (Goettems-Fiorin et al. 2016; Liu et al. 2014; Xu et al. 2010) and epidemiological evidence (Pearson et al. 2010) has also shown that environmental air pollution is associated with the development of metabolic diseases and is related to worsening metabolic dysfunction, suggesting that pathophysiological effects caused by air pollution exposure may be more severe in individuals who are already compromised.

Air pollution is considered a cause of many diseases attributed to environmental factors, causing an estimated 3.1 million pollution-related deaths per year (Miller et al. 2012). Of the pollutants found in urban areas, fine particulate matter (PM_{2.5}), a complex mixture of organic and inorganic substances (WHO 2003) with an aerodynamic size of less than 2.5 µm, has the greatest health impact. The main sources of PM_{2.5} are combustive emissions from industry and particles formed in vehicle emissions. Residual oil fly ash (ROFA), a metal-rich environmental PM_{2.5}, from engines has been used in animal or in vitro studies to evaluate the mechanisms involved in the development of many diseases related to PM_{2.5} exposure (Sacks et al. 2011).

The elderly, infants, and persons with chronic cardiopulmonary diseases are considered to be most susceptible to the mortality and morbidity effects of air pollution exposure (Pope 2000). Thus, people with T2DM exposed to PM_{2.5} present a higher risk of metabolic impairment, as well as the oxidative effects of PM_{2.5} (Goettems-Fiorin et al. 2016; Pearson et al. 2010; Sun et al. 2009). In human and animal studies, inflammation, oxidative stress, and 70 kDa heat-shock protein (HSP70) imbalance are described as mechanisms of impaired glucose homeostasis, mainly due to liver injury and dysfunction (Cangeri Di Naso et al. 2015). Similarly, cumulative evidence has shown that estrogen loss in menopause is associated with the development of metabolic diseases (Lizcano and Guzmán 2014) by mechanisms related to increased oxidative stress (Oliveira et al. 2018), inflammation, and impaired HSP70 balance (Crist et al. 2009; Heck et al. 2017). Furthermore, high plasma HSP70 levels represent a warning signal and oxidative biomarker under environmental challenges that can be used as a subclinical biomarker of the effects of environmental pollutant exposure (Baldissera et al. 2018). In this study, we investigated whether low estrogen levels affect hepatic tissue and alter glucose metabolism in female rats exposed to particulate air pollution.

Materials and methods

Animals

Female 8-week-old Wistar rats ($n = 24$) from the Regional University of the Northwestern Rio Grande do Sul State (UNIJUI) animal facility were kept under controlled temperature (24 ± 2 °C) and light-dark cycle (light from 7:00 a.m. to 7:00 p.m.) conditions. The animals received water and food ad libitum. This protocol was approved by the Animal Ethics Committee of UNIJUI (CEUA 076/15).

Experimental design

The rats ($n = 24$) were randomly assigned to two groups that received either an intranasal instillation of 50 µL of saline daily for 12 weeks (representing a clean environment) or particles suspended in saline (250 µg, representing a polluted environment) (Fig. 1, white background). Next, the animals received either ovariectomy (OVX) or false surgery (Sham) and continued to receive saline or particles for 12 additional weeks, comprising four groups: animals that received saline and received sham surgery (CTRL, $n = 6$) or ovariectomy (OVX, $n = 6$) and animals that received particles and were submitted to sham surgery (Polluted, $n = 6$) or ovariectomy (Polluted+OVX, $n = 6$) (Fig. 1, gray background). Throughout the study, biometric and consumption profiles, as well as glycemia and glucose tolerance tests, were performed every 4 weeks. After 24 weeks, the animals were euthanized, and blood and tissue were collected for biochemical and molecular analysis.

Particulate matter—particle characterization

Residual oil fly ash (ROFA) particles were collected from an electrostatic precipitator installed in the chimney of a large steel factory located in São Paulo (SP, Brazil). Animals in the polluted group received a daily intranasal dose (between 4:00 and 5:00 p.m.) of 50 µL of the ROFA suspension at the concentration of 5 µg/µL 5 days per week over a 24-week period, for a total daily dose of 250 µg. Since chronic exposure can be defined as repeated exposure via the inhalation route for more than 90 days is typical in laboratory studies using animal species (EPA 2010), we consider our chronic particle exposure regimen to be adequate. Control groups received 50 µL of a saline solution. The intranasal administration process was performed using an automatic pipette to deliver 50 µL of the solution into the animal's nostril; this procedure triggers the apnea reflex, which promotes the inhalation of the pollutant (Medeiros et al. 2004). The particles used in this study consist of Pb (3.1 ± 0.09), Al (789 ± 23), Zn (20.3 ± 0.04), Cd (0.04 ± 0.002), Ba (30.2 ± 0.31), Cu (9.7 ± 0.1), Ni (287 ± 10.8), As (4.1 ± 0.05), Se (7.5 ± 0.20), Mn

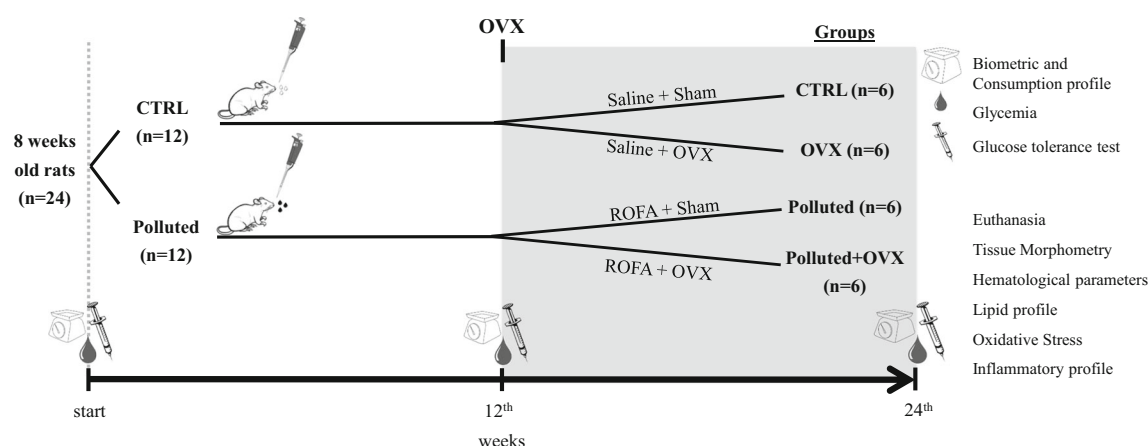


Fig. 1 Female Wistar rats were randomly assigned to receive either an intranasal dose of saline (representing a clean environment; CTRL) or ROFA particles suspended in saline (250 μ g, representing a polluted environment, Polluted) daily for 12 weeks (white box). The animals then received either ovariectomy or sham surgery and continued to receive saline or ROFA for an additional 12 weeks (gray box), comprising four groups: animals that received saline and received either

sham surgery (CTRL) or ovariectomy (OVX) and animals that received ROFA and received either sham surgery (Polluted) or ovariectomy (Polluted+OVX). Throughout the study, biometric and consumption profiles were collected, and glycemia and glucose tolerance tests were performed every 4 weeks. After 24 weeks, the animals were euthanized, and blood and tissue were collected for biochemical and molecular analysis

(48.3 ± 0.98), Sr (8.4 ± 0.16), Sb (2.3 ± 0.57), Fe ($20,397.2 \pm 283.3$), Mg (372.5 ± 1.93), P (388.5 ± 255.8), and Cr (7.6 ± 0.23) (mean $\pm$ SD, expressed by ng per m^3 of air). The particles have an average aerodynamic diameter of $1.2 \pm 2.2 \mu m$.

Ovariectomy

The animals were submitted to a surgical protocol. First, a pre-anesthetic medication, consisting of 5 mg/kg morphine intraperitoneal (i.p.), was administered. Next, 4% isoflurane (BioChimico®) was administered to induce anesthesia, followed by 2% isoflurane (BioChimico®) to maintain anesthesia. OVX corresponds to the bilateral removal of the ovaries and is achieved by a longitudinal incision in the lateral region of the abdomen, close to the hind limbs. The non-ovariectomized animals were submitted to false operation (Sham) where their ovaries were identified and surgically exposed and then repositioned for posterior suturing of the musculature and skin. After the surgical procedure, all animals (Sham and OVX) received appropriate postoperative treatment, which included body temperature and pain signal monitoring, in addition to pharmacological treatment: meloxicam (Ouro Fino®) 0.2% (2 mg/kg, subcutaneously) after 24 h (single dose).

Food and water consumption

The animals' water and food consumption, as determined by the volume of water offered minus the remaining volume in the bottle (mL) and the amount of food provided minus the remaining food in the box (g), was monitored 3 times per week during the 24-week study.

Biometric profiles and tissue morphometry

Each animal's biometric profile was monitored once every 4 weeks during the 24-week study. Body weight (g) and length (cm) measurements were taken and used to calculate a Lee index value (Lee 1929). The rats' body weight was checked with a semi-analytical scale, and their length was verified by naso-anal distance. The Lee index consists of dividing the cube root of the animal's body weight (g) by naso-anal distance (cm) (Lee 1929). At the end of the study, we also evaluated adiposity (i.e., % of visceral white adipose tissue [WAT]/body weight) and percent of the liver, pancreas, gastrocnemius, and soleus muscle weight in relation to body weight. Visceral white adipose tissue was collected from the perigonadal region surrounding the uterus and ovaries for analysis. Pulmonary and hepatic edema was evaluated using the wet/dry weight ratio.

Glycemia and the glucose tolerance test

Blood glucose levels were monitored before the start of the interventions, before the surgical procedure (12^a week), and at the end of the study (24^a week). Blood glucose was measured by Glucometer Optium Xceed (Abbott®) (5 μ L of tail blood) after 12 h of fasting. A glucose tolerance test was performed before the start of the interventions and at the 12th and 24th weeks of intervention in all animals. Food was withdrawn 12 h before the GTT. Glycemia was measured as described above immediately before and at 15, 30, and 120 min after glucose (1 g/kg in saline solution, i.p.) administration. The glycemic response during glycemia and the glucose tolerance

test (GTT) was evaluated via the area under the curve (AUC) method (Who 1998).

Blood collection and tissue preparation

At the end of the 24-week intervention, the animals were euthanized, and whole blood was immediately collected and stored in a vial containing 2 mg/mL of ethylenediaminetetraacetic acid (EDTA) anticoagulant for aliquots of total blood (blood count) and plasma (evaluation of IL-6, IL-10, and eHSP70). Blood was collected without EDTA to obtain serum used to obtain a lipid profile and determine the 17 β -estradiol concentration. Each blood sample was centrifuged at 3000 rpm for 15 min, and plasma was frozen with PMSF (Phenyl Methyl Sulfonyl Fluoride, Sigma® P7626, FW = 174.19 g/mol; 1.74 mg/mL = 100 mM) for the subsequent measurement of IL-6, IL-10, and eHSP70. The remainder was used for the aforementioned hematological analyses.

Metabolic-related tissues (MRT; i.e., WAT, liver, pancreas, gastrocnemius, and soleus muscle) were dissected and weighed. The liver was freeze-clamped in liquid nitrogen and stored for further homogenization. For the analysis of oxidative stress (i.e., thiobarbituric acid reactive substances (TBARS), antioxidant activity enzymes, superoxide dismutase (SOD), and catalase (CAT)), a portion of the liver was homogenized in a potassium phosphate buffer (KPi) pH 7.4. Another portion of the tissue was homogenized in a 0.1% (w/v) sodium dodecyl sulfate (SDS) buffer to determine iHSP70 expression by Western blotting.

The liver was homogenized (still frozen) in respective buffers, containing a protease inhibitor (PMSF—phenyl-methyl-sulfonyl fluoride, 100 μ M). Afterward, the homogenates were centrifuged for 10 min at room temperature, and the supernatant fractions were used for Bradford protein determination (Bradford 1976) using bovine serum albumin, as standard.

The measurement of 17 β -estradiol (E2)

To determine 17 β -estradiol (E2) levels, a serum sample was used, and a quantitative dosage was made through the automated system ADVIA Centaur XP® (Siemens Healthcare Diagnosis) by chemiluminescence methodology with sensitivity and in vitro test limits between 20 and 3000 pg/mL.

Hematological parameters

The blood was conditioned in an anticoagulant tube (EDTA) to determine hematological parameters (5 μ L of EDTA for each 500 μ L of blood) as previously described. For automatic determination, the hematology analyzer Micros 60® (Horiba) was used. Using this equipment, the following parameters can be obtained: the total red blood cell count, hematocrit,

hemoglobin, hematimetric indexes (VCM, HCM, and CHCM), erythrocyte distribution range—RDW; total counts of leukocytes, relative and absolute counts of leukocytes (neutrophils, eosinophils, basophils, lymphocytes, and monocytes), and platelet counts (Horiba-User Manual).

Samples were diluted 1:2 with 0.9% saline and analyzed in triplicate. Next, blood smear were performed on a blade, stained with panoptic staining (Newprov®), and analyzed by a professional, who counted 100 cells per sample.

To obtain the neutrophil-to-lymphocyte ratio (NLR), the number of neutrophils was divided by the number of lymphocytes, and to obtain platelet/lymphocyte ratio (PLR), the number of platelets was divided by the number of lymphocytes.

Lipid profiles

Triglycerides, total cholesterol, and lipoproteins (HDL-C) were performed by colorimetric methods with direct dosages. To obtain values related to the variables of the lipid profile, 100 μ L of serum per animal and 250 μ L of reagent were used for each analysis. Bioclin-Quibasa kits were used to perform these analyses using BS200 - Mindray® automation. The LDL-C dosage was estimated by the Friedewald formula ($LDL-C = CT - HDL-C - TG / 5$), where TG/5 represents the cholesterol bound to VLDL-C (Sposito et al. 2001).

Liver triglyceride content

Lipids from liver samples (50 mg) were extracted in 1 mL isopropanol. After centrifugation, to perform an enzymatic colorimetric determination of the triglyceride concentration, 10 μ L of the supernatant aliquots was added to 500 μ L of a reagent (Labtest®) (Oakes et al. 2001). The results were expressed in gram of lipids/100 g of liver tissue.

Oxidative stress, inflammatory profiles, and HSP70 content

Lipid peroxidation

Liver lipid peroxidation concentrations were analyzed using the thiobarbituric acid reactive substances method (TBARS; Buege and Aust 1978). 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 535 nm. The MDA standard was prepared from 1.1.3.3-Tetramethoxypropane (concentrations between 0.0005 and 0.016 mg/mL). The results were expressed in mmol MDA/mg of protein.

SOD and CAT activity

Liver SOD activity was analyzed by inhibiting the auto-oxidation of pyrogallol procedure (Marklund and Marklund 1974). Briefly, in a cuvette, 954 μ L of 50 mM Tris/1 mM EDTA buffer (pH 8.2), 4 μ L of catalase (CAT; 30 μ M), 10 μ L of homogenate, and 32 μ L pyrogallol (24 mM in HCl 10 mM) were added and mixed. SOD activity was determined at 36 °C in a spectrophotometer (420 nm) every 20 s for 120 s. Results were expressed in units of SOD/mg of protein.

Liver CAT activity was analyzed by the decomposition of hydrogen peroxide procedure (Aebi 1984). In a quartz cuvette, 955 μ L of phosphate buffer (50 mM, pH 7.4) and 35 μ L of hydrogen peroxide (0.01 M) were mixed. Then, 10 μ L of homogenate was added and mixed. CAT activity was determined at 36 °C in a spectrophotometer (240 nm) every 15 s for 120 s. The results were expressed in units of CAT/mg of protein.

Cytokines (IL-10 and IL-6) levels

Plasma and liver levels of IL-6 and IL-10 were measured using an ELISA kit (RayBio® Rat IL-6 and IL-10, Raybiotech, Inc., USA). The colorimetric optical density was detected at a wavelength of 450 nm. The cytokine levels were calculated based on a standard curve constructed for each assay, and each assay was performed in duplicate. All cytokines were reported as picogram per milliliter.

HSP70 tissue expression (iHSP70)

iHSP70 expression was evaluated in the liver by immunoblot analyses (Kolberg et al. 2006). Equivalent amounts of protein from each sample (~40 μ g) were mixed with Laemmli's gel loading buffer (50 mM Tris, 10% [w/v] SDS, 10% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, and 2 mg/mL bromphenol blue) in a ratio of 1:1, boiled for 5 min, and electrophoresed in a 10% polyacrylamide gel (5 h in 15 mA/gel). The proteins were then transferred onto a nitrocellulose membrane (GE Healthcare) by electrotransfer (1 h in 100 V), and subsequently, transferred bands were visualized with 3% (w/v) Red Ponceau S (Sigma-Aldrich).

The procedures were performed with a vacuum pump for rapid immunoblot. Membranes were washed with TEN-Tween 20 solution (0.1% w/v; TEN is 50 mM Tris, 5 mM EDTA, 150 mM NaCl, pH 7.4) and then blocked in 0.5% (w/v) nonfat dry milk in a washing buffer (TEN-Tween 20 solution). Membranes were incubated for 12 h with the monoclonal anti-HSP70 antibody (Sigma-Aldrich H5147, 1:1000). After three consecutive washings with the washing buffer, peroxidase-labeled rabbit anti-mouse IgG (Sigma-Aldrich A9044) was utilized as a secondary antibody at 1:15000 dilution. As a gel loading control, Coomassie Blue (0.1%

Coomassie blue, 40% methanol, 10% acetic acid) detection of 43 kDa β -actin region was used. Blot visualization was performed using ECL-Prime Western Blotting Reagent (GE Healthcare®). Quantification of bands was performed using Image J® software. The data were presented in arbitrary units of iHSP70.

Plasma HSP72 (eHSP72) levels

A highly sensitive EIA method (EKS-715 Stressgen, Victoria, BC, Canada) was used to determine the amount of eHSP72 protein in plasma as previously described (Hou et al. 2010). Absorbance was measured at 450 nm, and a standard curve constructed from known dilutions of HSP72 recombinant protein was used to allow a quantitative assessment of eHSP72 plasma concentrations. Quantification was made using a microplate reader (Mindray MR-96A®). The intra-assay coefficient of variation was identified as being less than 2%.

[eHSP72]/[iHSP70] ratio

After performing eHSP72 (plasma) and iHSP70 (liver) measurements, an extracellular to intracellular HSP70 ratio index (H-index) was established. This index reveals the rats' immunoinflammatory status (Heck et al. 2017). The rationale is that with higher eHSP70 amounts, there are more inflammatory signals because eHSP70 is pro-inflammatory in nature. However, for each particular "j" situation, the more the cells are able to respond to stressful stimuli by enhancing iHSP70 and the more such cells are facing a state of anti-inflammation.

Therefore, if one takes $R_c = (eHSP70)_c / (iHSP70)_c$ as the HSP70 ratio in a control situation, regardless of the techniques used to assess each eHSP70 and iHSP70, the H-index can be calculated as the quotient of any $R_j = (eHSP70)_j / (iHSP70)_j$ by R_c , which will, therefore, be considered the unity (CTRL = 1), normalizing all the remaining results in situation "j." Hence, $H\text{-index} = R_j / R_c$ may allow for comparisons between any stressful situation "j" and the assumed control condition. The H-index can be applied to estimate an animal's immunoinflammatory status in many different situations as responses to dietary and environmental challenges are present in each tissue.

Statistical analysis

The statistical analysis included a *t* test, as well as a one- and two-way analysis of variance (ANOVA). Post hoc multiple comparisons among groups were performed with the Tukey's test. All statistical analyses were performed using GraphPad for Windows, version 5.0. The level of significance was set at $P < 0.05$. Results were expressed as mean $\pm$ standard error or standard deviation.

Results

The effects of reduced estrogen levels on the weight gain of rats exposed to PM

We evaluated the effects of PM exposure and reduced estrogen levels on the biometric profile of female rats. First, we observed no significant weight gain or loss during the 12-week particle exposure period (left side of Fig. 2a and b, respectively). Second, we observed that ovariectomized rats, which had lower estrogen levels (17β -estradiol levels, CTRL = 34.6 ± 7.4 ; Polluted = 33.6 ± 11.5 ; OVX = $23.1 \pm 4.1^*$ and Polluted+OVX = $28.6 \pm 5.6^*$, pg/mL; * OVX and Polluted+OVX vs CTRL and Polluted; $P = 0.019$), experienced weight gains during the following 12 weeks (right side of Fig. 2a) when compared with the non-ovariectomized rats. The reduced estrogen levels seem to have induced weight gain in rats previously exposed to particulate matter (PM) (Fig. 2c). No alterations were observed in the rats' body length ($P = 0.685$) or body mass index (Lee index, $P = 0.312$).

To ensure that the amount of food consumed did not influence the weight gain result, we analyzed the rats' food intake behavior and observed that there was no difference between the groups in food consumption (food intake, g/week/animal,

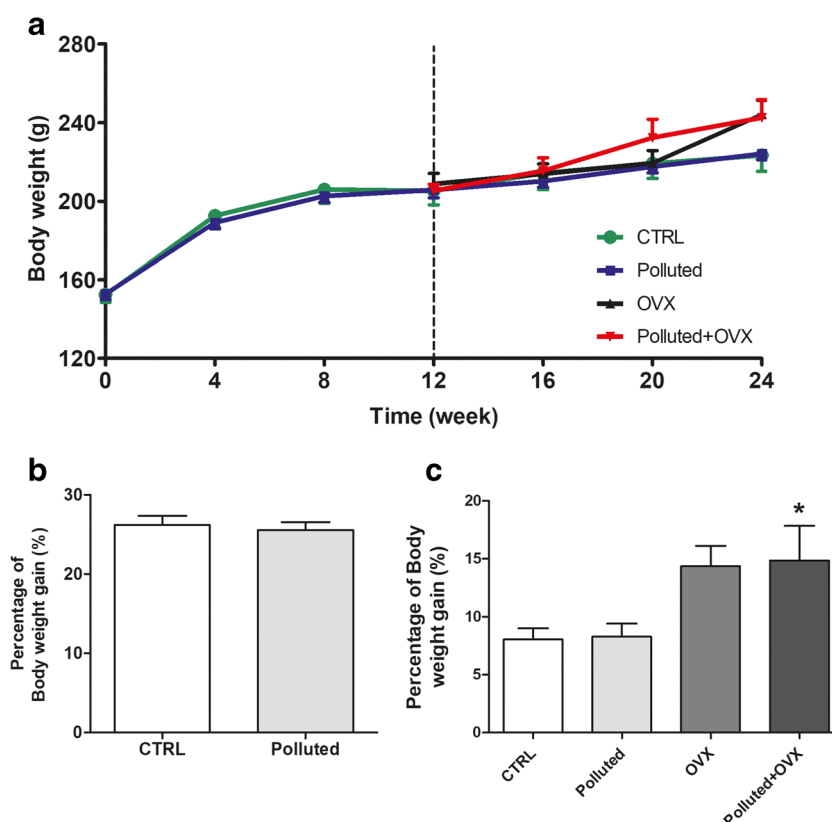
CTRL = 43.1 ± 6.5 ; Polluted = 44.8 ± 6.1 ; OVX = 43.7 ± 6.4 , and Polluted+OVX = 44.9 ± 6.5 . $P = 0.947$, $F_{3,20} = 0.119$).

The effects of reduced estrogen levels on the glycemic, lipidemic, and hepatic lipid profile of rats exposed to PM

The rats' glucose tolerance was evaluated prior to any intervention (Fig. 3a, b), after 12 weeks of pollution exposure (before ovariectomy; Fig. 3c, d), and at the end of the study (Fig. 3e, f). All animals presented a similar standard GTT response before the interventions (Fig. 4a, b), but 12 weeks of pollution exposure induced a glucose intolerance profile characterized by higher peak glucose levels in the GTT results (Fig. 3c) and by the area under the GTT curve (Fig. 3d). At the 24th week, the reduced estrogen levels induced glucose intolerance in the rats' GTT response, observed by increased glycemia levels 15 min after glucose administration in the OVX group (Fig. 3e) without alterations in AUC (Fig. 3f).

Reduced estrogen levels promoted an increase in total cholesterol levels (Table 1). No alterations in fasting glycemia, triacylglycerol, HDL-cholesterol, or LDL-cholesterol were observed (Table 1). The interventions also did not promote alterations in hepatic triglyceride content (Table 1).

Fig. 2 The effect of reduced estrogen levels on body weight in animals exposed to particulate matter. **a** Body weight before and after ovariectomy ($P = 0.657$). **b** Percentage of body weight gain during the first 12 weeks, before ovariectomy ($P = 0.680$). **c** Percentage of body weight gain from the 12th to the 24th week, after ovariectomy ($P = 0.023$; $F_{3,20} = 3.91$) * vs. CTRL. Data are presented as mean $\pm$ SEM. $n = 5$ –6 per group. A two-way ANOVA with repeated measures (**a**), Student's t test (**b**), and a one-way ANOVA followed by a post hoc Tukey test (**c**)



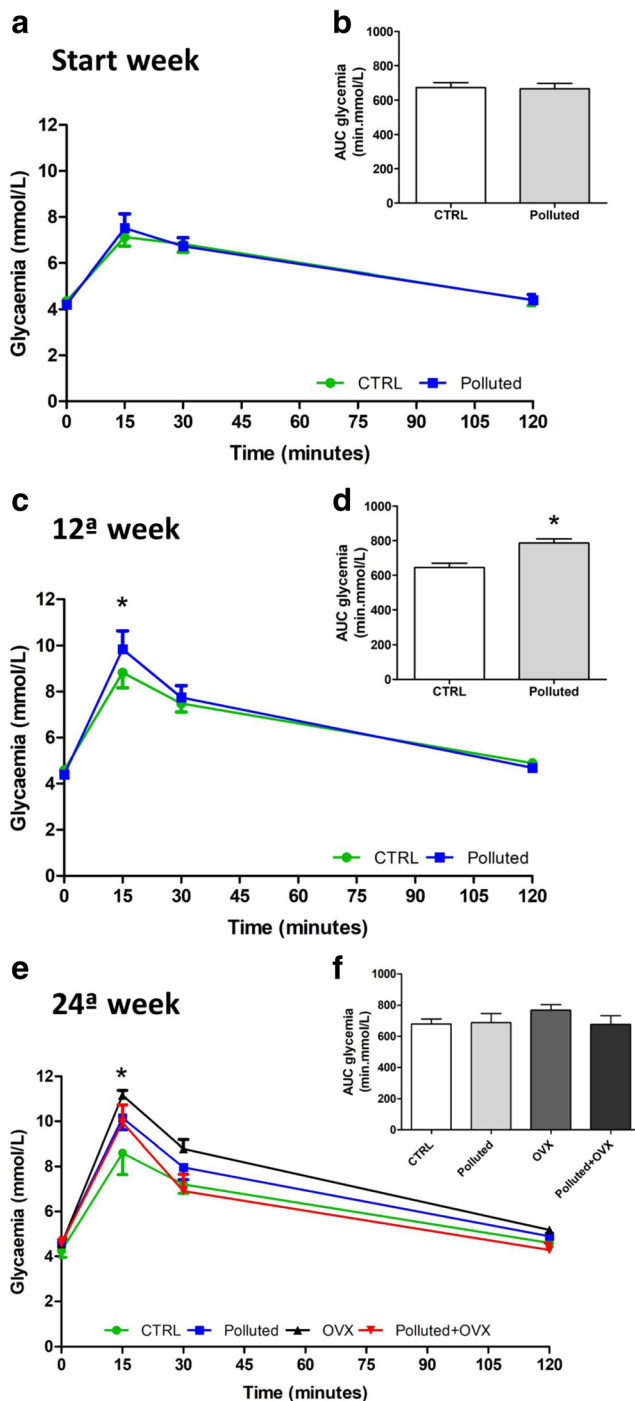


Fig. 3 The effect of reduced estrogen levels in animals exposed to particulate matter on the area under the curve in relation to the glucose tolerance test (GTT) response. **a** Baseline ($P=0.862$). **b** AUC Baseline ($P=0.877$; $F_{1,11}=1.13$). **c** 12th week ($P=0.474$). **d** AUC 12th week ($P=0.0004$; $F_{1,11}=1.14$) * vs CTRL. **e** 24th week ($P=0.001$) * OVX vs CTRL. **f** AUC 24th ($P=0.485$; $F_{3,20}=0.84$). Data presented as mean $\pm$ SEM. $n=6$ per group. A two-way ANOVA followed by a post hoc Tukey test for GTT (**a**, **c**, and **e**), Student's t test (**b** and **d**), and a one-way ANOVA followed by a post hoc Tukey test (**f**)

The effects of reduced estrogen levels on morphometric tissue alterations

We also evaluated the influence of reduced estrogen levels in polluted animals regarding morphometric alterations in metabolic-related tissues (Table 2). The OVX and Polluted+OVX groups both experienced an increase in adiposity, and the OVX group experienced a decrease in liver mass. No alterations were observed in the rats' pancreas mass or muscle mass (the gastrocnemius and soleus muscles, respectively). The Polluted+OVX group also experienced tissue edema in the liver and lung (Table 2).

Reduced estrogen levels modify the liver redox profile and inflammatory blood markers of rats exposed to PM

The Polluted+OVX group presented a reduction in SOD activity (Fig. 4a) without changes in CAT or lipoperoxidation levels (Fig. 4b, c, respectively). Additionally, since the liver CAT activity results showed a systematic decrease from the left bars (CTRL) to the right bars (Polluted+OVX), we supposed a decrease in estrogen levels is correlated with the decrease in liver CAT activity. Although there were no differences in liver CAT activity among the groups ($P=0.124$), there is a linear trend ($P=0.021$, $r=0.957$, evaluated by an ANOVA post hoc linear trend test) and a positive correlation between estrogen levels and CAT activity ($r=0.579$, $P=0.0059$). When analyzing intragroup correlations between estrogen and oxidative stress variables, in the Polluted+OVX group, we found that a decrease in estrogen levels is associated with a decrease in CAT activity ($r=0.927$, $P=0.023$) and an increase in lipid peroxidation levels in the liver ($r=0.977$, $P=0.004$).

We found no alterations in plasma IL-6 levels (Fig. 5a). However, OVX and particles associated with OVX decreased plasma interleukin-10 levels (Fig. 5b) and increased the IL-6/IL-10 ratio (Fig. 5c). The interventions did not modify the cytokine content in liver homogenates (Fig. 5d–f). We also found that IL-6 levels were negatively correlated with estrogen levels in the OVX group ($r=-0.981$, $P=0.0031$). Neutrophil levels and neutrophil/lymphocyte ratios were increased in the Polluted+OVX group at the end of the 24-week study (Table 3).

Reduced levels of estrogen were associated with PM and increased liver iHSP70 but not modified plasma eHSP70 levels

The heat-shock response (HSR) is performed by proteins in the HSP70 family, such as HSP72 (72-kDa, inducible isoform) and HSP73 (73-kDa, constitutive isoform), which constitute the total HSP70 content. The Polluted+

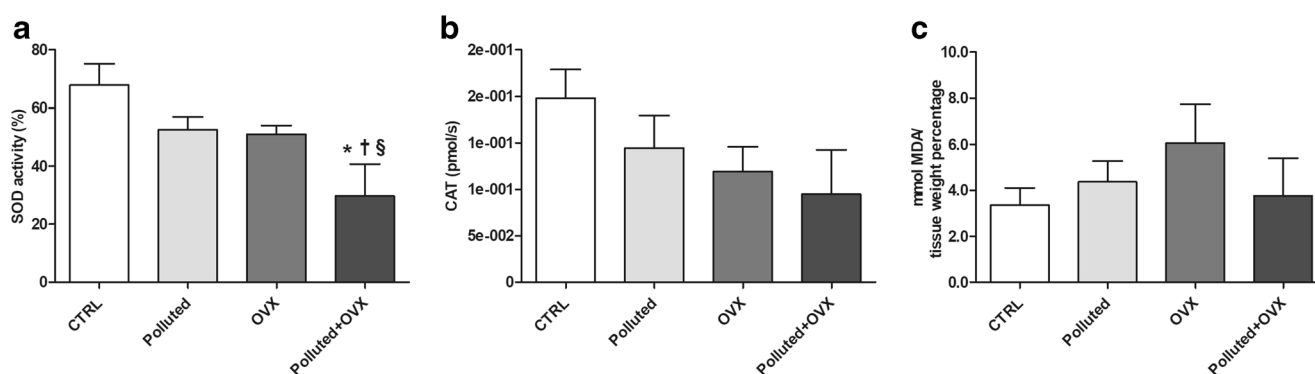


Fig. 4 The effect of reduced estrogen levels on the liver redox profile of animals exposed to particulate matter pollution. **a** SOD activity ($P = 0.0005$, $F_{3,20} = 9.34$) * vs CTRL, † vs Polluted, § vs OVX. **b** CAT

activity ($P = 0.124$, $F_{3,19} = 2.17$). **c** Lipoperoxidation ($P = 0.452$, $F_{3,16} = 0.92$). Data expressed as mean $\pm$ SEM. $n = 5$ –6 per group. A one-way ANOVA followed by a post hoc Tukey test

OVX group presented an increase in liver-inducible iHSP70 (iHSP72) and HSP70 total levels when compared with the CTRL group (Fig. 6a). None of the interventions modified plasma HSP70, as well as the extra-to-intracellular HSP70 ratio regarding the liver as demonstrated by the H-index values (Fig. 6b, c).

Discussion

In our study, ovariectomy promoted increased adiposity gain, reflected in increased body weight. Ovariectomized rats also presented with a pro-inflammatory profile and an altered heat-shock response in the liver when exposed to particle pollution. The data indicate that low estrogen levels represent a vulnerability to environmental air pollution challenges. Our study supports the hypothesis that menopausal women are at risk of developing metabolic diseases and that the risk increases with environmental air pollution exposure.

In our study, the animals were ovariectomized at 5 months of age; at the end of the study, all the animals were 8 months of age, and the non-ovariectomized groups presented hormonal levels characteristic of the estrus phase of the rat cycle (the mean 17β -estradiol level in the sham-operated groups was 34.1 pg/mL). The ovariectomy promoted a 24% reduction in these hormonal levels

(the mean 17β -estradiol level in the ovariectomized groups was 25.9 pg/mL). Irregular estrous cycles in rodents are characteristic of laboratory rodents with initiation occurring at approximately 8 months of age (Brinton 2014), whereas acyclicity begins in most laboratory rats between 12 and 16 months (Finch et al. 2014). Also, 60–70% of the aging rodents spontaneously transition into a polyfollicular anovulatory state of constant estrus characterized by sustained levels of plasma 17β -estradiol (Brinton 2014). The stages of reproductive senescence described above, from regular cycling to irregular cycling and then to acyclicity, are similar in humans and rodents. Although the transition is of shorter duration in rodents, it is characterized by multiple features found in humans, which include a decline in follicles, irregular cycling, steroid hormone fluctuations, and irregular fertility. Thus, if our ovariectomy occurred moderately early in the life cycle of the rat, it may be not representative of a senescence rat age; however, the age of the rats at the end of the study and the duration of low estrogen levels may adequately represent metabolic dysfunction (as observed by adiposity in our data) and changes in temperature regulation, which may affect the heat-shock response (Miragem et al. 2017), that typically occur 3–5 weeks after an ovariectomy, representing preclinical models of human menopause (Brinton 2014).

Table 1 The effects of reduced estrogen levels on the biochemical profile of rats exposed to PM

Parameter	CTRL	Polluted	OVX	Polluted+OVX	P value
Glycemia (mmol/L)	4.3 $\pm$ 0.3	4.2 $\pm$ 0.1	4.6 $\pm$ 0.2	4.6 $\pm$ 0.3	0.382 ($F_{3,20} = 1.07$)
Triacylglycerol (mg/dl)	120.3 $\pm$ 49.7	114.3 $\pm$ 42.9	126.2 $\pm$ 19.8	119.0 $\pm$ 9.0	0.958 ($F_{3,20} = 0.10$)
Total cholesterol (mg/dl)	59.2 $\pm$ 3.5	61.3 $\pm$ 3.6	75.8 $\pm$ 6.6*	76.7 $\pm$ 3.9*	0.020 ($F_{3,20} = 4.07$)
HDL-cholesterol (mg/dl)	22.5 $\pm$ 1.7	27.3 $\pm$ 3.4	25.8 $\pm$ 0.8	26.8 $\pm$ 1.2	0.349 ($F_{3,20} = 1.16$)
LDL-cholesterol (mg/dl)	16.0 $\pm$ 2.8	15.3 $\pm$ 3.8	24.8 $\pm$ 7.6	26.0 $\pm$ 3.7	0.082 ($F_{3,17} = 1.64$)
Liver triglycerides (g/100 g tissue)	2.5 $\pm$ 0.6	2.8 $\pm$ 0.6	3.6 $\pm$ 0.4	3.5 $\pm$ 0.6	0.479 ($F_{3,19} = 0.85$)

Data expressed as mean $\pm$ SEM. * $P < 0.05$ vs. CTRL. vs CTRL and OVX"?. One-way ANOVA followed by post hoc Tukey test. $n = 5$ –6 per group

Table 2 The effects of reduced estrogen levels on morphometric tissue alterations and tissue edema in rats exposed to PM

Parameter	CTRL	Polluted	OVX	Polluted+OVX	<i>P</i> value
White adipose tissue	0.91 ± 0.17	1.03 ± 0.15	1.39 ± 0.11*	1.19 ± 0.20*	0.019 ($F_{3,19} = 2.29$)
Liver	2.60 ± 0.08	2.60 ± 0.08	2.19 ± 0.06*	2.29 ± 0.09	0.002 ($F_{3,20} = 6.68$)
Pancreas	0.28 ± 0.03	0.29 ± 0.04	0.24 ± 0.02	0.23 ± 0.02	0.434 ($F_{3,20} = 0.95$)
Gastrocnemius	1.24 ± 0.05	1.28 ± 0.03	1.32 ± 0.12	1.24 ± 0.04	0.807 ($F_{3,20} = 0.32$)
Soleus	0.09 ± 0.005	0.08 ± 0.003	0.07 ± 0.003	0.08 ± 0.005	0.059 ($F_{3,20} = 2.09$)
Liver edema	29.6 ± 0.3	30.3 ± 0.3	31.3 ± 0.2*	31.5 ± 0.5*	0.001 ($F_{3,20} = 7.93$)
Lung edema	17.3 ± 0.4	18.8 ± 0.5	19.4 ± 0.3	20.2 ± 0.7*	0.008 ($F_{3,20} = 5.16$)

Percentage (%) of the tissue weight/body weight ratio. Data expressed as mean ± SEM. * $P < 0.05$ vs. CTRL. $n = 5$ –6 per group. A one-way ANOVA followed by a post hoc Tukey test

Ovariectomized rats experienced an increase in body weight and adiposity. Sex hormones, including the decline in estrogen levels due to ovariectomy, strongly influence body fat distribution and adipocyte differentiation (Min 2018). This decrease, which is also observed in menopausal women, is associated with a loss of subcutaneous fat and an increase in abdominal fat, evidencing why menopausal women are three times more likely to develop obesity and metabolic syndrome abnormalities than premenopausal women (Eshtiaghi et al. 2010; Lizcano and Guzmán 2014), as observed in the lipid profiles in our OVX and Polluted+OVX groups.

Estrogen deficiency was able to promote hepatic steatosis (Quinn et al. 2018) in the same way these effects are produced by the consumption of high-fat (HF) diets in studies. However, exposure to PM_{2.5} induces hepatic steatosis and hypertriglyceridemia in normal chow-fed mice but attenuates hepatic steatosis and hyperlipidemia in HF-fed mice (Qiu et al. 2017). We found decreased hepatic mass only in ovariectomized animals and edema in the liver tissue of ovariectomized animals regardless of pollution exposure. Cellular lesions, promoted by low estradiol levels, can lead to cellular edema or fat accumulation (i.e., steatosis; Oliveira et al. 2018), which

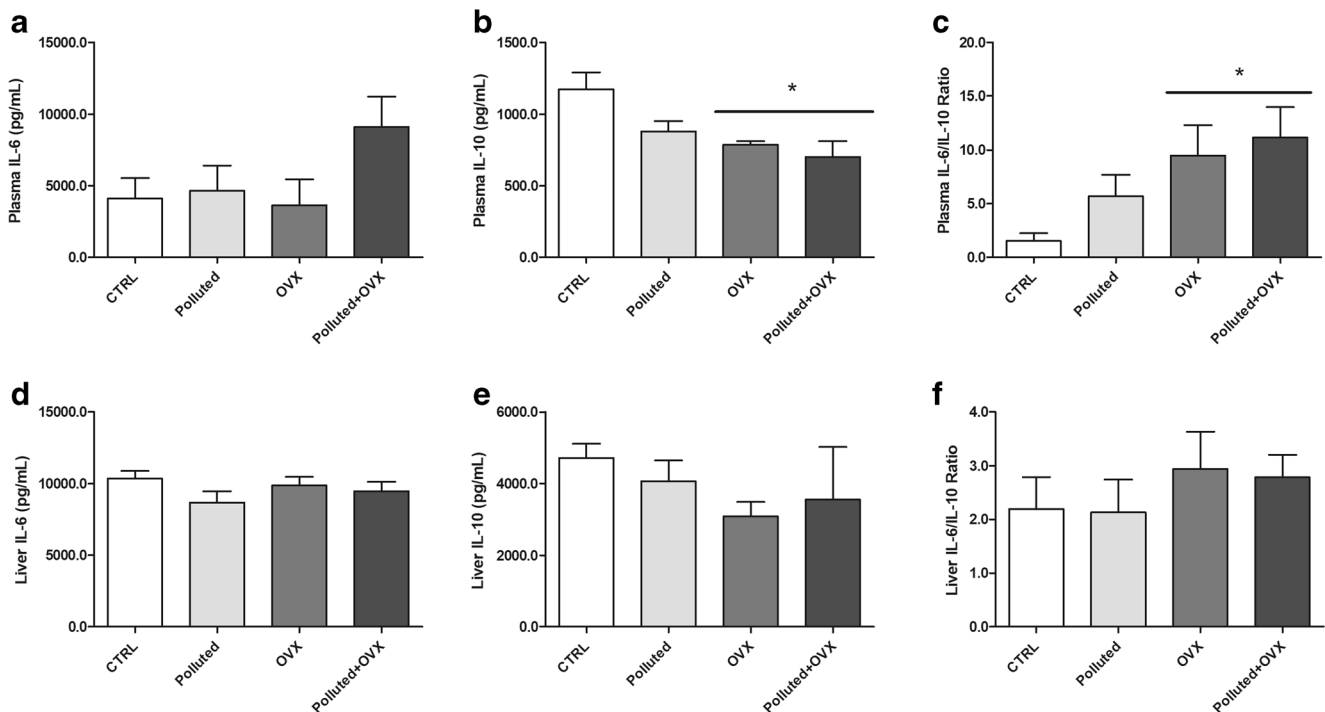


Fig. 5 The effect of reduced estrogen levels on the plasma and liver interleukin-6, interleukin-10, and the IL-6/IL-10 ratio at the 24th week in animals exposed to particulate matter pollution. **a** Interleukin-6 ($P = 0.228$, $F_{3,13} = 1.64$). **b** Interleukin-10 ($P = 0.010$, $F_{3,14} = 5.55$) * vs CTRL. **c** IL-6/IL-10 ratio ($P = 0.044$, $F_{3,11} = 3.75$) * vs CTRL. **d** Liver

interleukin-6 ($P = 0.346$, $F_{3,15} = 1.19$). **e** Liver interleukin-10 ($P = 0.181$, $F_{3,15} = 1.84$). **f** Liver IL-6/IL-10 ratio ($P = 0.696$, $F_{3,16} = 0.486$). Data expressed as mean ± SEM. $n = 3$ –6 per group. A one-way ANOVA followed by a post hoc Tukey test

Table 3 Effects of reduced estrogen levels on the hematological profile of rats exposed to PM

Cells count	CTRL	Polluted	OVX	Polluted+OVX	P value
RBC ($10^6/\text{mm}^3$)	2.5 ± 0.2	2.5 ± 0.1	2.8 ± 0.2	2.7 ± 0.1	$0.566 (F_{3,20} = 0.69)$
WBC ($10^3/\text{mm}^3$)	0.9 ± 0.03	1.1 ± 0.22	1.3 ± 0.07	1.1 ± 0.14	$0.336 (F_{3,20} = 1.19)$
Lymphocytes	83.0 ± 0.7	80.8 ± 1.3	81.8 ± 1.7	78.0 ± 1.1	$0.061 (F_{3,20} = 2.88)$
Neutrophils	11.2 ± 0.5	12.7 ± 1.3	12.5 ± 1.4	$15.5 \pm 1.2^*$	$0.014 (F_{3,20} = 5.41)$
Monocytes	5.8 ± 0.3	6.5 ± 0.2	5.7 ± 0.4	6.5 ± 0.2	$0.137 (F_{3,20} = 2.06)$
Platelets	227 ± 29	214 ± 27	238 ± 16	251 ± 19	$0.722 (F_{3,20} = 0.44)$
Other blood components					
Hemoglobin (Hgb) (g/dL)	4.7 ± 0.5	4.9 ± 0.3	5.2 ± 0.3	5.1 ± 0.1	$0.697 (F_{3,20} = 0.48)$
Hematocrit (%)	13.3 ± 1.2	13.4 ± 0.7	14.6 ± 1.0	14.5 ± 0.4	$0.642 (F_{3,20} = 0.56)$
Mean corpuscular volume (fL)	53.3 ± 0.7	54.0 ± 0.4	53.3 ± 0.4	53.2 ± 0.5	$0.650 (F_{3,20} = 0.55)$
Mean corpuscular Hgb (pg)	18.7 ± 0.5	19.6 ± 0.5	18.5 ± 0.2	18.8 ± 0.2	$0.220 (F_{3,20} = 1.60)$
Mean corpuscular Hgb conc. (%)	35.6 ± 0.4	36.2 ± 0.9	35.2 ± 0.5	35.1 ± 0.1	$0.435 (F_{3,20} = 0.95)$
Random distribution of RBC (%)	13.0 ± 0.3	13.3 ± 0.3	13.7 ± 0.4	13.1 ± 0.6	$0.675 (F_{3,20} = 0.51)$
Mean platelet volume ($10^3/\text{mm}^3$)	7.4 ± 0.5	7.3 ± 0.5	7.0 ± 0.1	6.5 ± 0.3	$0.372 (F_{3,20} = 1.10)$
Neutrophil/lymphocyte ratio	0.13 ± 0.01	0.16 ± 0.02	0.15 ± 0.02	$0.20 \pm 0.02^*$	$0.049 (F_{3,20} = 4.38)$
Platelet/lymphocyte ratio	2.72 ± 0.3	2.64 ± 0.3	2.92 ± 0.2	3.22 ± 0.2	$0.464 (F_{3,20} = 0.88)$

Data expressed as mean $\pm$ SEM. * $P < 0.05$ vs. CTRL. $n = 6$ per group. A one-way ANOVA followed by a post hoc Tukey test

occur when the cell is unable to maintain ionic homeostasis. Air pollution may be attenuating the development of hepatic steatosis and severe lipid abnormalities via the autophagy mechanism (Qiu et al. 2017).

Several liver dysfunctions significantly alter lipid metabolism. Lipids are necessary components that control cellular functions and homeostasis, and the liver plays an essential role

in lipid metabolism, as well as several stages of lipid synthesis and transportation (Ghadir et al. 2010). Our data show that the effects of ovariectomy on the liver promoted an increase in total cholesterol levels. Since estrogens and estrogen receptors regulate various aspects of glucose and lipid metabolism (Lizcano and Guzmán 2014), reduced estrogen levels promote hypercholesterolemia.

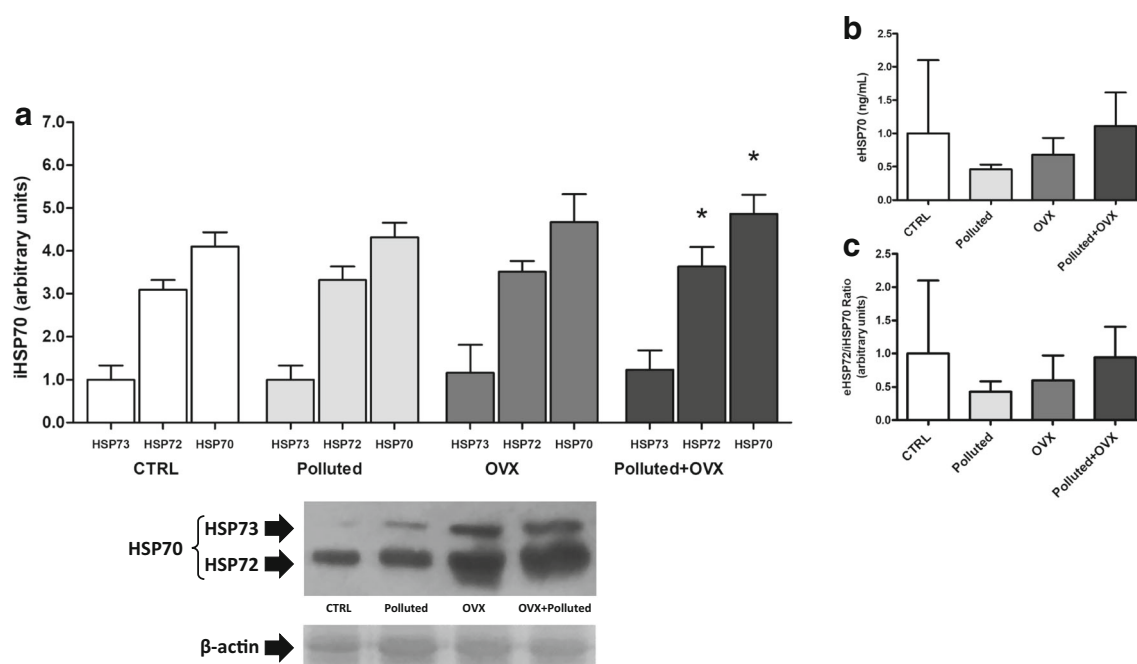


Fig. 6 The effect of low estrogen levels on hepatic iHSP70 expression, eHSP72 levels, and H-index ([eHSP72]/[iHSP70] ratio) values in animals exposed to particulate matter pollution. **a** Liver HSP73 ($P = 0.882$, $F_{3,7} = 0.21$), HSP72 ($P = 0.046$, $F_{3,16} = 2.62$), HSP70 ($P = 0.047$, $F_{3,16} = 2.77$)

(arbitrary units) * vs CTRL. **b** eHSP70 ($P = 0.647$, $F_{3,12} = 0.57$). **c** [eHSP72]/[iHSP70] ratio ($P = 0.727$, $F_{3,12} = 0.44$). Data expressed as mean $\pm$ SD. $n = 3-5$ per group. A one-way ANOVA followed by a post hoc Tukey test

While ovariectomy promoted direct effects on tissues that are related to metabolism, such as the liver, and consequently, in lipid metabolism, particulate matter pollution can directly affect lung tissue, causing pulmonary tissue edema. Exposure to PM can promote tissue injury by the direct effects of particle components, leading to oxidative and cellular stress, and by indirect effects, triggering the production of pro-inflammatory mediators, with consequent systemic low-grade inflammation (Goettems-Fiorin et al. 2016; Hoffmann et al. 2009; Miller et al. 2012).

Estrogen is known to prevent inflammatory gene transcription induced by inflammatory agents by inhibiting NF- κ B intracellular transport, an immediate-early event in the inflammatory signaling cascade (Ghisletti et al. 2005; Pelekanou et al. 2016). In our study, estrogen privation promoted a decrease in anti-inflammatory defense, with a reduction in IL-10 levels and an increase in the pro-inflammatory profile as indicated by the IL-6/IL-10 ratio. The decrease in plasma IL-10 may be causing changes in the leukocyte profile. Interleukin-10 is a crucial inhibitor of many aspects of the inflammatory response, primarily by inhibiting macrophage proliferation (O'Farrell et al. 1998). Once IL-10 can regulate their proliferation and, thus, the numbers of inflammatory cells and reduced IL-10 levels may result in increased neutrophilia and an increased ratio of neutrophils and lymphocytes (NLR). NLR is a simple parameter used to assess the inflammatory status of a subject and has the potential to be a sensitive prognostic marker (Faria et al. 2016). Air pollution is known to promote low-grade chronic inflammation, which can lead to severe cardiometabolic dysfunction in the long term (Sun et al. 2015; Wang et al. 2018). Furthermore, the relationship between estrogens and metabolism is reciprocal since the metabolic control favored by estrogens avoids the establishment of metabolic inflammation (Monteiro et al. 2014), although these effects cannot yet be evidenced directly in tissues related to metabolism, such as in the liver. The adiposity, liver edema, hypercholesterolemia, neutrophil-lymphocyte ratio (NLR), interleukins ratio (IL-6/IL-10), and increase in iHSP72 levels make it clear that the progression of these factors will further aggravate the glucose intolerance observed in particle-exposed animals, predisposing them to metabolic dysfunction, a condition that will develop into type 2 diabetes in later phases, primarily under low levels of estrogen as observed postmenopause.

In our study, we found a decrease in liver antioxidant enzyme activity (Fig. 4), an increase in the plasma pro-inflammatory profile (Fig. 5), and an increase in liver iHSP70 expression in the Polluted+OVX group. Decreased estrogen levels were also correlated with increased lipid peroxidation levels and lower antioxidant enzyme activity. Estrogen deficiency (Escalante-Gómez et al. 2009) and metabolic changes due to its decline predispose the female organism to an increase in the production of reactive oxygen species

(ROS) and reactive nitrogen species (NR), resulting in increased oxidative stress (OS; Crist et al. 2009). Oxidative stress may promote hepatic cellular stress, and at an early stage, the liver is able to evoke the heat shock response (HSR) and, consequently, increase the levels of iHSP70 to maintain homeostasis (Ludwig et al. 2014). Thus, the observed increase in liver iHSP72 content may represent an essential endogenous response to cellular stress and is sensitive to the occurrence of OS (Nakhjavani et al. 2010), related to the availability of estrogen (Hou et al. 2010). Thus, despite the decrease in antioxidant defense (SOD) in the Polluted+OVX group, the increase in the levels of iHSP70 as a cytoprotective response may avoid liver damage and dysfunction. Studies show that the absence of estrogens in rat favors fat accretion in the liver, which is significantly amplified by a high-fat diet (Shinoda and Lavoie 2007). However, our study demonstrated no difference in hepatic triglycerides profile, which may be related to the defense of heat-shock proteins.

It is well known that HSP70 expression is regulated by HSF1, which is, in turn, redox-regulated due to highly conserved cysteines (Ahn and Thiele 2003), in which HSP70 expression is induced following the oxidation of cysteine-containing GSH molecules. HSP70 expression in the liver represents a protective redox-mediated HSR induction by nitric oxide free radicals in rat hepatocytes that occurs when these cells are treated with a pro-inflammatory cytokine, a process mediated by nitric oxide signaling (Kim et al. 1997). Furthermore, decreased estrogen levels diminished the suppressive effect of this hormone in hypothalamic areas involved in thermoregulation, notably the infundibular nucleus in humans and the arcuate nucleus in other mammals, specifically in the kisspeptin-neurokinin B-dynorphin neurons (KNDy). The consequent rise in KNDy neuron activity leads to the stimulation of NO and HSP70 production, both directly and indirectly, via neuropeptide Y (NPY)-secreting neurons as a contra-regulatory action against the decrease in estrogen hormonal regulation (Miragem et al. 2017). Thus, ovariectomy may increase KNDy neuron function and, thus, enhance NF- κ B activity, predisposing the pro-inflammatory state observed in our study. Finally, liver HSP70 may be associated with adrenergic stimulation secondary to long-term estrogen deprivation (Freedman and Neurosciences 2015; Krause and Nakajima 2014) and should confer cytoprotection due to the stimulatory nature of adrenergic signaling over HSR and HSP70 production in the liver.

Although the H-index ([eHSP72]/[iHSP70] ratio) can reveal the context of an inflammatory process (Heck et al. 2017) and insulin resistance state (Krause et al. 2015), in our study, H-index was not altered by low levels of estrogen and exposure to particles. Our previous studies suggest that acute exposure to ROFA suspension at higher doses (a 1.5-fold dose—375 μ g/50 μ L) was able to produce an increase in plasma eHSP70 levels (primarily the 72-kDa inducible form) and

promoted alterations in plasma oxidative stress (Baldissera et al. 2018), although in chronic exposure studies, there was no increase in eHSP70 levels (Goettems-Fiorin et al. 2016; Mai et al. 2017). As estrogen is a physiological inducer of the heat-shock response, some estrogen-based protective effects could be related to its anti-inflammatory action via HSP70 expression. Studies have shown that in short-term estrogen deprivation, the HS response seems to be preserved, which an efficient HSP70-based anti-inflammatory response, thus avoiding oxidative stress and tissue damage (Miragem et al. 2015). Furthermore, in contrast with high-fat diet animal models with severe metabolic disease (Bruxel et al. 2019; Goettems-Fiorin et al. 2016) or human obesity studies (Rodrigues-Krause et al. 2012), which found an altered H-index, in our study, we did not find an imbalance in eHSP70 and iHSP70 levels. This difference may be due to the moderate nature of our experimental challenges, which promote only moderate biometric and metabolic effects without a greater increase in insulin resistance and obesity, in comparison with more aggressive metabolic disease studies.

While our study evidenced that ovariectomy promotes several metabolic, oxidative, pro-inflammatory, and heat-shock balance changes in the liver and predisposes female rats to fine particulate matter exposure effects, new studies may provide a more complete description of the effects of menopause and air pollution exposure on metabolic profiles. Thus, future animal studies that aim to investigate insulin levels and the insulin signaling pathway in the liver, lipogenesis and fatty acid oxidation enzymes metabolism, other hormones involved in metabolism, such as leptin, as well as a histopathological analysis of liver and adipose tissue are recommended. Studies involving hormonal replacement may help prove the role of estrogen levels in the effects observed in our study.

Conclusion

Ovariectomy promotes biometric and metabolic effects that, under environmental air pollution exposure conditions, represent an increased susceptibility to an oxidative stress state, a pro-inflammatory profile, and an altered liver heat-shock response. Our data indicate that alterations in the reproductive system predispose female organisms to particulate matter air pollution effects.

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Authors' contributions PBGF completed all the experiments described in this manuscript. LCCB, JBS, PTF, and LMS performed the biometric and metabolic profile procedures and performed the administration of

particles in the rats used in this study. LCCB, JBS, and LMS performed the experiments on oxidative stress parameters. JBS and LMS performed the Western blot analyses. MNF performed the hematological procedures. TGH performed eHSP70, cytokine, and eHSP70/iHSP70 ratio procedures. All authors were involved in analyzing the results. TGH and PBGF co-wrote the manuscript. Figure 1 was designed by TGH and PBGF. TGH, MSL, and CRR designed the study and provided experimental advice and helped with manuscript revision. All the authors had final approval of the submitted and published versions.

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Data availability The data used to support the findings of this study are available from the corresponding authors upon request.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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ARTIGO 2

PARA SUBMISSÃO

Título: Ovariectomy increases lipid deposition in the liver of HFD-rats exposed to particulate matter pollution independent of oxidative stress and inflammatory markers

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Objetivo: Investigar se a redução nos níveis de estrogênio altera perfil oxidativo e inflamatório hepático de ratas obesas expostas a poluição por material particulado fino.

Normas para submissão em: Environmental Science and Pollution Research

OVARECTOMY INCREASES LIPID DEPOSITION IN THE LIVER OF HFD-RATS INDEPENDENT OF PARTICULATE MATTER EXPOSURE AND OXIDATIVE STRESS OR INFLAMMATORY MARKERS

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ABSTRACT

BACKGROUND: The reduction of postmenopausal estrogen levels represents a metabolic risk factor for women. Also, inadequate diet consumption and exposure to particulate air pollution may increase the risk of development metabolic diseases in women. As well as hypoestrogenism induced by ovariectomy, high-fat diet (HFD) consumption and particulate matter exposure may increase the pro-oxidative and pro-inflammatory profile in metabolic tissues, as the liver. Here, we aim to investigate the influence of ovariectomy on oxidative and inflammatory liver profile of HFD-rats exposed to particulate matter. **METHODS:** First, 36 female Wistar rats received intranasal instillation of particles suspension in saline (ROFA 250µg) or saline for 12 weeks, 5x/week, and received a standard chow (11.4% of fat) or HFD (58.3% of fat). Following that, animals were submitted to bilateral ovariectomy (OVX) or false surgery (sham) and remained to receive particles (or saline) and HFD (or standard chow) for more 12 weeks. Thus, the female rats were assigned to one of the following five groups (n = 6-9 per group): CTRL, HFD, HFD+ROFA, HFD+OVX, and HFD+ROFA+OVX. **RESULTS:** high fat diet *per se* increased liver cholesterol content, but associated with OVX there was an increase in triglycerides and edema in the liver. These alterations were not accompanied by alterations in oxidative and inflammatory liver profile, or affected by particulate air pollution exposure. **CONCLUSION:** Ovariectomy predisposes fat liver disease independent of oxidative stress and inflammatory markers, and also independent of exposure to environmental pollution.

KEYWORDS: air pollution; high-fat diet; menopause; estrogen; liver; oxidative stress; inflammation

1. INTRODUCTION

Menopause is a typical and gradual process that occurs in middle-aged women and is due to the decline in circulating estrogen hormone production, resulting from the loss of ovarian function (Te Velde et al. 1998; Miquel et al. 2006). Estrogen regulates and coordinates multiple functions in organs, cells and genes through interaction with receptors and plays an important role in glucose and lipid metabolism (Camporez et al. 2013). In this way, experimental studies with ovariectomy in rodents have been shown that decreased levels of estrogen causes an increase in the body weight and intra-abdominal fat deposition (Barros and Gustafsson 2011). Visceral adiposity per se predisposes to the development of pro-oxidative and pro-inflammatory profile (Shen et al. 2014). However, reduced estrogen levels is not the only risk factor that women are exposed in the postmenopausal period. Eating behavior and environmental factors may also affect the women health.

When ovariectomy (OVX) is associated with high-fat diet (HFD) consumption in animal models, there there is an increase in adiposity and inflammatory markers, such as IL-6, reinforcing the hypothesis that eating behavior exacerbates complications of inflammation associated with hypoestrogenism (Ludgero-Correia et al. 2012). Also, the combination of OVX and HFD consumption causes disturbances in the metabolic profile, with increased serum levels of total cholesterol, triglycerides and in the oxidative profile, with an increase of hepatic lipoperoxidation and reduction of the level of antioxidant enzymes in the liver (Ludgero-Correia et al. 2012; Vuković et al. 2014).

Besides obesity is a well-known risk factor for metabolic diseases, it is also a predisposing factor to the harmful effects of some environmental factors, such as exposure to air pollutants (Goldberg et al. 2006). In experimental studies, the combination of obesity, (induced by HFD consumption) and inhalation of particulate matter (PM) has evidenced an early development of a pro-inflammatory and pro-oxidative profile, characteristic of the subclinical pathophysiological process of diabetes type 2 (Sun et al. 2009; Schuster 2010). Recently, our group identified that sub-chronic exposure (12 weeks) at low doses of fine particulate matter (PM_{2.5} 5µg intranasal daily) potentiated metabolic dysfunction in HFD-mice, promoting glucose intolerance, increased levels of serum glucose and triglycerides (Goettems-Fiorin et al. 2016). Also, animals exposed to PM_{2.5} reveal macrophage enlargement in adipose tissue, with a change to pro-inflammatory phenotype, characterized by increased TNF-α and IL-6 levels and decreased IL-10 levels (Mendez et al. 2013).

Thus, although the risk factors described above (HFD consumption, PM_{2.5} exposure and estrogen decreased levels) has similar effects regarding metabolic, oxidative and inflammatory parameters, few studies have investigated the synergistic effects of these conditions on the pathophysiological process of diseases related to metabolism. Thus, in our study, we propose to investigate the influence of ovariectomy on oxidative and inflammatory liver profile of HFD-rats exposed to particulate matter.

2. MATERIALS AND METHODS

2.1. Animals

Female 8 weeks old Wistar rats ($n = 36$), from Animal Facility of Regional University of the Northwestern Rio Grande do Sul State (UNIJUÍ) were kept under controlled conditions of temperature (24 ± 2 °C) and light-dark cycles (light from 7:00 a.m. to 7:00 p.m.). The animals received water and diet *ad libitum*. This protocol was approved by the Animal Ethics Committee of UNIJUÍ (CEUA 076/15).

2.2. Experimental Design

Rats ($n = 36$) were initially randomly allocated into three groups: CTRL ($n=6$), HFD ($n=15$), HFD+ROFA ($n=15$). Animals in CTRL group received standard diet (11.4 % calories from fat) for 24 weeks, while animals from HFD and HFD+ROFA groups received a diet containing 58.3% calories from fat for the same period (see below, please). During the first 12th week of study, CTRL and HFD groups received intranasal instillation daily of 50 μ L of saline (representing a clean environment) and HFD+ROFA group received particles suspension in saline (250 μ g, representing a polluted environment). At 12th week, animals were submitted to ovariectomy (OVX) or false surgery (Sham) and remained receiving standard or high fat diet, saline or particles for more 12 weeks, subdividing animals into five groups: Animals that received saline and were submitted to false surgery (CTRL, $n = 6$), animals that received saline, consumption high fat diet, and were submitted to false surgery (HFD, $n=6$), animals that received particles, consumption high fat diet and were submitted to false surgery (HFD+ROFA, $n=6$), animals that received saline, consumption high fat diet, and were submitted to ovariectomy (HFD+OVX, $n=9$), animals that received particles, consumption high fat diet and were submitted to ovariectomy (HFD+ROFA+OVX, $n=9$) (Fig 1, gray background).

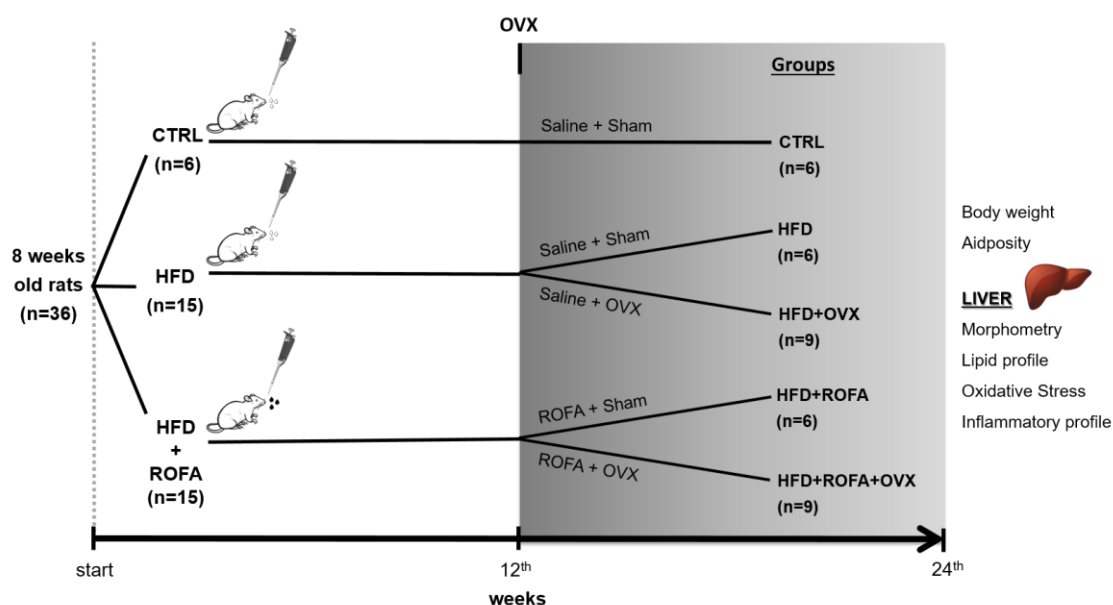


Fig 1 Female Wistar rats ($n = 36$) were initially randomly divided in animals that consumption of standard diet and received intranasal instillation daily for 12 weeks of saline (CTRL), animals that consumption of high fat diet and received intranasal instillation daily for 12 weeks of saline (HFD) and those who consumption of high fat diet and received ROFA particles suspension in saline (250 μ g, representing a polluted environment, HFD+ROFA) (white box). Thereafter, at 12th

week, animals were submitted to ovariectomy or false surgery and remained receiving standard or high fat diet, and saline or ROFA, and for more 12 weeks (gray box), subdividing animals into four groups: Animals that consumption of standard diet and received saline and were submitted to false surgery (CTRL), animals that consumption of high fat diet, received intranasal instillation daily for 12 weeks of saline and were submitted to false surgery (HFD) or ovariectomy (HFD+OVX), and animals in the same condition but were exposed to ROFA (HFD+ROFA and HFD+ROFA+OVX). Thus, after 24 weeks, animals were euthanized, and liver were collected for morphometric, biochemical and molecular analysis.

2.3. Diet

The standard pelleted diet (Nuvilab CR-1, commercially obtained from Nuvital Nutrientes SA.; total metabolizable energy: 16.6 MJ/kg, being 11.4 % as fats, 62.8% as carbohydrates and 25.8% as proteins) consisted of crude protein, mineral material, fibrous matter and minerals. The HFD groups was provided with a lard-based (37.4% w/w) high-fat diet (total metabolizable energy: 22.8 MJ/kg, being 58.3% as fats, 24.5% as carbohydrates, and 17.2% as proteins) work (Winzell and Ahrén 2004; Bock et al. 2015). All diet ingredients in the HFD (except for starch and lard) were adjusted to be present at the same amount as in the standard one. To meet this pattern, 13.7% egg albumin, 7.4% amino acid supplement (Aminomix™) and 1.1% dried bone and oyster flour were used to freshly prepare the experimental diet on a weekly basis.

2.4. Particulate Matter (PM) - Particles Characterization

Residual oil fly ash (ROFA) particles were collected from an electrostatic precipitator installed in the chimney of a large steel factory located in São Paulo (SP, Brazil). Particles consists of Pb (3.1 ± 0.09), Al (789 ± 23), Zn (20.3 ± 0.04), Cd (0.04 ± 0.002), Ba (30.2 ± 0.31), Cu (9.7 ± 0.1), Ni (287 ± 10.8), As (4.1 ± 0.05), Se (7.5 ± 0.20), Mn (48.3 ± 0.98), Sr (8.4 ± 0.16), Sb (2.3 ± 0.57), Fe ($20,397.2 \pm 283.3$), Mg (372.5 ± 1.93), P (388.5 ± 255.8), Cr (7.6 ± 0.23) (mean $\pm$ SD, expressed by ng per m³ of air). The particles have an average aerodynamic diameter of 1.2 ± 2.2 μ m.

Exposure to the pollutant was performed by the intranasal instillation method. Once a day (between 4:00 and 5:00 p.m.), five days a week, over 24 weeks, polluted groups (HFD+ROFA and HFD+ROFA+OVX) received 50 μ L of ROFA suspension at the concentration of 5 μ g/ μ L, performing a dose of 250 μ g per day by nasotropic instillation. Since chronic exposure can be defined as the repeated exposure by the inhalation route for more than 90 days in typically used laboratory animal species (EPA 2010), we consider chronic particle exposure regimen. Control groups (CTRL, HFD and HFD+OVX) received 50 μ L saline solution. The process of nasotropic instillation was performed with an automatic pipette, with 50 μ L of solution in the nostril of the animal, this procedure induces apnea reflex promoting the inhalation of the pollutant (Medeiros et al. 2004).

2.5. Ovariectomy (OVX)

The animals were submitted to the following surgical protocol: pre-anesthetic medication: 5 mg/kg morphine intraperitoneal (i.p.); induction of anesthesia: 4% isoflurane (BioChimico®); maintenance of anesthesia: 2% isoflurane (BioChimico®). The OVX corresponds to the bilateral removal of the ovaries achieved by longitudinal incision of the paralimbic region. The non-ovariectomized animals were submitted to false operation (Sham) where their ovaries were

identified and surgically exposed and then repositioned for posterior suturing of the musculature and skin. After the surgical procedures, all animals (Sham and OVX) received appropriate postoperative treatment, composed of monitoring of body temperature and pain signals, in addition to the pharmacological treatment: meloxicam (Ouro Fino®) 0.2% (2 mg/kg, subcutaneously) after 24 hours (single dose).

2.6. Measurement of 17 β -estradiol

For the determination of 17 β -estradiol, a serum sample was used, and a quantitative dosage was made through the automated system ADVIA Centaur XP® (Siemens Healthcare Diagnosis) by chemiluminescence methodology with sensitivity and in vitro test limits between 20 and 3,000 pg/mL. Ovariectomized rats presented lower estrogen levels compared to non-ovariectomized groups (17 β -estradiol levels: Sham groups = 31.3 ± 9.9 ; OVX groups = $24.7 \pm 7.6^*$ pg/mL; $P = 0.037$).

2.7. Tissue collection and Preparation

At the end of the study, we evaluated the body weight of the animals, and after being euthanized, white adipose tissue and liver were collected, and we evaluate these tissue weight in relation to body weight, and hepatic edema, using the wet/dry weight ratio. After, the tissue was freeze-clamped in liquid nitrogen and stored for further homogenization. Liver tissues (50 mg) was extracted in 1ml isopropanol for analysis of lipid content. For the analysis of oxidative stress [Thiobarbituric acid reactive substances] (TBARS), antioxidant activity enzymes superoxide dismutase (SOD) and catalase (CAT)], a portion of the liver was homogenized in potassium phosphate buffer (KPi) pH 7.4, containing protease inhibitor (PMSF - Phenyl-Methyl-Sulfonyl Fluoride, 100 μ M). Afterward, the homogenates were centrifuged for 10 min at room temperature, and the supernatant fractions were used to Bradford protein determination (1976) (Bradford 1976) using bovine serum albumin as standard.

2.8. Liver triglyceride and cholesterol content

Liver samples (50 mg) were extracted in 1ml isopropanol. After centrifugation, to perform an enzymatic colorimetric determination of the triglyceride and cholesterol concentration, 10 μ L and 20 μ L of the supernatant aliquots were added to 500 μ L of a reagent (Triglicérides Liquiform, Labtest Diagnóstica® and Cholesterol Liquiform, Labtest ®), respectively (Oakes et al. 2001). Results were expressed in g of lipids/100g of liver tissue.

2.9. Oxidative Stress and Inflammatory profile

2.9.1. Liver Lipid Peroxidation levels

Liver lipid peroxidation were analyzed using the thiobarbituric acid reactive substances method (TBARS) (Buege and Aust 1978). Homogenates were precipitated with 10% trichloroacetic acid, centrifuged, and incubated with thiobarbituric acid for 15 min at 100°C. After, the absorbance was measured at 535 nm. The MDA standard was prepared from 1.1.3.3-Tetramethoxypropane (concentrations between 0.0005 – 0.016 mg/mL). Results were expressed in mmol MDA/mg of protein.

2.9.2. Liver SOD and CAT activity levels

Liver SOD activity were performed by inhibition of auto-oxidation of pyrogallol (Marklund and Marklund 1974). Briefly, in a cuvette, 954 μ L of 50 mM Tris/ 1mM EDTA buffer (pH 8.2), four μ L of catalase (CAT; 30 μ M), ten μ L of homogenate and 32 μ L pyrogallol (24 mM in HCl 10 mM) were added and mixed. SOD activity was determined at 36°C in a spectrophotometer (420 nm), every 20 seconds, during 120 s. Results were expressed in units of SOD/mg of protein.

Liver CAT activity were performed by the decomposition of hydrogen peroxide (Aebi 1984). In a quartz cuvette, 955 μ L of phosphate buffer (50 mM, pH 7.4) and 35 μ L of hydrogen peroxide (0.01 M) were mixed, and after, ten μ L of homogenate was added and mixed. CAT activity was determined at 36°C in a spectrophotometer (240 nm), every 15 seconds, during 120 s. The results were expressed in units of CAT/mg of protein.

The SOD/CAT ratio was calculated by dividing USOD/grams of tissue and UCAT/grams of tissue.

2.9.3. Liver cytokines (IL-10 and IL-6) levels

The liver levels of IL-6 and IL-10 were measured using an ELISA kit (RayBio® Rat IL-6 and IL-10, Raybiotech, Inc., USA). The colorimetric optical density was detected at a wavelength of 450 nm. The cytokine levels were calculated based on a standard curve constructed for each assay, and each assay was performed in duplicate. All cytokines were reported as pg/ml.

2.10. Statistical Analysis

Statistical analysis was developed using T-test (for 17 β -estradiol levels) and One-way analysis of variance (ANOVA; for other parameters). Post hoc multiple comparisons among groups were performed with the Tukey's test. All statistical analyzes were performed using GraphPad for Windows, version 5.0. The level of significance was set to $P < 0.05$. Results were expressed as mean $\pm$ standard error.

3. RESULTS

The consumption of high-fat diet *per se* did not increase the body weight of female rats (Control vs HFD groups, $P > 0.05$). However, ovariectomy (HFD+OVX) increased body weight in comparison with the CTRL, HFD group, and ROFA exposure avoids this effect (Table 1). Also, all high-fat and ovariectomized groups presented an increase in adiposity in comparison with CTRL group, without the influence of ROFA exposure (Table 1).

Table 1. Effects of reduced estrogen levels on body weight and adiposity of HFD-rats exposed to PM

Parameter	CTRL	HFD	HFD+ROFA	HFD+OVX	HFD+ROFA+OVX	<i>P value</i>
Body weight (g)	223.3 ± 8.3	225.7 ± 5.6	233.8 ± 11.4	288.6 ± 14.8 *§ #	262.2 ± 8.0	0.0005 ($F_{4,31}=6.81$)
Adiposity (%)	0.91 ± 0.17	1.79 ± 0.22	1.80 ± 0.13	2.69 ± 0.33 *	2.61 ± 0.22 *	0.0001 ($F_{4,31}=8.42$)

Data expressed as mean ± SEM. * vs. CTRL. § vs. HFD. # vs. HFD+ROFA. One-way ANOVA followed by *post-hoc* Tukey test. $n=6-9$ per group.

High-fat diet consumption promoted an increase in the hepatic levels of cholesterol content, and this effect was not influenced by ovariectomy or ROFA exposure (Fig 2A). However, ovariectomy increased the hepatic levels of triglycerides (Fig 2B) edema (Fig 2C) in high-fat treated rats, also without the influence of ROFA exposure. These effects listed in hepatic tissue was not accompanied by alterations in relative liver-to-body weight mass (Fig 2D).

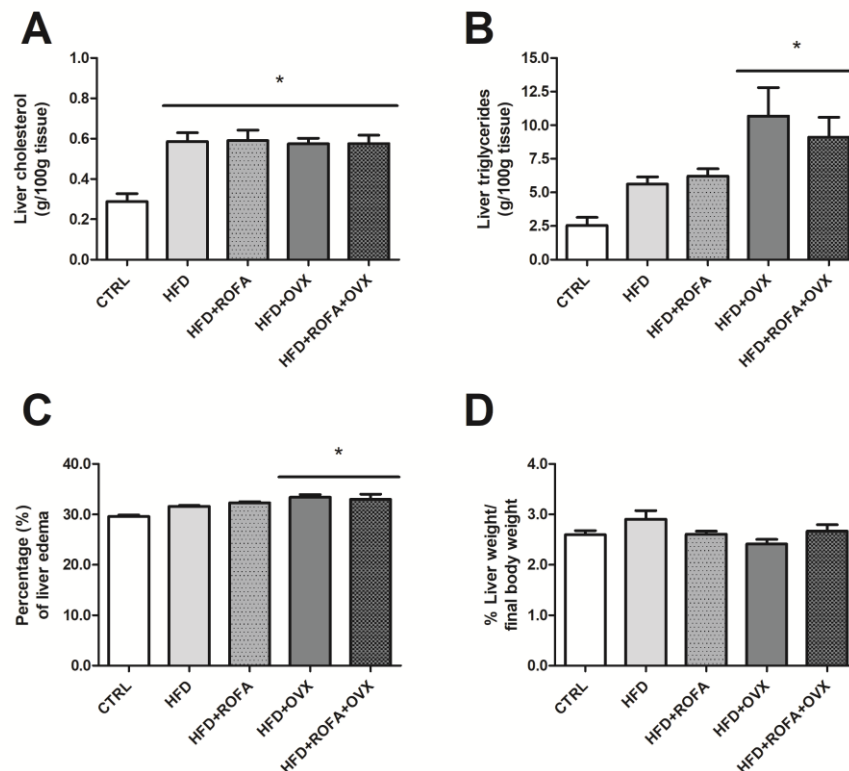


Fig 2 Effect of reduced estrogen levels in HFD-animals exposed to particulate matter on liver tissue. **(A)** Liver cholesterol content ($P < 0.0001$; $F_{4,35} = 9.16$) * vs. CTRL. **(B)** Liver triglyceride content ($P = 0.0054$; $F_{4,35} = 4.52$) * vs. CTRL. **(C)** Percentage of liver edema ($P < 0.0001$; $F_{4,34} = 4.54$) * vs. CTRL. **(D)** Percentage (%) of the tissue weight/final body weight ($P=0.076$; $F_{4,35} = 2.35$). Data presented as mean $\pm$ SEM. $n=6-9$ per group. One-way ANOVA followed by *post-hoc* Tukey test.

At the end of the study, we evaluated liver oxidative and inflammatory profiles. We found no alterations in the liver lipid peroxidation levels (Fig 3A) and in the antioxidant enzyme activity of CAT (Fig 3 B) and SOD (Fig 3C). Also, we found no alterations on IL-6 (Fig. 3D) and IL-10 (Fig 3E) cytokines content in the liver homogenates. Therefore, the pro-inflammatory/anti-inflammatory balance (IL6/IL-10 ratio) was not altered in our study (Fig 3F).

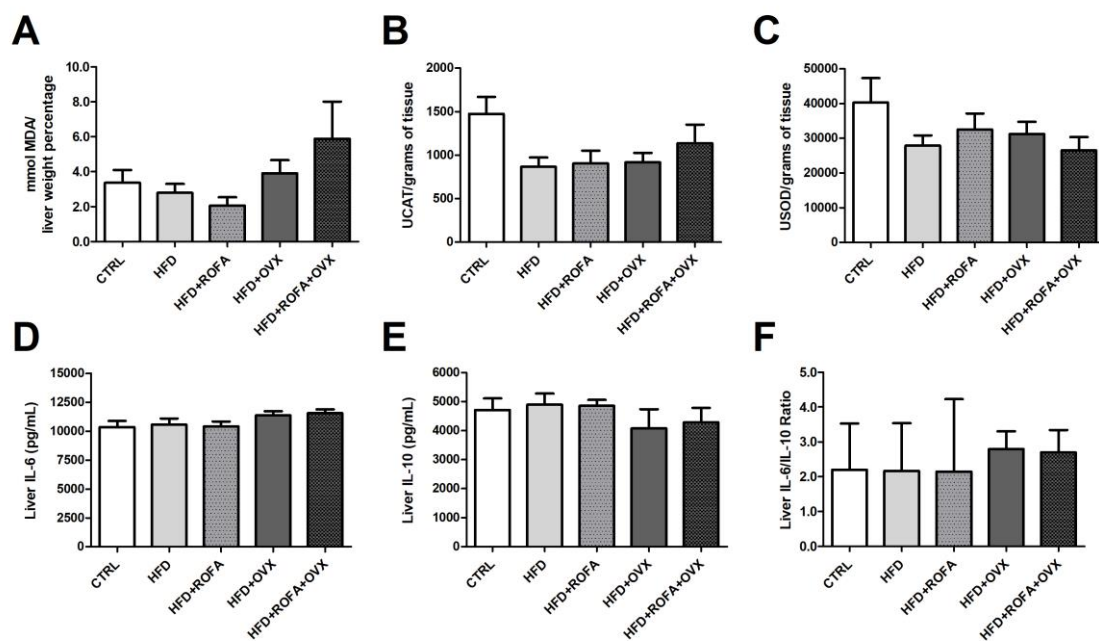


Fig 3 Effect of reduced estrogen levels in HFD-animals exposed to particulate matter on liver redox profile and liver levels of interleukin-6, interleukin-10 and IL-6/IL-10 ratio at the 24^a week. **(A)** Lipoperoxidation ($P = 0.308$ $F_{4,31}=1.25$) **(B)** Catalase ($P = 0.120$ $F_{4,34}=1.99$) **(C)** Superoxide dismutase ($P = 0.256$ $F_{4,35}=1.40$) **(D)** Interleukin-6 ($P= 0.194$; $F_{4,24}= 1.67$) **(E)** Interleukin-10 ($P= 0.642$; $F_{4,24}= 0.63$) **(F)** IL-6/IL-10 Ratio ($P=0.880$; $F_{4,24}= 0.29$). Data expressed as mean $\pm$ SEM. $n=5-9$ per group. One-way ANOVA followed by *post-hoc* Tukey test.

4. DISCUSSION

In our study, we propose to investigate the influence of ovariectomy on oxidative and inflammatory profile of HFD-rats exposed to particulate matter. The main findings of our study were: 1) HFD consumption increased hepatic cholesterol levels; 2) HFD effects become more evident when associated with lower estrogen levels induced by ovariectomy as showed by the increased adiposity, hepatic triglycerides levels and edema in the liver; 3) None of these effects were modified by association with air pollution exposure and 4) the hepatic lipid profile modification was not accompanied by liver oxidative or inflammatory markers.

First, to simulate female organism conditions, we used ovariectomy surgery procedure in animal model for induction of hypoestrogenism which characterizes a postmenopausal condition, an animal model widely used in many studies because of its simplicity and reliability (Ye et al. 2018). Thus, animals were ovariectomized at 5 months old, and at the end of the experimental design, all rats were 8 months old. This age was chosen because around 9 to 12 months of age, rodents usually experience irregular cycles, called estropause. When the estrus phase is prolonged, anovulatory cycles may occur and the animals may then transit to an anestrus state where ovulatory cycles cease and low levels of gonadal steroids are present, which may alter the assessment of estrogen levels in sham and OVX animals. Rodents generally enter persistent estrus during reproductive senescence (estropause), characterized by moderate to high levels of 17β -estradiol, whereas in human reproductive senescence (climacteric) the levels of 17β -estradiol are low or undetectable (Koebele and Bimonte-Nelson 2017). Considering the ovariectomy model detail described above, we investigated HFD and air pollution effects based on groups with different hormonal conditions, as high levels of estrogen (which characterizes the reproductive phase) and low levels of estrogen (which characterizes postmenopausal condition). Thus, since 17β -estradiol reaches low or non-detectable plasmatic levels within 1-2 wk (Brinton, 2014), the time of 12 weeks after OVX was sufficient for a significant reduction in estrogen levels (17β -estradiol levels: Sham groups = 31.3 ± 9.9 ; OVX groups = $24.7 \pm 7.6^*$ pg/mL; $P = 0.037$), and was adequate to analyze the consequences of this reduction in metabolic and inflammatory profile of female rats.

We observed that consumption of HFD increases the fat content of liver. This increase, which occurs mainly in obese individuals, is closely associated with the development of liver diseases, such as nonalcoholic fatty liver disease (NAFLD). NAFLD is defined as either excessive fat accumulation of excess liver triglyceride ($>5\%$ of liver weight) (El-kader et al. 2015; Hart-unger et al. 2017), often asymptomatic at presentation but may progress with time to steatohepatitis and cirrhosis and is associated with the metabolic syndrome (central adiposity, hypertension, insulin resistance, and dyslipidemia), which predisposes to type 2 diabetes mellitus and coronary artery disease (Hart-unger et al. 2017). All HFD-rats of our study presented an increase of cholesterol liver content, in addition, in relation to triglyceride hepatic levels, the HFD effects were aggravated by ovariectomy.

Study of Hart-unger and colleagues (2017) showed that ovariectomized mice, lacking estrogen production, develop increased liver fat content, and proposed that hepatic steatosis is controlled

by a balance between non-hepatic estrogenic signaling and androgenic hepatic or non-hepatic actions. The balance between estrogen levels and liver enzymes in perimenopause and menopause period may be an important parameter for controlling the risk of metabolic syndrome and NAFLD (Hart-unger et al. 2017). In our study, animals submitted to ovariectomy presented edema in the hepatic tissue, although there were no changes in tissue weight. In the same way, Ludgero-Correia and colleagues (2012) highlighted that HFD and OVX animals although did not present an increase of hepatic mass, had the worst tissue structure, with much more lipid droplets within the hepatocytes. Moreover, was previously demonstrated that ovariectomy increased body weight and adiposity and promoted edema in hepatic tissue, hypercholesterolemia, glucose intolerance, and a pro-inflammatory profile (reduced IL-10 levels and increased IL-6/IL-10 ratio levels), independent of particulate matter exposure (Goettems-Fiorin et al. 2019).

We investigated if particulate matter exposure (using ROFA particle instillation model) could influence the liver lipid metabolism. Liver is one of the most vulnerable target organs to be impacted by the effects of air pollution particles. Since liver microvasculature allows almost free access to hepatocytes, the inhaled particles can be translocated from the alveolar space into the bloodstream and reach the hepatocyte, promoting direct effects by induction of oxidative stress and DNA strand breaks (Kim et al. 2014). In this way, our group showed that low levels of PM_{2.5} exposure associated with HFD consumption promoted and increase in mice liver iHSP70 expression, highlighting the evoked cell stress response of this tissue against dangerous extracellular particles and injuring signals, such as ROS and adipokines (Goettems-Fiorin et al. 2016). Corroborant with this observation, previous study showed that PM can activate Kupffer cells, resident hepatic macrophages, and subsequently exacerbates NAFLD via production of pro-inflammatory cytokines. Thus, PM exposure may induced interleukin-6 (IL-6) secretion from isolated Kupffer cells in a concentration dependent manner and this effect was reduced if Toll-like receptor 4 (TLR4) was absent, indicating that PM acts like a TLR4 ligand (Kim et al. 2014). However, in our study exposure to ROFA, a surrogate for PM_{2.5}, did not promoted liver effects in our animals, which can be explained by the fact that the metabolic effects of the air pollutants are only visible in the long term, since they generate a low-grade chronic inflammation (Sun et al. 2009). Also, studies have been shown that repeated exposures, at high doses of PM_{2.5}, in the long term, appear to cause larger and more persistent cumulative effects (Air et al., 2006). Finally, we investigate the effects of the combination of the risk factors described above (HFD consumption, PM_{2.5} exposure and estrogen decreased levels) on liver oxidative and inflammatory markers. Even we previously demonstrated that the reduction of estrogen levels predispose female organisms to particulate matter air pollution effects by affecting metabolic, oxidative, pro-inflammatory, and heat-shock protein expression (Goettems-Fiorin et al. 2019), in this work we did not found alterations in the oxidative and inflammatory profile of female rats. Since animals were ovariectomized after a HFD consumption period, and remained with this obesogenic diet until the end of the study, probably, HFD alone promoted the major impact on

liver metabolism, and in this model it was not possible to evidence synergism between the three factors.

5. CONCLUSION

Loss of estrogen may predispose fat liver disease independent of oxidative stress and inflammatory markers in this tissue, and also independent of exposure to environmental pollution. Our study reinforces the risk of liver fat disease in an obese organism during the decline in estrogen levels process.

6. AUTHOR CONTRIBUTION

PBGF completed all the experiments described in this manuscript. LCCB, JBS, PTF and LMS performed the feed supply, the administration of ROFA in rats and the biometric profile. PRF and KSO performed experiments on oxidative stress parameters. TGH performed cytokine procedures. All authors were involved in analyzing the results. TGH, PBGF and co-wrote the paper. Figure 1 was designed by TGH and PBGF. TGH and CRR designed the study and provided experimental advice and helped with manuscript revision. All the authors had final approval of the submitted and published versions.

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9. DECLARATION OF INTEREST

The authors declare that they do not have competing financial interests.

10. DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

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8. CONSIDERAÇÕES FINAIS

Nosso estudo evidenciou os efeitos da exposição crônica ao ROFA em ratas obesas submetidas à ovariectomia.

O declínio nos níveis de estrogênio, em decorrência da ovariectomia, promove efeitos biométricos e metabólicos que, em condições de exposição à poluição atmosférica, representam aumento da suscetibilidade a um perfil pró-oxidativo, pró-inflamatório e resposta ao estresse celular no fígado. Nossos dados indicam que alterações hormonais no climatério, como a redução dos níveis de estrogênio, podem predispor o organismo feminino aos efeitos da poluição atmosférica.

Quando associado ao consumo de dieta hiperlipídica, a redução nos níveis de estrogênio predispõe ao desenvolvimento de doença hepática gordurosa e doenças metabólicas, independente de alterações no perfil oxidativo e inflamatório hepático, e também independente da exposição à poluição atmosférica.

Concluimos que, em condição de declínio nos níveis de estrogênio, no climatério, ocorre um maior risco para desenvolvimento de doenças metabólicas, quando associado a fatores de risco isolados, como poluição atmosférica ou consumo de dieta hiperlipídica.

9. PERSPECTIVAS

Como perspectivas deste estudo, propomos realizar:

- Avaliação do perfil inflamatório (iHSP70) hepático dos animais submetidos ao consumo de dieta hiperlipídica.
- Avaliação histológica dos tecidos hepático e adiposo brando.
- Avaliação de Estresse Oxidativo e Perfil Inflamatório em outros tecidos metabólicos, como, adiposo branco, pâncreas e músculo esquelético (gastrocnêmio e sóleo).

10. ANEXOS

10.1. NORMAS DO PERÍODICO: ENVIRONMENTAL SCIENCE AND POLLUTION RESEARCH

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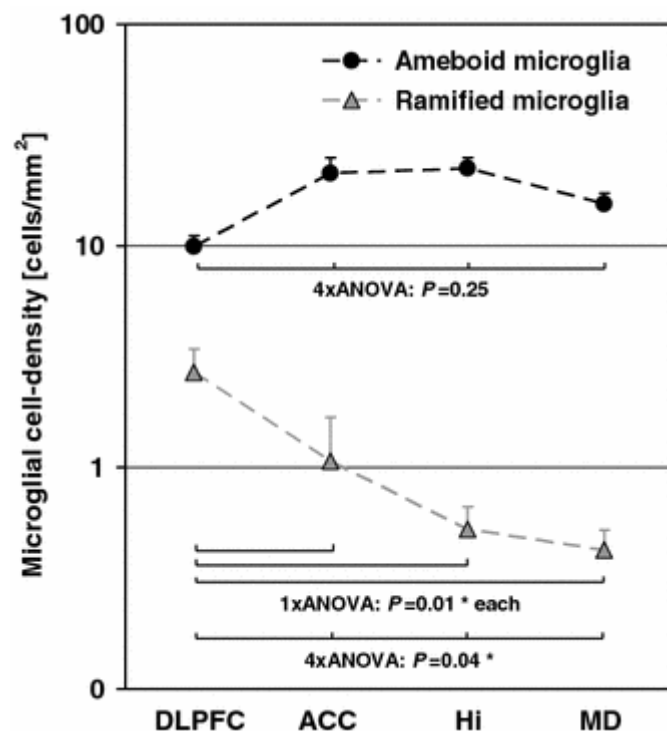
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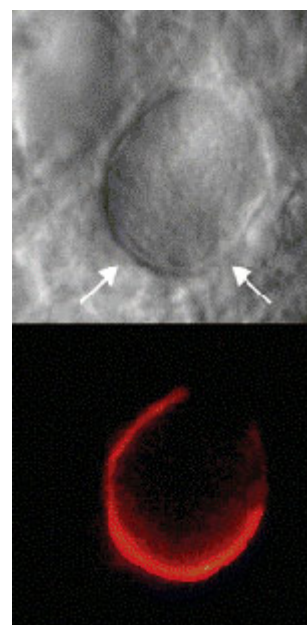
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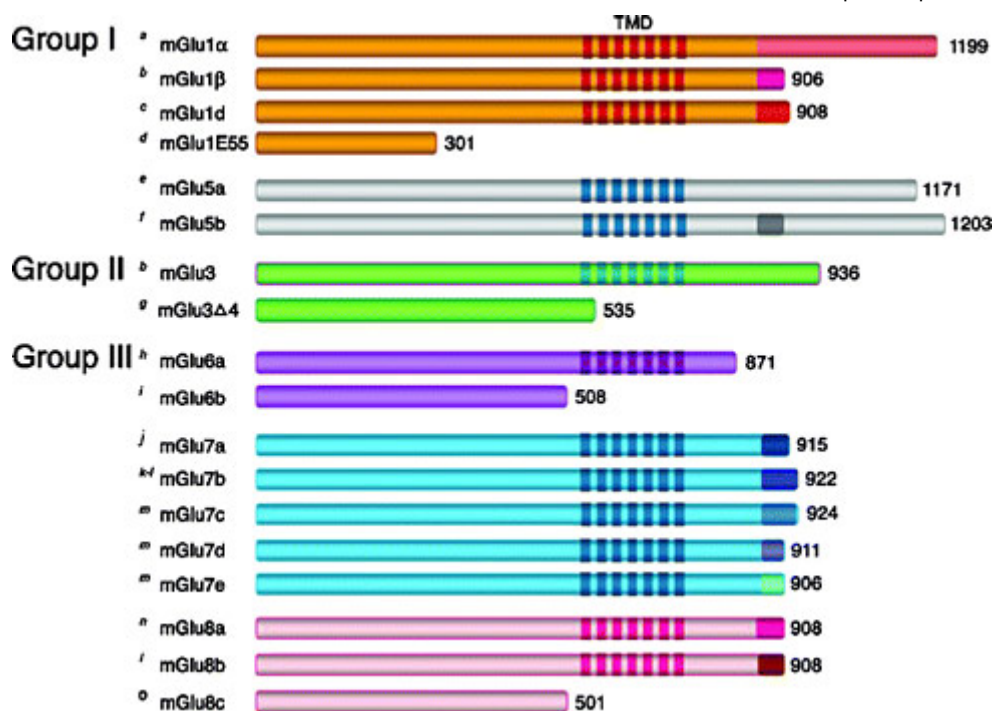
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A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006

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10.2. PARECER CEUA



Comissão de Ética
no Uso de Animais
da UNIJUI

PARECER CONSUBSTANCIADO CEUA - 076/2015

Comissão de Ética no Uso de Animais da UNIJUI

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
DCVDA – UNIJUI
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,  Prof. Dr. Fernando Silvério Ferreira da Cruz Coordenador de CEUA UNIJUI
Ijuí, 10 de dezembro de 2015.

Comissão de Ética no Uso de Animais - CEUA/UNIJUI

Telefone: (55) 3332-0301

E-mail: ceua@unijui.edu.br

Rua do Comércio, 3000, Bairro Universitário, Ijuí/RS - Brasil CEP 98700-000.

10.3. EMENDA PARA CEUA



Comissão de Ética
no Uso de Animais
da UNIJUI

COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA/UNIJUI

FORMULÁRIO PARA APRESENTAÇÃO DE “EMENDA” OU EXTENSÃO DO CRONOGRAMA

PARECER Nº 076/15

Título do Projeto: 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

Pesquisador Responsável: PAULINE BRENDLER GOETTEMES FIORIN

TIPO DE DOCUMENTO: PROTOCOLO DE PESQUISA

1. Quais fatores influenciaram na emissão deste documento?

A emissão deste documento se deve à proposta de alteração de metodologias no protocolo experimental referente ao projeto de título acima descrito. O referido projeto tem anuência do Núcleo de Pesquisa do DCVida e possui aprovação do CEUA pelo Parecer Consubstanciado CEUA de nº 076/2015. As modificações propostas e suas respectivas justificativas são apresentadas a seguir:

Modificação nº1:

Onde esta descrito no projeto: ...serão utilizadas 64 ratas Wistar, no período pós desmame...

Substitui-se por: ...serão utilizadas 64 ratas Wistar, com 2 meses (60 dias de vida)...

Justificativa: Outros estudos realizados pelo grupo de pesquisa em fisiologia (GPef/UNIJUI) evidenciaram que o período ideal para realização da ovariectomia é no período adulto e não desde o desmame.

Referência: Dissertação de mestrado: "EFEITO DA TERAPIA DE CHOQUE TÉRMICO SOBRE PARÂMETROS BIOMÉTRICOS, HEMATOLÓGICOS, BIOQUÍMICOS E DE ESTRESSE CELULAR, EM RATAS WISTAR OVARECTOMIZADAS" de Yana Picinin Sandri Lissarassa no PROGRAMA DE PÓS-GRADUAÇÃO STRICTO SENSU EM ATENÇÃO INTEGRAL À SAÚDE - UNIJUI/UNICRUZ).

Modificação nº2:

Onde esta descrito no projeto: ...os animais serão expostos ao material particulado fino (MP_{2,5}).

Substitui-se por:...os animais serão expostos ao ROFA (Residual oil fly ash).

Comissão de Ética no Uso de Animais - CEUA/UNIJUI

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Justificativa: No momento não foi possível a obtenção de material particulado fino (MP_{2,5}) em coletas realizadas na USP. Sendo o ROFA um dos componentes do MP_{2,5}, o projeto não será prejudicado pela alteração de poluente, uma vez que os principais efeitos que se pretende analisar, como o estresse oxidativo, são gerados pelo ROFA, que promove reações de Fenton, produzindo espécies reativas de oxigênio (Ghio, et al. Biologic effects of oil fly ash. Environ Health Perspect. 2002 Feb; 110(Suppl 1): 89-94).

A dose de ROFA utilizada será de 1mg/kg de animal, conforme já estabelecido por pesquisadores parceiros do GPeF (Prof Paulo Hilário Nascimento Saldiva da USP, referência nacional em estudos com poluição atmosférica - Ferraro, et al. Air particulate matter exacerbates lung response on Sjögren's Syndrome animals. Experimental and Toxicologic Pathology 67 (2015) 125-131).

Modificação nº3:

Serão coletados mais tecidos para estudo.

Justificativa: Os tecidos descritos na coleta de material biológico no projeto são: músculo sóleo e gastrocnêmio, pâncreas, fígado, adiposo branco, adiposo marrom, encéfalo, hipotálamo, rins, coração, pulmões. **Solicitamos a coleta de aorta**, pois inúmeros estudos têm demonstrado os efeitos da poluição atmosférica, bem como, da deficiência de estrogênio sob efeitos vasculares (Marchini, et al. Acute exposure to air pollution particulate matter aggravates experimental myocardial infarction in mice by potentiating cytokine secretion from lung macrophages. Basic Res Cardiol (2016) 111:44; e outro estudo publicado em parceria do com o GPeF: Miragem, et al. Estrogen deprivation does not affect vascular heat shock response in female rats: a comparison with oxidative stress markers. Mol Cell Biochem. 2015 Sep;407(1-2):239-49). O aproveitamento de maior número de material biológico diminui a necessidade de uso futuro de novos animais para um novo estudo.

Modificação nº4:

Extensão do Cronograma, a vigência do Projeto é Agosto de 2018, solicita-se que o prazo seja estendido até agosto de 2020.

Justificativa: Temos como estimativa de que para concretizar todas as fazeres experimentais, análise de dados e publicação dos resultados sejam necessários mais 3 anos.

2. O que se propõe no documento apresentado?

Solicitar aprovação do CEUA para realização das modificações metodológicas e para extensão de cronograma do referido projeto.

As modificações solicitadas para a realização do projeto contribuirão significativamente com os resultados obtidos e evitarão que novos projetos com delineamento semelhante necessitem ser realizados.

3. Anexar os documentos que compõe a emenda.

Os artigos, bem como a dissertação de mestrado, que justificam as alterações propostas estão anexadas junto ao encaminhamento desta emenda.

Comissão de Ética no Uso de Animais - CEUA/UNIJUI

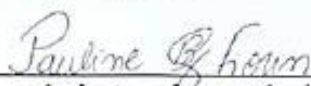
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Declaro estar ciente e de acordo com as informações presentes neste formulário

Local e data: Ijuí, 07 de Junho de 2017


Assinatura do pesquisador

Comissão de Ética no Uso de Animais - CEUA/UNIJUI

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10.4. ANUÊNCIA EMENDA CEUA



Pauline Fiorin <paulinebgfiorin@gmail.com>

Modificações Projeto Parecer 076/15

Comissão de Ética em Uso com Animais <ceua@unijui.edu.br>
Para: Pauline Fiorin <paulinebgfiorin@gmail.com>

19 de junho de 2017 13:54

Boa tarde Pauline,

Comissão está ciente de acordo com as modificações.

Atenciosamente,

Comissão de Ética no Uso de Animais da UNIJUI
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