UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Pauline Brendler Goettems Fiorin

EXPOSIÇÃO CRÔNICA À $MP_{2,5}$ AGRAVA DISFUNÇÃO METABÓLICA INDUZIDA POR DIETA HIPERLIPÍDICA EM CAMUNDONGOS

Porto Alegre

2014

Pauline Brendler Goettems Fiorin

Exposição crônica à $MP_{2,5}$ agrava disfunção metabólica induzida por dieta hiperlipídica em camundongos

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

Orientadora: Dra. Claudia Ramos Rhoden

Co-orientador: Dr. Thiago Gomes Heck

Porto Alegre

2014

Catalogação na Publicação

Goettems Fiorin, Pauline Brendler
Exposição crônica à MP2,5 agrava disfunção metabólica induzida por dieta hiperlipídica em camundongos / Pauline Brendler Goettems Fiorin. -- 2014.
94 f. : il., graf., tab. ; 30 cm.

Dissertação (mestrado) -- Universidade Federal de Ciências da Saúde de Porto Alegre, Programa de Pós-Graduação em Ciências da Saúde, 2014.

Orientador(a): Claudia Ramos Rhoden ; coorientador(a): Thiago Gomes Heck.

1. Obesidade. 2. Material Particulado. 3. Estresse Oxidativo. 4. HSP70. 5. Tecido Adiposo. I. Título.

Sistema de Geração de Ficha Catalográfica da UFCSPA com os dados fornecidos pelo(a) autor(a).

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

ATA 520- 2014

De acordo com o estabelecido previamente pela Comissão Coordenadora do Curso de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre, realizou-se aos quatro dias do mês de julho de dois mil e quatorze, às 09:00h, na sala 508 no Prédio do Anexo II da UFCSPA, a defesa da Dissertação de Mestrado da aluna **Pauline Brendler Goettems Fiorin,** orientada pela Profa. Dra. Cláudia R. Rhoden e co-orientada pelo Prof. Dr. Thiago G. Heck no PPG em Ciências da Saúde da UFCSPA. O trabalho defendido intitula-se "Exposição crônica à $MP_{2,5}$ agrava disfunção metabólica induzida por dieta hiperlipídica em camundongos", e faz parte da área de concentração em Farmacologia e Toxicologia. A Banca Examinadora foi composta pelos professores Anderson Z. Ulbrich (UFP), Raul C. Maranhão (USP) e Helena T. Barros (UFCSPA). Após a abertura da sessão a candidata dispôs de 45 minutos para expor seu trabalho. Ao término da apresentação, foi franqueada à platéia a possibilidade de dirigir perguntas a autora. Ao término da Sessão foi anunciada a **aprovação** da candidata, conferindo-lhe o grau de Mestre em Ciências da Saúde: Farmacologia e Toxicologia. Nada mais havendo a tratar, foi encerrada a sessão e lavrada a presente ata, que será assinada pela orientadora da aluna e pelo Vice-Coordenador do Programa.

Porto Alegre, 04 de julho de 2014.

Profa. Cláudia Ramos Rhoden
Orientadora

Prof. Sérgio L. Amantéa
Vice-Coordenador

AGRADECIMENTOS

À orientadora Prof. Dra. Claudia Ramos Rhoden, por confiar em nosso trabalho e proporcionar a oportunidade de realizar um sonho. Por fazer do Laboratório de Estresse Oxidativo e Poluição Atmosférica um lugar agradável de trabalhar, instigando seus alunos a aprender.

Ao líder do GPeF, co-orientador e amigo Prof. Dr. Thiago Gomes Heck, sempre com palavras de incentivo, pelo otimismo constante, obrigado pela confiança, oportunidade e por tornar o GPeF um grupo sólido e acolhedor.

A "chefe" e amiga Prof. Dra. Mirna Stela Ludwig, que acreditou no meu trabalho no começo de tudo, que sempre nos engradece intelectualmente com grandes discussões e bons conselhos.

As colegas/amigas de mestrado, Bethânia e Fernanda, pelas grandes lutas que superamos juntas, as inúmeras idas à Porto Alegre, pelo companheirismo vivido e momentos compartilhados em cada etapa do mestrado.

Ao querido amigo Maciel, com quem aprendi e sempre aprendo muito, obrigada pela amizade, conversas e desabafos.

Aos queridos amigos e colegas do GPeF, Analu, Eloisa, Eliara, Maicon, Renan, Yohanna e Iberê pela incansável ajuda, amizade, companheirismo e boas risadas, sem vocês não seria possível e não teria sido por muitas vezes divertido. E aos colegas que aos poucos vêm se agregando ao grupo, obrigada por acreditarem em nosso trabalho.

Aos colegas e amigos do Laboratório de Estresse Oxidativo e Poluição Atmosférica, Gabi, Marlise, Marcella, Alan, Ale, Maurício, Grazi, Karen, Paulo, Prica, não tenho palavras para agradecer o quão acolhedor foi cada momento que passamos em Porto Alegre, o carinho de todos vocês com certeza foi uma motivação para que voltássemos toda a semana.

As minhas amigas, que apesar de minha ausência, sempre demonstraram orgulho de minhas conquistas, o que pra mim é um grande incentivo.

Ao meu maior presente de Deus: minha família!

Aos meus pais por me fortalecer nos momentos de fraqueza, pela paciência nas horas de crise, por estar sempre prontos e felizes em patrocinar e acreditar nesta conquista.

Por me ensinaram que com dignidade e muito trabalho um sonho nunca é distante. Por compreenderem minha "presente" ausência, respeitarem meu momento, sempre com espaço para um abraço, um cuidado, um carinho. Obrigado por todo amor e confiança!!!

À minha muito mais que irmã Bruna, minha amiga e confidente fiel, que com muito amor, sempre me dizia "Que mania você tem de subestimar tua capacidade"! Obrigada pelo incentivo e amor.

À minha segunda família, a família Fiorin, que me recebeu de braços abertos, e sempre me acolheu e apoiou com o maior carinho.

Ao meu avô Bruno, pelos inúmeros ensinamentos, e pelo exemplo de humildade e luta.

À minha nova família, meu esposo Diego, pela paciência, pelas palavras de carinho, por me compreender nas horas mais difíceis e respeitar meus momentos. Obrigada por me escutar, me aconselhar e me apoiar em tudo, sempre!!!

E agradeço, principalmente, a Deus que escuta minhas aflições e sempre me ilumina com sabedoria e me guia para o melhor caminho.

RESUMO

Estudos epidemiológicos destacam efeitos nocivos à saúde da associação entre obesidade e exposição a material particulado ($MP_{2,5}$). Entretanto, poucos estudos têm documentado o efeito da exposição à longo prazo à $MP_{2,5}$ nos tecidos relacionados ao metabolismo, em uma condição de obesidade pré-existente. Nesse sentido, procuramos investigar os efeitos da exposição à longo prazo à $MP_{2,5}$ nos tecidos relacionados ao metabolismo de nutrientes (NMRT – *nutrient metabolism related tissues*) em modelo de obesidade induzida por dieta hiperlipídica (HFD - *high fat diet*). Camundongos B6129SF2/J machos foram alimentados com dieta padrão ou HFD durante 12 semanas, e depois foram expostos aleatoriamente à instilação intranasal de salina ou solução de $MP_{2,5}$ diariamente, durante 12 semanas subsequentes. Os animais foram divididos em quatro grupos: CONTROL (dieta padrão + salina; n=15), $PM_{2.5}$ (dieta padrão + $MP_{2,5}$; n=15), HFD (dieta hiperlipídica + salina; n=14) e HFD + $PM_{2.5}$ (dieta hiperlipídica + $MP_{2,5}$; n=15). Foram avaliadas medidas biométricas, perfil glicêmico, lipídico e teste de tolerância à glicose (GTT) durante o período experimental. Após 24 semanas de intervenção, os tecidos foram avaliados em relação ao peso tecido/peso corpóreo, perfil oxidativo e expressão de HSP70. A exposição à longo prazo à $MP_{2,5}$ promoveu prejuízo na resposta glicêmica durante sobrecarga de glicose em animais obesos induzidos por HFD. A associação HFD e $MP_{2,5}$ promoveu diferentes respostas oxidativas e expressão de HSP70 em NMRT. Esta associação também desenvolveu uma diminuição da massa muscular e pancreática. Danos nos NMRT levaram a alterações na glicemia e triglicerídeos, ao longo das 24 semanas de estudo. Conclui-se que a exposição à longo prazo à $MP_{2,5}$ promove prejuízo na resposta glicêmica durante sobrecarga de glicose em animais previamente tratados com dieta hiperlipídica, altera o perfil oxidativo e expressão de HSP70 em NMRT, fornecendo evidências para uma importante interação entre meio ambiente e fatores dietéticos.

Palavras-chave: Obesidade. Material Particulado. Estresse Oxidativo. HSP70. Tecido Adiposo.

ABSTRACT

Epidemiological studies demonstrate an important health risk by association among obesity and exposure to fine particulate matter ($PM_{2.5}$). However, few studies have documented the effect of long-term exposure to $PM_{2.5}$ in metabolism involved tissues in a pre-existing obesity condition. We seek to investigate the long-term effects of $PM_{2.5}$ exposure on nutrient metabolism related tissues (NMRT) for high fat model of diet-induced obesity. Male mice were fed with standard or high fat diet (HFD) for 12 weeks. After the diet protocol they were randomly exposed to daily intranasal instillation of saline or $PM_{2.5}$ solution for subsequent 12 weeks, divided into four groups: CONTROL (n=15), $PM_{2.5}$ (n=15), HFD (n=14) e HFD + $PM_{2.5}$ (=15). Biometric and metabolic measurements were evaluated during the experimental time. After 24 weeks, it was evaluated liver, pancreas, gastrocnemius muscle and epididymal white adipose tissue (EWAT) weights, their respective oxidative profile and HSP70 expression. The long-term exposure to $PM_{2.5}$ promoted impaired glycemic response during glucose overload in obese animals induced by HFD. HFD and $PM_{2.5}$ association promotes different oxidative responses and HSP70 expression in NMRT. This association also promoted a decrease in muscle and pancreatic mass. Impaired NMRT lead to changes in blood glucose and triglyceride levels, along the 24 weeks of study. Long-term exposure to $PM_{2.5}$ worsens the glycemic response to glucose overload in animals previously treated with HFD, and altered oxidative profile and HSP70 expression in NMRT, providing evidence for an important interaction between environmental and dietary factors.

Keywords: Obesity. Particulate Matter. Oxidative Stress. HSP70. Adipose Tissue.

LISTA DE ILUSTRAÇÕES

Figura 1. Instilação intranasal realizada em camundongo B6. 25
Figura 2. Ração Padrão (4% de gordura - Nuvilab CR-1) e Ração Hiperlipidica (60% de gordura) preparada para consumo de camundongo B6. 27

LISTA DE ABREVIATURAS

CAT: Catalase

CO: Monóxido de carbono

CoQ10: ubiquinona, Coenzima Q10

DHL: Dieta Hiperlipídica

DM: Diabetes mellitus

DM2: Diabetes mellitus tipo 2

DNA: Ácido Desoxirribonucleico

EDTA: *ethylenediaminetetraacetic acid*

eHSP70: Proteína de Choque térmico extracelular

EO: Estresse oxidativo

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

EWAT: *Epididymal White Adipose Tissue* - Tecido Adiposo Branco Epididimal

GPx: Glutationa Peroxidase

GR: Glutationa Redutase

GSH: glutationa reduzida

GSSG: glutationa oxidada

GTT: *Glucose Tolerance Test* - Teste de Tolerância a Glicose

H_2O: Água

H_2O_2: Peróxido de Hidrogênio

HCl: Ácido Cloridríco

HSPs: *Heat Shock Proteins* - Proteínas de Choque térmico

HSP70: Proteína de Choque térmico de 70 KDa

i.n.: Intranasal

i.p.: Intraperitoneal

IAUC: *Incremental Area Under the Curve* - Área incremental sob a Curva

IAUC-GTT: Área incremental sob a curva em resposta ao teste de tolerância à glicose

IMC: Índice de Massa Corporal

IR: *Insulin Resistance* - Resistência à insulina

KPi: *Potassium Phosphate Buffer* - Tampão Fosfato de Potássio

LPO: Lipoperoxidação

MDA: Malondialdeido

MP: Material Particulado

MP_{10}: Material Particulado Grosso <10μM

$MP_{2,5}$: Material Particulado Fino <2,5μM

$MP_{0,1}$: Material Particulado Ultrafino <0,1μM

NO_2: Dióxido de Nitrogênio

O_2: Oxigênio

$^1O_2$: Oxigênio *singlet*

$O_2^{\bullet-}$: Radical Superóxido

O_3: Ozônio

OH^-: Radical Hidroxila

PM: *Particulate Matter* - Material Particulado

$PM_{2.5}$: *Fine Particulate Matter* - Material Particulado Fino <2,5µM

PMSF: *Phenylmethanesulfonyl Fluoride*

PTS: Partículas Totais em suspensão

ROS: *Reactive Oxigen Species* - Espécies reativas de oxigênio

SDS: *Sodium Dodecyl Sulfate*

SO_2: Dióxido de Enxofre

SOD: Superóxido Dismutase

TBARS: *Thiobarbituric Acid Reactive Substances* - Teste de Substâncias Reativas ao Ácido Tiobarbitúrico

TLCK: *Tosyl-L-Lysine Chloromethyl Ketone Hydrochloride*

TRIS: *2-amino-2-hydroxymethyl-propane-1,3-diol*

SUMÁRIO

Introdução **10**
1. POLUIÇÃO ATMOSFÉRICA 10
1.1 Fontes Poluidoras 10
1.2 Tipos de Poluentes 11
1.3 Efeitos da Poluição na Saúde 12
1.4 Níveis Recomendados para Exposição à Poluição do Ar 12
2. OBESIDADE 14
3. ESTRESSE OXIDATIVO 16
3.1 Espécies Reativas de Oxigênio 16
3.2 Defesas Antioxidantes 18
3.3 Estresse Oxidativo, Obesidade e Poluição Atmosférica 20
4. PROTEÍNAS DE CHOQUE TÉRMICO – Heat Shock Proteins (HSPs) 21
4.1 HSP70, Estresse Oxidativo, Obesidade e Poluição Atmosférica 22
5. MODELOS EXPERIMENTAIS DE EXPOSIÇÃO À POLUIÇÃO ATMOSFÉRICA . 24
6. MODELOS EXPERIMENTAIS DE OBESIDADE 25
Justificativa **28**
Objetivo Geral **29**
Objetivos específicos 29
Referências **30**
Artigo **36**
ABSTRACT 38
INTRODUCTION 39
MATERIALS AND METHODS 39
RESULTS 46
DISCUSSION 49
CONCLUSION 53
REFERENCES 54
TABLES 57
FIGURES 58
SUPPLEMENTAL MATERIAL 64
Conclusão **65**
Anexos **66**
1. Normas do periódico Environmental Health Perspectives 66
2. Parecer da Comissão de Ética no Uso de Animais (CEUA) 94

INTRODUÇÃO

1. 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 (EPA, 2010). A poluição atmosférica, por sua vez, pode ser definida como qualquer forma de matéria ou energia com intensidade, concentração, tempo ou características que possam tornar o ar impróprio e nocivo à saúde, sendo inconveniente ao bem-estar público e à qualidade de vida da comunidade (MMA, 2012).

Os poluentes são classificados de acordo com a fonte poluidora, com o tipo de emissão e em função de suas transformações quando em contato com o meio ambiente.

1.1 Fontes Poluidoras

Uma fonte de poluição é caracterizada por exercer uma atividade que realiza a emissão de poluente para a atmosfera. Existem dois tipos de fontes poluidoras, classificadas em fonte naturais ou biogênicas e fontes antropogênicas. Exemplo de fontes naturais são vulcões, matéria orgânica em decomposição, árvores e outras vegetações que liberam grandes quantidades de poeira e pólen na nossa atmosfera (EPA, 2010).

As fontes antropogênicas se originam do processo de modernização, incluindo indústrias, agricultura, mineração, transportes, construção e habitações. Estas podem ser classificadas em: fontes móveis e fixas (estacionárias), entre as quais, fontes móveis incluem a maioria das formas de transporte, como automóveis, caminhões e aviões, e as fontes fixas ocupam uma área relativamente limitada, como instalações industriais e habitacionais (EPA, 2010).

Do mesmo modo, podemos classificar a poluição de acordo com o ambiente onde os poluentes se concentram. Sabe-se que, hoje em dia, as pessoas estão expostas a poluentes do ar em ambientes externos, chamado de poluição *outdoor,* mas também em ambientes internos, chamada de poluição *indoor*.

Em relação a poluição *outdoor*, destacam-se poluentes originários de fontes fixas (indústrias e residências) e fontes móveis (carros, ônibus e caminhões), que emitem misturas complexas de substâncias no ar. Condições como localização geográfica, temperatura, vento e

fatores climáticos influenciam na dispersão destes poluentes e, portanto, no tipo de exposição e agravo à condição de saúde (Lawrence Berkeley National Lab, 2012).

A poluição *indoor* também tem recebido ênfase recentemente, a partir do momento que se compreendeu que os efeitos da poluição *indoor* podem ser tão prejudiciais quanto aos causados pela poluição *outdoor*. Podemos considerar como fontes poluidoras *indoor*: a fumaça produzida pela combustão de biomassa (lenha, folhas, esterco, etc) e os produtos gerados pela combustão do gás de cozinha, uso de pesticidas e produtos de limpeza (EPA, 2010).

1.2 Tipos de Poluentes

A identificação dos poluentes presentes no ar contribui para elucidar os riscos à saúde e para a criação de políticas para o manejo de questões ambientais e sociais. Os produtos emitidos diretamente pelas fontes poluidoras são chamados de poluentes primários, como o Material Particulado (MP), Dióxido de Enxofre (SO_2), Dióxido de Nitrogênio (NO_2) e Monóxido de Carbono (CO), enquanto os que resultam de transformação com o meio ambiente são poluentes secundários, como é o caso do Ozônio (O_3) (Ministério do Meio Ambiente).

Dentre estes poluentes, o Material Particulado é o que tem recebido maior ênfase em pesquisas cientificas, tanto epidemiológicas, como experimentais
Sob a denominação geral de Material Particulado se encontra um conjunto de poluentes formados a partir dos processos de combustão, incluindo fontes emissoras como veículos, usinas, incêndios, queimadas agrícolas e processos industriais, constituídos de poeira, fumaça e todo o tipo de material sólido e líquido que se mantém suspenso na atmosfera (EPA, 2011).

O tamanho das partículas está diretamente associado ao seu potencial para causar efeitos adversos à saúde, sendo que, quanto menor a partícula, maiores são os efeitos produzidos (Xu et al., 2011). O material particulado pode ser classificado em Partículas Totais em Suspensão (PTS), que são aquelas cujo diâmetro é menor que 50 μm, e em Partículas Inaláveis, cujo diâmetro é menor que 10 μm e que também podem ser classificadas em partículas grossas – MP_{10} (2,5 a 10 μm), partículas finas – $MP_{2,5}$ (<2,5 μm) ou ultrafinas – $MP_{0,1}$ (<0,1 μm) (Ministério do Meio Ambiente).

1.3 Efeitos da Poluição na Saúde

A poluição do ar pode afeta 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 (Lawrence Berkeley National Lab, 2012).

Mais especificamente, podemos caracterizar como efeitos de exposição à curto prazo como: irritação dos olhos, nariz e garganta, e infecções respiratórias superiores, tais como bronquite e pneumonia. Como efeitos à longo prazo sobre a saúde podemos incluir: doença respiratória crônica, câncer de pulmão, doenças cardíacas, e até mesmo danos ao sistema nervoso central (Lawrence Berkeley National Lab, 2012).

Emmerechts e Hoylaerts (2012) destacam que a exposição à poluentes do ar constitui um importante fator de risco para desenvolvimento de doenças numa escala global. Estudos epidemiológicos e experimentais vêm fornecendo, ao longo das últimas décadas evidências de que a poluição atmosférica, derivada dos processos de modernização global, tem contribuído para o desenvolvimento de inúmeras patologias cardiorrespiratórias e consequentemente aumentando a morbidade associada a essas doenças (Janghorbani et al., 2014). Foram observados efeitos deletérios da poluição do ar sobre a saúde humana, que podem causar tanto aumento da mortalidade, como na morbidade de graves doenças, incluindo doenças cardiovasculares, pulmonares e câncer (Pope et al., 2002).

Nesse sentido, destaca-se que as PTS podem causar problemas à saúde ou afetar desfavoravelmente a qualidade de vida da população, por interferir nas condições climáticas do ambiente e prejudicar as atividades normais da comunidade. Em relação ao MP_{10}, as principais preocupações sobre à saúde humana incluem: efeitos sobre o sistema respiratório, danos ao tecido pulmonar, câncer e morte prematura (EPA, 2012). Sobre a exposição à $MP_{2,5,}$ destaca-se que estas partículas devido ao seu tamanho diminuto, têm a capacidade de atingir tanto vias aéreas respiratórias como a corrente sanguínea, consequentemente levando a uma ação sistêmica. Seus efeitos podem levar à alteração do tônus vasomotor, inflamação sistêmica, adiposidade, aterosclerose e resistência insulínica (Xu et al., 2011).

1.4 Níveis Recomendados para Exposição à Poluição do Ar

A poluição do ar continua a representar uma ameaça significativa para a saúde mundial, segundo a OMS (2014), em 2012, cerca de 7 milhões de pessoas morreram em

resultado à exposição à poluição atmosférica. Portanto, o estabelecimento e o controle de padrões de qualidade do ar podem contribuir para realização de estimativas que auxiliem na administração de problemas ambientais e da saúde da população. Nesse sentido, o padrão americano de qualidade do ar para concentrações de MP_{10} ficou mais rigoroso no ano de 2005, passando o limite máximo permitido para um período de 24 horas de 150 $\mu g/m^3$ para 50 $\mu g/m^3$ em média, e para o período de um ano de 50 $\mu g/m^3$ para 20 $\mu g/m^3$ em média(OMS, 2005). Quando consideramos o $MP_{2,5}$, o padrão estabelecido para concentrações deste passou de 65 $\mu g/m^3$ para 35 $\mu g/m^3$, durante o período de 24 horas e de 15 $\mu g/m^3$ para 12 $\mu g/m3$, durante o período de um ano (EPA, 2012).

No Brasil, os padrões de qualidade do ar foram estabelecidos pela resolução do Conselho Nacional do Meio Ambiente (CONAMA) nº 03/90, sendo classificados em padrões primários e secundários. São padrões primários de qualidade do ar as concentrações de poluentes que, ultrapassadas, poderão afetar a saúde da população, e padrões secundários as concentrações de poluentes atmosféricos abaixo das quais se prevê o mínimo efeito adverso sobre o bem estar da população. Esses parâmetros podem ser entendidos como níveis desejados de concentração de poluentes, constituindo-se em meta de longo prazo (Ministério do Meio Ambiente, 2012)

O Brasil segue os mesmos valores estabelecidos pelo padrão americano para MP_{10}, em relação ao período de 24 horas (média 50 $\mu g/m^3$) e de um ano (média 20 $\mu g/m^3$). Sendo que para PTS foi adicionado um padrão de qualidade do ar de 240 $\mu g/m^3$ para 24 horas e 80 $\mu g/m^3$ para um ano (CONAMA) nº 03/90). Entretanto, não há ainda padrão estabelecido para $MP_{2,5}$ no Brasil.

A exposição à poluição atmosférica leva ao aumento de estresse oxidativo (Capítulo 3) e tem ação pró-inflamatório (Capítulo 4), sendo potencial agente nocivo para o surgimento de inúmeras patologias. Nesse sentido, enfatiza-se a importância da realização de pesquisas que tenham como intuito elucidar os mecanismos pelos quais a exposição à poluição agrava à saúde, buscando alertar para a necessidade do desenvolvimento de políticas de saúde pública, e de padrões de qualidade do ar. Que tenham como objetivo a prevenção dos danos à saúde causados por poluentes atmosféricos, mantendo a poluição em valores que não se constituam um risco à população.

2. OBESIDADE

Sobrepeso e obesidade são definidos como acúmulo de gordura anormal ou excessiva que apresenta um risco para a saúde (OMS, 1998). A epidemia da obesidade também têm se tornado um risco mundial, segundo as estatísticas da Organização Mundial da Saúde (2012) a obesidade é a causa de morte de 2,8 milhões de pessoas por ano, sendo que, atualmente, 12% da população mundial apresenta obesidade.

No Brasil, um levantamento realizado pela Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico (VIGITEL, 2012), com a população adulta das 27 capitais brasileiras, aponta que a frequência de adultos obesos em nosso país é de 15,8%. Sendo que as maiores frequências de obesidade entre homens foram observadas em Macapá (24,2%), Natal (23,5%) e Manaus (20,2%); e, entre mulheres em Porto Alegre (21,5%), Maceió (18,9%) e Macapá (18,6%).

A etiologia para o aumento das taxas de obesidade tem sido associada a uma piora no estilo de vida, com excesso de calorias consumidas e falta de atividade física. Globalmente, tem acontecido um aumento da ingestão de alimentos altamente energéticos que são ricos em gordura, e um aumento na inatividade física devido à natureza cada vez mais sedentária de muitas formas de trabalho (WHO, 2013).

Condições de sobrepeso e obesidade são estabelecidas como principais fatores de risco para uma série de doenças crônicas não transmissíveis (DCNT), incluindo diabetes *mellitus* (DM), doenças cardiovasculares e câncer. Considerado há alguns anos como um problema apenas em países de alta renda, hoje em dia o sobrepeso e a obesidade encontram-se dramaticamente em ascensão em países de baixa e média renda, especialmente em regiões urbanos.

Este aumento nos casos de obesidade pode estar também relacionado ao surgimento de números crescentes de casos de diabetes no Brasil. Nesse sentido, a Sociedade Brasileira de Endocrinologia e Metabologia alerta para resultados de estudos epidemiológicos obtidos na última década, que apontam a obesidade como importante condição que predispõe à maior morbidade e mortalidade, uma vez que, a obesidade é comumente associada com o aumento de estresse oxidativo (Capítulo 3), quadro pró-inflamatório (Capítulo 4), resistência à insulina e alterações na distribuição de gordura corporal. Sendo assim, busca-se elucidar, os efeitos de condições alimentares, principalmente de dietas ricas em gordura, no desenvolvimento do

quadro de obesidade, e quais os mecanismos fisiopatológicos que tornam estes indivíduos mais susceptíveis a esta condição de morbimortalidade.

3. ESTRESSE OXIDATIVO

O estresse oxidativo (EO) é um desequilíbrio bioquímico, que se apresenta quando a produção de radicais livres ou espécies reativas de oxigênio (ROS) excede a capacidade antioxidante natural do organismo, resultando em dano oxidativo (Halliwell e Gutteridge, 2007). Este desequilíbrio pode ocorrer após a exposição a agentes pró-oxidantes, como poluentes do ar (Delfino et al., 2011) ou em condições crônicas como na obesidade (Furukawa et al., 2004). A própria resposta antioxidante ou anti-inflamatória do organismo aos efeitos destas condições (poluição do ar e obesidade) se constitui como importante mecanismo de defesa do organismo para auxiliar na manutenção do equilíbrio homeostático e oxidativo (Brook et al., 2010). O estresse oxidativo torna o organismo susceptível e contribui para o desenvolvimento de inúmeras patologias, especialmente aqueles de natureza crônica ou degenerativa (Wexler, 2007), como podemos salientar, no caso de indivíduos obesos (Furukawa et al., 2004; Van Gaal et al., 2006) ou em uma condição de exposição à poluição atmosférica (Miller et al., 2012).

3.1 Espécies Reativas de Oxigênio

Em condições fisiológicas do metabolismo celular aeróbio, o O_2 sofre redução tetravalente, com aceitação de quatro elétrons, resultando na formação de H_2O. Durante esse processo são formados intermediários reativos, designados radicais livres ou espécies reativas de oxigênio (Ferreira e Matsubara, 1997).

Radical livre é qualquer átomo que contém um ou mais elétrons desemparelhados na sua órbita. Um elétron não emparelhado é aquele que ocupa uma órbita atômica ou molecular sozinho. Um ponto sobrescrito após a fórmula química é utilizado para designar radicais livres (Halliwell e Gutteridge, 2007).

No entanto, radical livre não é o termo ideal para designar os agentes reativos patogênicos, pois alguns deles não apresentam elétrons desemparelhados em sua última camada (Ferreira e Matsubara, 1997), nesse sentido, utiliza-se o termo espécies reativas de oxigênio (ROS), que inclui não apenas radicais livres, mas também algumas espécies não-radicalares derivadas do O_2, como por exemplo, o peróxido de hidrogênio, que é capaz de gerar outros radicais livres (Halliwell e Gutteridge, 2000). Entre os principais ROS, destacam-se os radicais superóxido ($O_2^{\bullet -}$), hidroxila (OH^-) e o peróxido de hidrogênio (H_2O_2) (Ferreira e Matsubara, 1997).

3.1.1 Radical Superóxido ($O2^{\bullet-}$)

Ocorre em quase todas as células aeróbicas (Ferreira e Matsubara, 1997), é formado após a primeira redução do O_2, sendo eliminado por ação da enzima superóxido dismutase, que catalisa a dismutação de duas moléculas de radical superóxido ($O_2^{\bullet-}$) em oxigênio e peróxido de hidrogênio. Este último, quando não eliminado do organismo por ação das enzimas peroxidases e catalase, pode gerar radical hidroxila (Barreiros et al., 2006).

O radical superóxido participa de certos processos químicos importantes no contexto biológico e apesar de seus efeitos danosos ao organismo, o mesmo tem importância vital para as células de defesas, na ação contra infecções causadas por vírus, bactérias e fungos (Barreiros et al., 2006). Apesar de ser considerado pouco reativo em soluções aquosas, tem sido observada lesão biológica secundária a sistemas geradores de $O_2^{\bullet-}$ (seja enzimático, fagocítico ou químico) (Ferreira e Matsubara, 1997).

3.1.2 Radical Hidroxila (OH^{-})

O radical hidroxila é considerada a ROS mais reativa em sistemas biológicos. A combinação extremamente rápida do OH^{-} com metais ou outros radicais no próprio local onde foi produzido confirma sua alta reatividade. Além disso, o OH^{-} pode inativar várias proteínas, ao oxidar seus grupos sulfidrilas (-SH) a pontes dissulfeto (-SS), e iniciar a oxidação dos ácidos graxos poli-insaturados das membranas celulares, processo chamado de lipoperoxidação (LPO) (Ferreira e Matsubara, 1997).

3.1.3 Peróxido de hidrogênio (H_2O_2)

O peróxido de hidrogênio é gerado pela dismutação do ânion-radical superóxido ($O_2^{\bullet-}$) por enzimas oxidases ou pela β-oxidação de ácidos graxos (Barreiros et al. 2006). O H_2O_2 é um metabólito do oxigênio extremamente deletério, pois participa da reação que produz radical hidroxila (OH^{-}) (Ferreira e Matsubara, 1997). O peróxido de hidrogênio em si, é pouco reativo frente às moléculas orgânicas na ausência de metais de transição. No entanto, exerce papel importante no estresse oxidativo por ser capaz de transpor as membranas celulares facilmente e gerar o radical hidroxila. Ele somente oxida proteínas que apresentem

resíduos de metionina ou grupos tiol muito reativos, como por exemplo, a glutationa reduzida (GSH (Barreiros et al., 2006).

3.1.4 Oxigênio *singlet* (^{1}O2)

A forma mais deletéria do oxigênio ao organismo é o oxigênio *singlet* (^{1}O2), pois é a causa ou o intermediário da toxicidade fotoinduzida do O_2 em organismos vivos. O oxigênio *singlet* se difere do O_2 em seu estado molecular porque não apresenta transferência de elétrons, sendo altamente reativos, ele causa danos às proteínas devido a oxidação de grupos essenciais de aminoácidos, sendo responsável também por iniciar o processo de LPO (Barreiros et al., 2006).

3.2 Defesas Antioxidantes

Antioxidantes são substâncias que reagem com radicais livres impedindo ou diminuindo o estresse oxidativo e a consequente destruição tecidual (Halliwell e Gutteridge, 2007). O metabolismo aeróbio envolve a produção de ROS, mesmo em condições basais, e, portanto, há uma necessidade permanente de inativar estas espécies reativas de oxigênio, papel fundamental dos antioxidantes. O estado de equilíbrio de pró-oxidantes e antioxidantes pode ser interrompido, e o desbalanço em favor dos pró-oxidantes, leva potencialmente à danos, esse desequilíbrio é chamado de "estresse oxidativo" (Sies, 1993).

Os sistemas de defesa antioxidantes, tem a capacidade de inibir a oxidação de outras moléculas, transferindo elétrons de uma substância para um agente oxidante (ROS). Antioxidantes podem agir em diferentes níveis no processo oxidativo, pela eliminação de radicais primários, quelação de íons metálicos, eliminação de radicais peroxilas ou remoção de biomoléculas oxidadas, entre outros tipos de ação. Para proteger-se, as células possuem um sistema de defesa antioxidante que pode atuar em duas linhas, divididas em defesas em enzimáticas e não-enzimáticas.

3.2.1 Defesas antioxidantes enzimáticas

As enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT), e glutationa peroxidase (GPx), servem como linha de defesa primária na neutralização de ROS,

protegendo as células e os tecidos do dano oxidativo (Halliwell e Gutteridge, 2007). A avaliação dessas enzimas quanto à atividade e expressão é bastante utilizada como marcadores do desequilíbrio que leva ao estresse oxidativo (Zanchi et al., 2008; Heck, 2007).

As superóxido dismutase (SOD) são metaloenzimas abundantes nas células aeróbias e uma das mais importantes enzimas antioxidantes. Nos sistemas eucariontes existem duas formas de SOD, SOD-cobre-zinco que está presente principalmente no citosol, e a SOD-manganês que está localizada primariamente na mitocôndria. Ambas tem função antioxidante, já que catalisam a dismutação do radical superóxido ($O_2^{\bullet -}$) em H_2O_2 e O_2 (Ferreira e Matsubara, 1997).

A catalase (CAT) é uma hemeproteína citoplasmática, encontrada em todos os tecidos, principalmente no fígado. Essa enzima é um tetrâmetro formado por unidades idênticas, sendo que monômero contém um grupo prostético heme no centro catalítico, ela catalisa a redução do peróxido de hidrogênio (H_2O_2) em H_2O e O_2 (Halliwell e Gutteridge, 2007).

As peroxidases, como a glutationa peroxidase (GPx) são enzimas que utilizam uma variedade de redutores celulares para inativar peróxidos. Em mamíferos as principais peroxidases são as glutationas dependentes, como a GPx, uma seleno-enzima (utiliza selênio como co-fator), que catalisa a redução do peróxido de hidrogênio (H_2O_2) e peróxidos orgânicos para seus correspondentes álcoois às custas da conversão da glutationa reduzida (GSH) a glutationa oxidada (GSSG) (Halliwell e Gutteridge, 2007).

3.2.2 Defesas antioxidantes não enzimáticas

Existem poucos antioxidantes enzimáticos (e nem todos são universalmente aceitos), entretanto, há inúmeros antioxidantes não enzimáticos, que aumentam a capacidade do organismo para se defender contra as ROS (Olinescu e Smith, 2002).

A interceptação antioxidante tem como propriedade reagir com radicais livres primários, e transferir a função radical para alvos que sofram dano em menor potencial (Sies, 1997), ação realizada também pelos antioxidantes não-enzimáticos.

Os principais antioxidantes não enzimáticos incluem vitaminas A, C e E; glutationa, ácido α-lipóico; carotenoides, ubiquinona ou coenzima Q10 (CoQ10), e cofatores como o ácido fólico, vitaminas B1, B2, B6 e B12, que atuam impedindo processos de LPO e o dano oxidativo (Johansen et al., 2005).

3.3 Estresse Oxidativo, Obesidade e Poluição Atmosférica

O estresse oxidativo é o mecanismo molecular e celular que promove danos a diversos tecidos, levando ao desenvolvimento de um amplo espectro de doenças humanas (Halliwell, 1994). Inúmeros fatores já são conhecidos por estimular o desequilíbrio pró e antioxidante, que leva ao desenvolvimento de patologias associadas ao estresse oxidativo, como, por exemplo, a obesidade (Furukawa et all., 2004; Vincent e Taylor, 2006) e distúrbios causados por exposição à poluição atmosférica (Demetriou et al., 2012; Miller et al., 2012).

A obesidade, que pode ser resultante de distúrbios alimentares, como alto consumo de dietas hiperlipídicas, é componente central e causal de disfunções metabólicas. O acúmulo de tecido adiposo está intimamente relacionado com marcadores de estresse oxidativo sistêmico, por dois fatores: o aumento do estresse oxidativo no tecido adiposo leva à produção desregulada de adipocinas; e consequentemente, o aumento da produção de ROS no tecido adiposo leva à estresse oxidativo sistêmico (Furukawa et al., 2004).

Estas disfunções relacionadas à obesidade, mediadas pelo estresse oxidativo, podem levar a coexistência de vários fatores de risco para desenvolvimento de aterosclerose, dislipidemia, hipertensão e também prejudicar a captação de glicose no músculo e no tecido adiposo, podendo desencadear DM (Furukawa et al., 2004). A maioria destas condições também está associada a um quadro pró-inflamatório crônico, o que pode aumentar a susceptibilidade ao estresse oxidativo adicional causado por exposição à poluição do ar (Romieu et al., 2008).

O estresse oxidativo mediado por poluição, principalmente por material particulado (MP), pode surgir a partir da geração direta de ROS, da alteração na função mitocondrial e na atividade da enzima NADPH oxidase, bem como, do dano oxidativo ao DNA (Romieu et al., 2008). O estresse oxidativo, resultante da exposição à oxidantes, como a poluição, ou a presença de defesas antioxidantes prejudicada, por condição de susceptibilidade pré-existente, como na obesidade, pode desencadear uma série de sinalizações do estado redox, que atuam como biomarcadores (Kelly, 2013).

Estudos demonstram a importância efeitos da exposição à $MP_{2,5}$ sobre o estresse oxidativo, que promove disfunções no tecido adiposo. Estes resultados ressaltam os efeitos adversos de poluição do ar, especialmente no contexto de desenvolvimento de obesidade, doenças cardiometabólicas e resistência à insulina (Xu, 2011), e apresentam o estresse oxidativo como biomarcador do desbalanço metabólico.

4. PROTEÍNAS DE CHOQUE TÉRMICO – *Heat Shock Proteins* (HSPs)

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 (De Maio, 2011). Nesse sentido, proteínas de choque térmico (HSPs), funcionam a nível celular para proteger as células contra muitas condições estressantes (Chung et al., 2008).

Ao longo dos últimos anos, tornou-se evidente que em condições de estresse (ex. exposição a níveis elevados de poluição) as células respondem com a síntese de um conjunto de proteínas conhecidas como proteínas de choque térmico, situação designada de *stress response* (resposta ao estresse) (Mukhopadhyay et al., 2003).

Proteínas de choque térmico são proteínas altamente conservadas durante a evolução das espécies e são encontradas em organismos eucarióticos e procarióticos (Robert, 2003). A primeira demonstração da indução celular de HSP, em resposta a um estresse celular, foi relatada em 1962 por Ferrucio Ritossa, que observou o surgimento de um novo padrão de espessamento cromossomal em células de glândulas salivares de *Drosophila buskii,* fato que representava a transcrição para a síntese de HSPs após a exposição celular a temperaturas altas (De Maio, 2011).

Embora a descoberta das HSPs tenha ocorrido sob choque térmico, outros estímulos estressores, como obesidade e poluição atmosférica podem promover alterações na expressão destas proteínas (Chung et al., 2008; Kido et al., 2011).

As HSPs podem ser agrupadas de acordo com suas massas moleculares em famílias (HSP110, HSP100, HSP90, HSP70, HSP60 e HSP30). Estas proteínas, especificamente, as pertencentes à família de HSPs de 70 kDa (HSP70) são proteínas muito conservadas (Smock et al., 2011) e possuem funções extremamente importantes para a manutenção do bom funcionamento celular, atuando como chaperonas moleculares e prevenindo o dano celular. Nesse sentido, pode-se considerar que a indução da expressão HSP70, aumentando o conteúdo intracelular desta proteína, pode representar um potencial fator citoprotetor e anti-inflamatório, em diversas condições estressantes (Heck et al., 2011).

4.1 HSP70, Estresse Oxidativo, Obesidade e Poluição Atmosférica

Os estresses oxidativo, térmico, hemodinâmico e osmótico induzem a expressão de HSP70, que por sua vez, é responsável por proteger as células contra estes desafios (Kim et al., 2007). Altos níveis plasmáticos desta proteína estão correlacionados, simultaneamente com alteração no status pró/anti-inflamatório e com o desequilíbrio dos sistemas pró/antioxidante do organismo.

Devido às suas funções versáteis, as HSPs podem intervir no estresse oxidativo em vários níveis (Kalmar e Greensmith, 2009). Em primeiro lugar, algumas HSPs, principalmente a HSP70 e suas co-chaperonas, desempenham um papel crucial na depuração de proteínas danificadas. Por outro lado, podem regular negativamente a apoptose, por ligação e inibição da cascata apoptótica, e mesmo se as vias de morte celular forem ativadas, as HSPs podem intervir para resgatar as células do processo de apoptose. Desta forma, o aumento na síntese de HSPs, pode apresentar um aumento na tolerância ao estresse subsequente, o que pode prevenir a danos proteicos relacionados ao estresse oxidativo (Kalmar e Greensmith, 2009).

No entanto, a primeira linha de defesa contra o dano oxidativo ocorre por meio de eventos moleculares, que reconhecem mudança redox no ambiente intracelular, e aumentam a expressão das HSPs (Kalmar e Greensmith, 2009). Obesidade, sedentarismo e consumo de dietas ricas em gordura produzem um ciclo vicioso de inflamação e estresse oxidativo, que leva a perda do equilíbrio homeostático e podem produzir efeitos sobre a expressão de HSP70. A redução na expressão de HSPs pode tornar os tecidos vulneráveis ao estresse (Hooper e Hooper, 2009) e ser indicativo de possível dano tecidual e desenvolvimento de patologias.

As HSP70 também possuem a capacidade de proteger as células de dano oxidativo induzido por exposição à poluição atmosférica. 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), induzidos pela exposição crônica à poluição do ar. 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 (Mukhopadhyay et al., 2003).

Sugere-se que a expressão de genes de estresse (HSP70, em particular) são potenciais indicadores de qualquer agressão química ou física, e tem sido usado com sucesso recentemente como um biomarcador precoce para a avaliação de condições ambientais. Alterações na expressão de HSP70 por meio da regulação do estado redox celular e de processos inflamatórios, em resposta a estímulos estressantes (obesidade e poluição atmosférica, por exemplo) é que determinam sua função de chaperona molecular intracelular, sendo, portanto, um potencial biomarcador de inúmeras condições adversas (Fittipaldi et al., 2014; Kido et al., 2011).A ligação direta da expressão de HSP70 com danos celulares, juntamente com a sua natureza altamente conservada entre espécies permite a aplicação de ensaios em uma vasta gama de organismos.

5. MODELOS EXPERIMENTAIS DE EXPOSIÇÃO À POLUIÇÃO ATMOSFÉRICA

Vários estudos têm demonstrado efeitos da exposição à poluição atmosférica (Fajersztajn et al., 2013; Pearson et al., 2010; Rajagolapan e Brook, 2010), para tal, muitos modelos são utilizados para avaliar o grau de exposição *versus* efeito produzido.

O desenvolvimento de modelos para avaliar a exposição à poluição do ar dentro das cidades, no intuito de atribuir os riscos produzidos à saúde, é prioritário para futuras pesquisas. No entanto, os mecanismos fisiológicos dos efeitos nocivos à saúde relacionados à exposição à poluição não são totalmente elucidados em estudos epidemiológicos, levantando questionamento sobre como ocorre o desenvolvimento de patologias e porque indivíduos expostos se tornam mais susceptíveis a certas condições.

Para tal, modelos que mimetizam a exposição à poluição atmosférica, são apropriados para elucidar os mecanismos fisiopatológicos pelos quais o organismo desenvolve inúmeras doenças relacionadas à poluição atmosférica, estudos que vêm sendo desenvolvidos tanto *in vivo*, como *in vitro*.

Como modelo de exposição *in vivo*, em experimentação animal, podemos destacar métodos de exposição em câmara, em que o ar é propulsionado do ambiente externo 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 (Sun et al., 2009; Xu et al., 2011). Além do método que utiliza um concentrador de partículas de poluição atmosférica, desenvolvido pela Universidade de Harvard, os quais são utilizados para exposição à concentração real de partículas no ambiente, durante um período de tempo (Rhoden et al., 2004).

Outros estudos utilizam métodos de instilação intratraqueal e intranasal (Yan et al., 2011; Zanchi et al., 2008). O método de instilação intratraqueal é usado com frequência para a exposição de animais, tanto à partículas solúveis e insolúveis, é um método relativamente barato, que permite administração instantânea e diretamente na traqueia, de concentração conhecida do poluente em teste diretamente para o pulmão (Osier e Oberdorster, 1997). No modelo de instilação intranasal, conforme utilizado em nosso estudo, o animal é contido pelo manipulador (suspenso 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 (Osier e Oberdorster, 1997). (conforme Figura 1).

Estudos que utilizam estes modelos de exposição têm sido eficientes em relacionar os prejuízos da poluição atmosférica, no desenvolvimento de doenças crônicas, sejam estas respiratórias, cardiometabólicas ou neurodegenerativas, destacando principalmente os efeitos fisiológicos produzidos para o agravo destas condições (Brook et al., 2004).

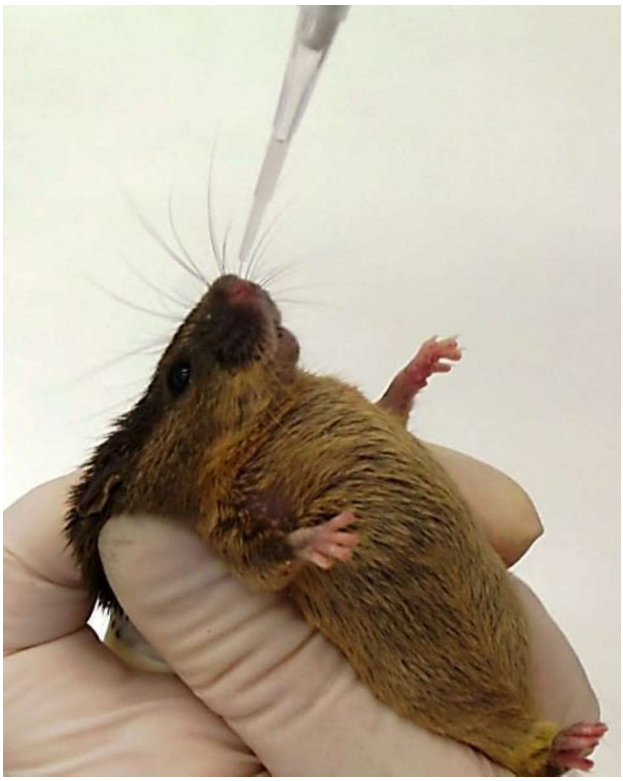


Figura 1. Instilação intranasal realizada em camundongo B6.

6. MODELOS EXPERIMENTAIS DE OBESIDADE

Modelos animais também têm sido utilizados para estudar condições relacionadas à obesidade. Os mecanismos moleculares através dos quais a obesidade é desenvolvida e induz problemas à saúde ainda não são claras. Para entender melhor os mecanismos patológicos de doenças humanas, modelos animais são essenciais (Kanasaki e Koya, 2001). Neste sentido, podem ser utilizados modelos genéticos, animais com alguma mutação gênica, animais transgênicos ou aqueles em que se produziu *knock-out* de um ou mais genes; e também modelos nos quais a obesidade é induzida por meio do consumo de dietas ricas em gordura ou dietas de cafeteria (Cesaretti et al., 2006).

O modelo que mais se assemelha ao desenvolvimento da obesidade humana é o modelo de obesidade exógena, no qual é oferecido ao animal um maior aporte calórico, mediante uma sobrecarga de carboidratos ou de gordura. Neste modelo experimental, acrescenta-se, ou associa-se à ração padrão substâncias altamente calóricas (Cesaretti et al., 2006).

Dietas hiperlipídicas (DHL), principalmente constituídas de gordura animal, têm apresentado maiores efeitos sobre o ganho de peso quando comparado a outros tipos de dieta (Buettner et al. 2007). Dietas contendo mais de 40% de energia com base em gordura animal, introduzido no período pós-desmame, com um consumo contínuo por várias semanas, são adequados para o estudo dos mecanismos fisiopatológicos do desenvolvimento da obesidade (Matsuzawa-Nagata et al., 2008; Buettner et al., 2007).

Linhagens de ratos quando alimentados com DHL têm respostas diferentes no que diz respeito ao desenvolvimento de obesidade, no entanto, camundongos (principalmente da linhagem C57BL/6J) se comportam como modelos apropriados para mimetizar distúrbios metabólicos humanos que são observadas na obesidade, pois estes quando alimentados com DHL desenvolvem adiposidade, hiperinsulinemia, hiperglicemia e hipertensão, podendo ter seus efeitos comparado ao mesmo modelo alimentado apenas com dieta padrão, que permanecem sem anormalidades metabólicas (ração padrão e hiperlipídica conforme Figura 2) (Wang e Liao, 2012).

Desta forma, enfatiza-se que a intervenção com dietas hiperlipídicas em camundongos apresenta-se como modelo adequado para elucidar os mecanismos fisiopatológicos do desenvolvimento de um quadro de obesidade, bem como das condições que agravam esta pré-condição e resultam no surgimento de doenças crônicas, como o diabetes.

Figura 2. Ração Padrão (4% de gordura - Nuvilab CR-1) e Ração Hiperlipidica (60% de gordura) preparada para consumo de camundongo B6.

JUSTIFICATIVA

Estudos epidemiológicos têm demonstrado forte associação entre obesidade e poluição atmosférica, no desenvolvimento de doenças crônicas não transmissíveis, como diabetes *mellitus* tipo 2 (DM2) (Pearson et al., 2010; Sun et al., 2009). A obesidade está associada ao desenvolvimento de doenças metabólicas, por vias pró-inflamatórios e pró-oxidantes, tornando indivíduos susceptíveis a outros fatores de risco, como aos efeitos da poluição atmosférica. A exposição crônica à poluição atmosférica, principalmente por Material Particulado Fino ($MP_{2,5}$) também promove modificações no perfil inflamatório e aumento do estresse oxidativo, provocando agravos à saúde, principalmente em condições de susceptibilidade pré-existente. Nesse sentido, justifica-se o desenvolvimento deste trabalho, na busca por elucidar os mecanismos fisiopatológicos relacionados ao agravo das condições de obesidade por exposição à poluição atmosférica.

OBJETIVO GERAL

Avaliar o efeito da associação entre consumo de dieta hiperlipídica e exposição crônica ao $MP_{2,5}$ no perfil biométrico, metabólico e na resposta celular ao estresse em camundongos B6129SF2/J (B6).

Objetivos específicos

Avaliar o efeito da associação entre consumo de DHL e exposição à $MP_{2,5}$ no(s):

- Desenvolvimento da adiposidade, a partir da relação entre Peso Corpóreo e Tecido Adiposo Branco Epididimal;

- Perfil Glicêmico e Lipídico;

- Tecidos relacionados ao metabolismo dos camundongos (Fígado, Pâncreas, Músculo Gastrocnêmio e Tecido adiposo Branco Epididimal) quanto ao dano oxidativo (TBARS), Atividade das enzimas antioxidantes (CAT e SOD) e expressão de HSP70.

REFERÊNCIAS

BARREIROS, A.; DAVID, J.M.; DAVID, J.P. Estresse Oxidativo: Relação entre geração de espécies reativas e defesa do organismo. **Quim Nova,** v. 29, n. 1, p. 113-123, 2006.

Brasil. Ministério da Saúde. Secretaria de Vigilância em Saúde. Vigitel Brasil 2011: Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico. **Secretaria de Vigilância em Saúde – Brasília: Ministério da Saúde**, 2012.

BROOK, R.D. et al. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. **Circulation,** v.121, n.21, p.2331-78, 2010.

BUETTNER, R.; SCHÖLMERICH, J.; BOLLHHEIMER, L.C. High fat diets: modeling the metabolic disorders of human obesity in rodents. **Obesity**, v. 15, n. 4, p. 798-808, 2007.

CESARETTI, M.; KOHLMANN JUNIOR, O. Modelos experimentais de resistência à insulina e obesidade: lições aprendidas. **Arq Bras Endocrinol Metab**, v. 50, n. 2, p. 190-197, 2006.

CHEN, J.C.; SCHWARTZ, J. Metabolic syndrome and inflammatory responses to long-term air pollutants. **Environ Health Perspect,** v. 16, n. 5, 2008.

CHUNG J.; NGUYEN, A.K.; HENSTRIDGE, D.C.; HOLMES, A.G.; CHAN, M.H. et al. HSP72 protects against obesity-induced insulin resistance. **Proc Natl Acad Sci USA**, v. 105, n. 5, p. 1739-44, 2008.

CONAMA (Conselho Nacional Do Meio Ambiente). Resolução nº 3, de 28 de junho de 1990.

DE MAIO, A. Extracellular heat shock proteins, cellular export vesicles, and the Stress observation System: A form of communication during injury, infection, and cell damage. **Cell Stress Chaperones**, v. 16, n. 3, p. 235-249, 2011.

DELFINO, R.J.; STAIMER, N.; VAZIRI, N.D. Air pollution and circulating biomarkers of oxidative stress. **Air Qual Atmos Health**. v.4, p.37–52, 2011.

DEMETRIOU, C.A. et al. Biomarkers of ambient air pollution and lung cancer: a systematic review. **Occup Environ Med**, v. 69, p.619–627, 2012.

EMMERECHTS, J.; HOYLAERTS, M.F. The effect of air pollution on haemostasis. **Hamostaseologie**, v. 32, n. 1, p.5-13, 2012.

FAJERSZTAJN, L. et al. Air pollution: a potentially modifiable risk factor for lung cancer. **Nat rev cancer**, v. 13, n. 9, p.674-8, 2013.

FERREIRA, A.L.A.; MATSUBARA, L.S. Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo. **Rev Ass Med Brasil**, v. 43, n. 1, p. 61-68, 1997.

FITTIPALDI, S. et al. Role of exercise-induced reactive oxygen species in the modulation of heat shock protein response. **Free Radic Res**, v. 48, n. 1, p. 52-70, 2014.

FURUKAWA, S. et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. **J clin invest**, v. 114, n. 12, p. 1752-61, 2004.

GUPTE, A.A. et al. Heat Treatment Improves Glucose Tolerance and Prevents Skeletal Muscle Insulin Resistance in Rats Fed a High-Fat Diet. **Diabetes**, v. 58, n. 3, p. 567-78, 2009.

HALLIWELL B: Free radicals, antioxidants, and human disease: cause or consequence? **Lancet**, v. 344, p. 721-724, 1994.

HALLIWELL, B.; GUTTERIDGE, J. M. C. **Free Radicals in Biology and Medicine**. 3 ed. New York: Oxford University Press inc., 2007.

HECK, T.G. **HSP70 como Marcador de Intensidade de Exercício: Razão entre o Conteúdo Extracelular e Intracelular de HSP70 como um Sinal de Alerta Imunológico.** 2011. 143f. Tese (Doutorado Fisiologia). Universidade Federal Do Rio Grande Do Sul, UFRGS, Porto Alegre, 2011.

HECK, T.G.; SCHOLER, C.M.; BITTENCOURT JR, P.I.H. HSP70 expression: does it a novel fatigue signaling factor from immune system to the brain? **Cell Biochem Funct.**, v. 29, p.215-226, 2011.

HECK, TG. **Estudo do Estresse Oxidativo Cardiopulmonar e da Resposta Hemodinâmica em Ratos Expostos Agudamente ao Material Particulado durante o exercício.** 2007. 127f. Tese (Mestrado em Ciências da Saúde). Universidade Federal De Ciências da Saúde, UFCSPA, Porto Alegre, 2007.

HOOPER, P.L.; HOOPER P.L. Inflammation, heat shock proteins, and type 2 diabetes. **Cell Stress Chaperones,** v.1, n. 2, p. 113-5, 2009.

JANGHORBANI, M. et al. Systematic review and meta-analysis of air pollution exposure and risk of diabetes. **Eur J Epidemiol,** 29:231–242. 2014.

JERETT, M. et al. A review and evaluation of intraurban air pollution exposure models. **J Expo Anal Environ** Epidemiol, v.15, n.2, p. 185-204, 2005.

JOHANSEN, J.S. et al. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. **Cardiovasc Diabetol,** v. 4, n.1, p.5, 2005.

JORDAN, M. Oxidative Stress: What is Oxidative Stress? **Kindle Ed.**, 1ed., 2012.

KALMAR, B.; GREENSMITH, L. Induction of heat shock proteins for protection against oxidative stress. **Advanced Drug Delivery Reviews**, v. 6, p. 310–318, 2009.

KANASAKI, K.; KOYA, D. Biology of obesity: lessons from animal models of obesity**. J Biomed Biotechnol.** v. 2011, 2011.

KELLY, F.J. Oxidative stress: Its role in air pollution and adverse health effects. **Occup Environ Med**, v. 60, p.612–616, 2003.

KIDO, T.; BAI, N.; YATERA, K.; SUZUKI, H.; MEREDITH, A. et al. Diesel Exhaust inhalation induces heact shock protein 70 expression in vivo. **Inhal Toxicol.**, v. 23, n. 10, p. 593-601, 2011.

KIM, H.; HWANG, N.R.; LEE, K. Heat shock responses for understanding diseases of protein**. Mol. Cells,** v. 23, n. 2, p. 123-131, 2007.

KRAUSE, M.da S. et al. Physiological concentrations of interleukin-6 directly promote insulin secretion, signal transduction, nitric oxide release, and redox status in a clonal pancreatic β-cell line and mouse islets. **J Endocrinol**. v. 214, n. 3, p. 301-11, 2012.

MATSUZAWA-NAGATA, N. et al. Increased oxidative stress precedes the onset of high-fat diet–induced insulin resistance and obesity. **Metabolism,** v. 57, n. 8, p. 1071-7, 2008.

MENDEZ, R. et al. Exposure to fine airborne particulate matter induces macrophage infiltration, unfolded protein response, and lipid deposition in white adipose tissue. **Am J Transl Res.**, 5(2):224-34, 2013.

MILLER, M.R. et al. From particles to patients: oxidative stress and the cardiovascular effects of air pollution. **Future Cardiol. v**., n.4, p.577–602, 2012.

MUKHOPADHYAY, I. et al. Heat shock response: hsp70 in environmental monitoring. **J Biochem Mol Toxicol,** v. 17, n. 5, p. 249-54, 2003.

OLINESCU, R.; SMITH, T.L. Free radicals in medicine. **Nova Science Publ**, 1ed, 2002.

OSIER, M.; OBERDORSTER, G. Intratracheal Inhalation vs Intratracheal Instillation: Differences in Particle Effects. **Fundam Appl Toxicol.**, v. 40, p. 220-227, 1997.

PEARSON J.F.; et al. Association Between Fine Particulate Matter and Diabetes Prevalence in the U.S. **Diabetes Care***,* v.33, n.10, p. 2196-2201, 2010.

POPE, C.A. et al. Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. **JAMA***,* v.287, n.9, p. 1132-1141, 2002.

RAJAGOPALAN, S. et al. Air pollution and type 2 diabetes: mechanistic insights. **Diabetes**, v. 61, n. 12, p.3037-45, 2012.

RHODEN, C.R. et al. N-Acetylcysteine prevents lun inflammation after short-term ihalation to concentrated ambient particles. **Toxicol Sci,** v. 79, n. 2, p.296-303, 2004.

ROBERT, J. Evolution of heat shock protein and immunity. **Developmental and Comparative Immunology**, v. 27, p. 449–464. 2003.

ROMIEU, F.; CASTRO-GINER, N.; KUNZLI, N.; SUNYER, J. Air pollution, oxidative stress and dietary supplementation: a review. **Eur Respir J**, v. 31, p. 179–196, 2008.

SCHUSTER D. Obesity and the development of type 2 diabetes: the effects of fatty tissue inflammation Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. **Diabetes Metab Syndr Obes**., v. 3, p. 253–262. 2010;

SIES, H. Oxidative stress: oxidants and antioxidants. **Exp Physiol**, v. 82, p. 291-295, 1997.

SIES, H. Strategies of antioxidant defense. **Eur. J. Biochem.** v. 215, 213-219, 1993.

SMOCK, R.G.; BLACKBURN, M.E.; GIERASCH, L.M. Conserved, disordered C terminus of DnaK enhances cellular survival upon stress and DnaK in vitro chaperone activity. **J Biol Chem,** v.286, n.36, p.31821-31829, 2011.

SUN, Q. et al. Ambient air pollution exaggerates adipose inflammation and insulin resistance in a mouse model of diet-induced obesity. **Circulation,** v. 119, n. 4, p. 538-46, 2009**.**

US Environmental Protection Agency (EPA). Disponível em: http://www.epa.gov/ Acesso em: 30 de Agosto de 2013.

VAN GAAL, L.F. et al., Mechanisms linking obesity with cardiovascular disease. **Nature**, v.444, p.875-880, 2006.

VINCENT, H.K.; TAYLOR, A.G. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. **International Journal of Obesity**, v.30, p.400–418, 2006.

WANG, C.Y.; LIAO, J.K. a mouse model of diet-induced obesity and insulin resistance. **Methods Mol Biol.** v. 821, p.421-33, 2012.

WEXLER, B. Antioxidants: Natural Defense Against Oxidative Stress. **Woodland Publishing Inc.** 1ed., 2007. p.32.

WHO, 2013. Obesity and overweight. Avaiable from: http://www.who.int/mediacentre/factsheets/fs311/en/

WHO, world Health Organization, 2006. Fact Sheet for worldwide prevalence of obesity. Available from: http://www.who.int/mediacentre/factsheets/fs311/en/index.html

WHO. Division of Noncommunicable Diseases. Programme of Nutrition Family and Reproductive Health. Obesity: preventing and managing the global epidemic: report of a WHO consultation on obesity. Geneva: WHO; 1998.
WORLD HEALTH ORGAN TECH REP SER. Obesity: preventing and managing the global epidemic. **Report of a WHO consultation.** 894:i-xii, p. 1-253, 2000.

WORLD HEALTH ORGANIZATION. Air Quality Guidelines, global update. Europe: WHO, 2005.

WORLD HEALTH ORGANIZATION. WHO Air quality guidelines for particulate matter, ozone, nitrogen dioxide and sulfur dioxide; Global update 2005; Summary of risk assessment. Geneva: **WHO,** 2006.

XU, X. et al. Effect of early particulate air pollution exposure on obesity in mice. Role of $p47^{phox}$. **Arterioscler Thromb Vasc Biol**, v. 30, n. 12, p. 2518-27, 2010.

XU, Z. et al. Ambient particulate air pollution induces oxidative stress and alterations of mitochondria and gene expression in brown and white adipose tissues. **Part. Fibre Toxicol.**, v. 8, n. 20, p. 1-14, 2011.

YAN, Y.H. et al. Enhanced insulin resistance in diet induced obese rats exposed to fine particles by instillation. **Inhal Toxicol,** v. 23, n. 9, p. 507-19, 2011.

ZANCHI, A. C. et al. Hippocampus lipid peroxidation induced by residual oil fly ash intranasal instillation versus habituation to the open field. **Inhal Toxicol,** v.22, n.1, p.84-88, 2010.

ZANCHI, A.C. et al. Chronic Nasal Instillation of Residual-Oil Fly Ash (ROFA) Induces Brain Lipid Peroxidation and Behavioral Changes in Rats. **Inhal Toxicol,** v.20, p. 795–800, 2008.

http://www.cetesb.sp.gov.br/ar/Informa??es-B?sicas/21-Poluentes. Acesso em: 01 de Novembro de 2013.

http://www.endocrino.org.br/obesidade-introducao/ Acesso em: 28 de Outubro de 2013.

http://www.epa.gov / Acesso em: 01 de Novembro de 2013.

http://www.epa.gov/airquality/particlepollution/2012/decfsstandards.pdf

http://www.epa.gov/airtrends/aqtrnd95/pm10.html Acesso em: 02 de Dezembro de 2013.

http://www.epa.gov/heasd/research/emi.html Acesso em: 05 de Dezembro de 2013.

http://www.lbl.gov/. Acesso em: 01 de Novembro de 2013.

http://www.mma.gov.br/cidades-sustentaveis/qualidade-do-ar. Acesso em: 28 de Outubro de 2013.

http://www.mma.gov.br/cidades-sustentaveis/qualidade-do-ar/padroes-de-qualidade-do-ar. Acesso em: 14 de Julho de 2014.

http://www.who.int/en/ Acesso em: 30 de Outubro de 2013.

http://www.who.int/topics/obesity/en/ Acesso em: 28 de Outubro de 2013.

http://www.who.int/mediacentre/news/releases/2014/air-pollution/en/. Acesso em: 14 de Julho de 2014.

ARTIGO

(Periódico Internacional: Environmental Health Perspectives)

Long-term exposure to $PM_{2.5}$ worsens metabolic dysfunction induced by high fat diet in mice

Pauline Brendler Goettems-Fiorin[1,2] Bethânia Salamoni[1,2], Fernanda Giesel Baldissera[1,2], Analu Bender dos Santos[1], Paulo Ivo Homem de Bittencourt Jr[3], Mirna Stela Ludwig[1,5], Claudia Ramos Rhoden[2] and Thiago Gomes Heck[1,5]

[1]Research Group in Physiology. Department of Life Sciences, Regional University of Northwestern State's Rio Grande do Sul (UNIJUI), Ijuí, RS, Brazil.

[2]Laboratory of Oxidative Stress and Atmospheric Pollution, Postgraduate Program in Health Sciences, Federal University of Health Sciences of Porto Alegre (UFCSPA), Porto Alegre, RS, Brazil.

[3]Department of Physiology, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

[5]Postgraduation Program in Integral Attention to Health (PPGAIS-UNIJUI/UNICRUZ), Ijuí, RS, Brazil.

Corresponding author

Pauline Brendler Goettems Fiorin, Department of Life Sciences, Regional University of Northwestern State's Rio Grande do Sul

Rua do Comércio, 3000 – Bairro Universitário - Ijuí-RS. Brazil.

ZIP code: 98700-000.

Phone: +55 (55) 3332-0476

E-mail: pauline_goettems@yahoo.com.br

Running title: Long-term exposure to $PM_{2.5}$ worsens metabolic impairment

Acknowledgements

The authors would like to thank E.G.de P., Basso, E.T.C., Martins, M.M., Sulzbacher, R.D.B., Basso (All from UNIJUI), P.H.N. Saldiva (USP) and colleagues for Laboratory of Oxidative Stress and Atmospheric Pollution (UFCSPA) for their technical support.

Financial support

This work was supported by Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) and by grants from the Rio Grande do Sul State Foundation for Research Support (PqG-2013 - FAPERGS, process: 002106-2551/13-5 T. G. HECK). P. B.G., Fiorin and F.G., Baldissera were supported by a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and A.B. dos Santos was supported by fellowship from FAPERGS.

Competing financial interests

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

ABSTRACT

Introduction: Epidemiological studies demonstrate an important health risk by association among exposure to fine particulate matter ($PM_{2.5}$), obesity and type 2 diabetes (T2DM). However, few studies have documented the effect of long-term exposure to low levels of $PM_{2.5}$ in nutrient metabolism related tissues (NMRT) in a pre-existing adiposity condition. We seek to investigate the long-term effects of $PM_{2.5}$ exposure on nutrient metabolism related tissues (NMRT) for high fat diet (HFD)-induced obesity model.

Materials and Methods: Male mice (n=59) were fed with standard or HFD for 12 weeks. After the diet protocol they were randomly exposed to daily intranasal instillation of saline or $PM_{2.5}$ solution for subsequent 12 weeks, divided into four groups: CONTROL, $PM_{2.5}$, HFD and HFD+$PM_{2.5}$. Biometric and metabolic measurements were evaluated during the experimental time. After 24 weeks, it was evaluated NMRT weights, their respective oxidative profile and HSP70 expression.

Results: The long-term exposure to $PM_{2.5}$ promoted impaired glycemic response during glucose overload in obese animals induced by HFD. HFD and $PM_{2.5}$ association promotes different oxidative responses and HSP70 expression in NMRT. This association also promoted a decrease in muscle and pancreatic mass. Impaired NMRT lead to changes in blood glucose and triglyceride levels, along the 24 weeks of study.

Significance: Long-term exposure to $PM_{2.5}$ worsens the glycemic response to glucose overload in animals previously treated with HFD, and altered oxidative profile and HSP70 expression in NMRT, providing evidence for an important interaction between environmental and dietary factors.

Keywords: Adiposity, particulate matter, oxidative stress, HSP70.

INTRODUCTION

Accumulated evidence suggests that exposure to air pollutant as one of major risk factor for the development of diseases on a global scale (Fajersztajn et al. 2013; Emmerechts and Hoylaerts 2012). Epidemiological studies show important links between exposure to Particulate Matter (PM) and risk for development of chronic diseases, as obesity and diabetes (Chen and Schwartz 2008; Pearson et al. 2010; Rajagolapan and Brook 2010).

As is known, the current lifestyle with HFD consumption might induce metabolic dysfunctions, making individuals more susceptible to adverse environmental conditions (Yan et al. 2011). In the same way, the fine particulate matter ($PM_{2.5}$), due to their size, have a systemic effect, and are able to induce damage in several tissues, including metabolic tissues (Pearson et al. 2010; Xu et al. 2010; Yan et al. 2011), additionally associated to inflammatory and oxidative markers (Jacobs et al. 2010). Moreover, Yan et al. (2011) demonstrated that exposure to $PM_{2.5}$ enhanced insulin resistance (IR) in HFD treated rats, seems a risk factor for subsequent development of type 2 diabetes mellitus (T2DM), and suggest effects that obese subjects with IR may be a susceptible population to $PM_{2.5}$.

The pathophysiological effects of $PM_{2.5}$ may occur via activation of intracellular stress responses, similarly as other risk factors (such as HFD consumption), leading to modulation of cell metabolism in the whole body (Mendez et al. 2013). The oxidative stress induced by $PM_{2.5}$, mediated by transition metals, impairs target cells/tissue (Bae et al. 2010; Rhoden et al. 2004).

In addition from oxidative stress, both $PM_{2.5}$ and HFD can induce other ways of stress responses, such as the expression of heat shock proteins (HSPs). Under stressful conditions, cells respond by synthesizing a suite of proteins known as HSPs (Mukhopadhyay et al. 2003), that plays a fundamental role in providing cytoprotection for many types of disorders (De Maio 2011).

Since the imbalance of oxidative profile marks the beginning of a tissular injury and HSP70 expression represents the activation of cytoprotective mechanisms, we used these variables to investigate the HFD consumption associated with $PM_{2.5}$ exposure to challenge body homeostasis, specifically on NMRT. All epidemiological studies suggest a elevate risk to T2DM development by combination of high levels of $PM_{2.5}$ exposure and HFD metabolic complications. Thus, we hypothesized that low concentrations of $PM_{2.5}$, however during long

periods, is able to promote metabolic alterations and contribute to T2DM development, marked by alterations in oxidative profile and HSP70 expression in NMRT.

MATERIALS AND METHODS

Animal Care

All animals used in this research were treated humanely, with due consideration for the relief of suffering and discomfort. This protocol was approved by the Animal Ethics Committee of UNIJUÍ (CEUA 009/13).

Animals

Male (n=59) B6129SF2/J mice, 21 days old (weight ± 14g) post-weaning, from Animal Facility of Regional University of Northwestern State´s Rio Grande do Sul, were kept in semi-metabolic cages, under controlled conditions of temperature (22±2ºC), relative humidity (50% - 60%) and light-dark cycles (light from 7:00 a.m. to 7:00 p.m.). The animals received water *ad libitum*.

Experimental Design

The mice were randomly divided into four groups: Control (CONTROL), Polluted ($PM_{2.5}$), High Fat Diet (HFD) and High Fat Diet Polluted (HFD+$PM_{2.5}$). Animals in CONTROL and $PM_{2.5}$ groups received standard diet (4% fat) and animals from HFD and HFD+$PM_{2.5}$ groups received HFD (60% fat diet) for 24 weeks. At 12th week, $PM_{2.5}$ and HFD+$PM_{2.5}$ groups received intranasal instillation of $PM_{2.5}$ (5μg/10μL) daily, while CONTROL or HFD groups received saline solution, for a subsequent period of 12 weeks (up to 24 weeks). Treatments are summarized in Table 1.

Diet

The standard diet (Nuvilab CR-1) (4% fat) consisted of crude protein, mineral material, fibrous matter and minerals. The HFD (60% fat) was composed by 40.4% of standard diet (Nuvilab CR-1), 37.4% of lard, 13.7% of albumin, 7.4% of aminomix, 1.1% of bone and oyster meal, prepared once a week in our laboratory. The animals received standard diet or HFD for 24 weeks.

Characterization of Particulate Matter

The pollutant used in the experiment was $PM_{2.5,}$ contained in polycarbonate filter, which was collected through a gravimetric collector, on the terrace of the Faculty of Medicine, University of São Paulo (USP) in São Paulo, Brazil. After the exposure to the urban air pollution (24 hours), the filter was removed and retained particles were obtained by

sonication, with ultrasound bath in seven sessions (50 min) in H_2O. The particles were resuspended in saline solution at a dose of 5μg/10μL. The process of intranasal instillation was performed daily, once a day (at 1:00 and 2:00 p.m.), for 12 weeks with an automatic pipette, with 10μL of solution in the nostril of the animal, which reflects apnea inhales the pollutant.

Food and Water Consumption

The consumption of water [volume offered *minus* remaining volume in the bottle (mL)] and food [diet offered *minus* remaining diet in the box (g)] were monitored three times per week during the 24 weeks study.

Biometric Profile

The biometric profile of animals was monitored once a week for 24 weeks. It was measured body weight (g), length (cm) and carried out the calculation of the Lee Index, the body mass index (BMI) for rodents. Body weight was checked with semi-analytical scale and length was verified by naso-anal distance. The Lee Index consists to divide the cube root of body weight (g) by naso-anal distance (cm), the result of the weight/length division is multiplied by 1000 (Lee 1928). We also evaluate adiposity [% of epididymal white adipose tissue (EWAT)] and % of liver, gastrocnemius muscle and pancreas.

Glucose and Lipid levels

Blood glucose, triglycerides and cholesterol were monitored every 2 weeks, with 12 hours of fasting. Blood glucose was measured by Glucometer Optium Xceed (Abbott) (5μL of blood) and the triglycerides and total cholesterol levels was assessed with Accutrend ® Plus System (Roche) (25μL of blood) in a puncture of the distal part of the tail of mice. The results were expressed in mg/dL of blood. The response of blood glucose, triglycerides and cholesterol over 24 weeks was assessed by calculating the incremental area under the curve (IAUC; glycaemia is the y-axis of the graph, and weeks represents the x-axis of graph), calculated geometrically by applying the trapezoid rule, ignoring in the area the first measurement of each variable at time zero of the study (USA-FAO 1997).

Glucose Tolerance Test (GTT)

Glucose tolerance test were performed in 4^{th}, 8^{th}, 12^{th}, 16^{th}, 20^{th} and 24^{th} weeks of intervention. Food was withdrawn from the animals in the night before experiments (12 hours before). Mice were restrained and blood samples were obtained by a puncture of the distal

part of their tails. Glycaemia was measured with Glucometer Optium Xceed (Abbott) immediately before and at 30 and 120 min after an glucose administration (1g/kg in saline solution, *i.p*). The glycemic response during GTT was evaluated by calculating the incremental area under the curve [IAUC; glycaemia is the y-axis of the graph, and time (minutes) represents the x-axis of graph], calculated geometrically by applying the trapezoid rule, ignoring in the area the fasting concentration values of glycaemia (USA-FAO 1997).

Tissue Preparation

At the end of the 24 weeks of intervention, the animals were euthanized. NMRT: liver, pancreas, gastrocnemius muscle and EWAT were dissected, weighed, frozen in liquid nitrogen with *freeze clamp* and stored for further homogenization.

For analysis of thiobarbituric acid reactive substances (TBARS) and activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), a portion of the tissues was homogenized in potassium phosphate buffer (KPi, pH 7.4) containing protease inhibitor cocktail: leupeptin (2 μg/mL = 4.2 μM), aprotinin (2 μg/mL = 0.31 μM), TLCK (tosyl-L-lysine chloromethyl ketone hydrochloride, 0.74 μg/mL = 20 μM) and PMSF (phenylmethanesulfonyl fluoride, 17.42 μg/mL = 100 μM). To determine the HSP70 expression by Western blotting, another portion of the tissues were homogenized in 0.1% (w/v) SDS buffer containing protease inhibitor cocktail, consisting of leupeptin (4.2 μM), aprotinin (0.31 μM), TLCK (20 μM) and PMSF (100 μM).

Determination of Protein Concentration Content

The protein concentration in tissues was determined by the spectrophotometric method of Bradford (1976) at 595nm, using bovine serum albumin as standard (points from 0.04 – 3.0 mg/mL).

Evaluation of Oxidative Stress and HSP70 Expression

Determination of Lipid Peroxidation

The lipid peroxidation was analyzed using TBARS method (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, it was measured by spectrophotometry at 535 nm. The MDA standard was prepared from 1.1.3.3-Tetramethoxypropane (points from 0.0005 – 0.016 mg/mL). Results were expressed in nmol MDA/mg of protein.

Determination of SOD and CAT Activity

SOD activity was performed by inhibition of auto-oxidation of pyrogallol (Marklund and Marklund 1974). In a cuvet, 930 µl Tris-EDTA Buffer (TRIS 2-amino-2-hydroxymethyl-propane-1,3-diol, 50 mM, EDTA ethylenediaminetetraacetic acid, 1 mM, pH 8.2), 4 µl of catalase (CAT; 30 µM) and 50 µl of homogenate were added and mixed. After, pyrogallol (24 mM in HCl 10 mM) was added and SOD activity determined at 25°C in spectrophotometer (420 nm) for 120 sec. Results were expressed in Unit SOD/mg of protein.

CAT activity was performed accordingly to Aebi (1984). In a quartzo cuvet, 30 µl of homogenate, 2865 µl of phosphate buffer (50 mM, pH 7.4) were mixed, and after, 105 µl of hydrogen peroxide (0.01 M) was added and mixed. The decomposition of hydrogen peroxide by CAT activity was determined at 25°C in spectrophotometer (240 nm - ultraviolet) for 120 sec. The results were expressed in pmol/mg of protein.

HSP70 Expression

The HSP70 expression was evaluated in NMRT by immunoblot analyses (Laemmli 1970). Equivalent amounts of protein from each sample (~ 40 µg) were prepared in sample buffer [Tris 50 mM, SDS 10%, glycerol 10%, 2-mercaptoethanol 10% and 2mg/ml bromphenol blue]. The samples were boiled for 5 min and electrophoresed in a 10% polyacrylamide gel (5h in 15 mA/gel). After, the proteins were transferred to a nitrocellulose membrane (GE HealthCare) by electrotransfer (1h in 100 V) and subsequently, transferred bands were visualized with 0.3% (w/v) Red Ponceau S (Sigma-Aldrich).

The procedures were performed with SNAP i.d. (Merck Millipore), vacuum system for rapid immunoblot. Membranes were washed with water and then blocked in 0.5% (w/ v) nonfat dry milk in washing buffer [TEN-Tween 20 solution (0.1% w/v); TEN is 50 mM Tris, 5 mM EDTA, 150 mM NaCl, pH 7.4]. Membranes were washed three times with wash buffer and incubated for 15 min with monoclonal anti-HSP70 antibody (Sigma-Aldrich H5147, 1:1000). After three consecutive washings with wash buffer, peroxidase-labeled rabbit anti-mouse IgG (Sigma-Aldrich A9044) was utilized as secondary antibody, at 1:15,000 dilution. As a gel loading control, was used anti β-actin containing peroxidase (Sigma-Aldrich A3854, dilution 1:15000) or C*oomassie Blue* (0.1% *Coomassie blue*, 40% methanol, 10% *acetic acid*). Blot visualization was performed using ECL-Prime Western blotting Reagent (GE Healthcare). Quantification of bands was performed using the Image J® software. The data are presented in arbitrary units HSP70, normalized in terms of β-actin.

Statistical Analysis

Statistical analysis was developed using One-way analysis of variance (ANOVA). Post hoc multiple comparisons among groups were performed with the Tukey's test. The Pearson's correlation test was employed to test the associations between the variables in each experimental group (EWAT weight/body weight, Body weight and IAUC response to GTT at the 24^{th} week). All statistical analyzes were performed using SPSS for Windows, version 19.0. The level of significance was set at 5%. Results were expressed as mean ± standard deviation.

RESULTS

Along 24 weeks, the water consumption (P=0.747; $F_{1,10}$=1.154), food intake (P=0.105; $F_{1,10}$=2.015) and kcal consumption (P=0.966; $F_{1,10}$=1.152) was similar among the groups. We did not observe differences in body length (P=0.169; $F_{3,58}$=1.742) and Lee Index (P=0.289; $F_{3,52}$=1.286) over 24 weeks of intervention between groups. However, in IAUC of body weight (Figure 1A-C) we found an increase only in HFD group (Figure 1B). At the end of the treatments (24^{th} week) we did not observed any difference among the groups in these variables (body length P=0.537, $F_{3,58}$=0.732; Lee index P=0.478; $F_{3,55}$=0.840; and body weight P=0.371, $F_{3,55}$=1.066).

HFD and $PM_{2.5}$ association promotes a decrease in muscle and pancreatic mass

As expected, HFD consumption promoted an increase in adiposity (in HFD and HFD+$PM_{2.5}$ groups) as showed by EWAT weight/body weight percentage (Figure 1D and Supplemental Material Figure S1). In the other hand, the association between HFD and $PM_{2.5}$ promoted a decrease in pancreas and muscle mass (Figure 1E and 1F, respectively). The liver mass was not influenced by any treatment (Figure 1G).

HFD and $PM_{2.5}$ association change oxidative responses in nutrient metabolism related tissues

The improvement in antioxidant response in the EWAT was able to protect to the challenges imposed by the interventions (HFD and air pollution). The EWAT showed lower MDA concentration, in both HFD and HFD+$PM_{2.5}$ groups (Figure 2A). Was observed an increase in the CAT and SOD activity in EWAT in groups receiving HFD, with greater response in the HFD+$PM_{2.5}$ group (Figure 2B and 2C).

The association HFD +$PM_{2.5}$ promoted an increase in antioxidant response in the pancreas (CAT activity, Figure 2E), thus, we observed a decreased in MDA concentration (Figure 2E). However, no changes were observed in the SOD activity in this tissue (Figure 2F).

In the liver, we did not observed lipid peroxidation alterations in any groups (Figure 2G), while, HFD +$PM_{2.5}$ intervention showed lower CAT activity (Figure 2H). Inversely, groups that received HFD showed higher SOD activity, when compared with groups that was exposure to air pollution (Figure 2I).

Gastrocnemius muscle did not show any alterations in oxidative profile (Figure 2J, 2K and 2L).

HFD and $PM_{2.5}$ promotes different HSP70 expression in nutrient metabolism related tissues

We evaluated the HSP70 expression in NMRT (Figure 3A-D) and observed that the EWAT responded to all treatments showing higher HSP70 expression compared to the CONTROL group (Figure 3A).

On the other hand, the pancreas showed an ineffective response to the effects of HFD consumption (HFD and HFD+$PM_{2.5}$ groups), as demonstrated by lower HSP70 expression (Figure 3B).

The HFD+$PM_{2.5}$ group showed higher HSP70 expression in liver, evidencing the ability of this tissue to respond with a classical stress protection (Figure 3C). Gastrocnemius muscle showed no alterations in the expression of HSP70 (Figure 3D).

The association of HFD and $PM_{2.5}$ increased blood glucose and triglyceride levels over the 24 weeks of intervention

We observed in the HFD group and mainly in HFD+$PM_{2.5}$ group an increase in glycaemia, when evaluated the IAUC of blood glucose measurements along the experimental period (Figure 4A-B). In the same way, the association of treatments (HFD and $PM_{2.5}$) promoted an increase in triglycerides levels over the 24 weeks of intervention (Figure 4D-E). No modifications were observed in the IAUC cholesterol (Figure 4G-H), or final values (at 24^{th} week) of glycaemia, triglycerides and cholesterol in blood (Glycemic levels P=0.324, $F_{3,35}$=1.204; Triglycerides levels P=0.132, $F_{3,25}$=2.084; Cholesterol levels P=0.285, $F_{3,25}$=1.346).

The long-term exposure to low levels of $PM_{2.5}$ worsens the response to a glucose overload in animals pre-treated with HFD

Long-term exposure to $PM_{2.5}$ worsens the response to glucose overload in the glucose tolerance test in animals previously treated with HFD (Figure 5A-K). The results of GTT before the $PM_{2.5}$ intervention (at the 4^{th}, 8^{th}, 12^{th} weeks, Figure 5A-C) and after the $PM_{2.5}$ intervention (at the 16^{th}, 20^{th} and 24^{th} week, Figure 5D-F), evaluated by IAUC-GTT, showed that until the 8^{th} week there were no effect of any treatments (4^{th} week in the Figure 5A and 8^{th} week in the Figure 5B). From the 12^{th} week to the 20^{th} week of the study, the groups that received HFD showed impaired response to a glucose overload (12^{th} week in Figure 5C, 16^{h} week in Figure 5D, 20^{th} week in Figure 5E, respectively). However, at the 24^{th} week, the

effect of the HFD+$PM_{2.5}$ association becomes evident, by high IAUC GTT response only in this group (Figure 5F). This results, suggests that long-term exposure to $PM_{2.5}$ worsens the response to glucose overload in obese mice.

Furthermore, the Figure 5G, shows important correlations between body weight and adiposity. We observed that only the animals that received HFD showed 2% or more of adiposity (% EWAT weight/Body weight) (right points from vertical lines) and has simultaneously more than 30g of body weight (horizontal line) (points situated at right and above the vertical line on Figure 5G). Since the body weight is correlated to adiposity, in our study ($P<0.001$; $r=0.446$; Figure 5G), the effect of the association of HFD and exposure to $PM_{2.5}$ was evidenced by the higher positive correlation between final body weight and adiposity only in the HFD+$PM_{2.5}$ group ($p=0.005$; $r=0.702$; Figure 5G), this correlation was not observed in other treatments (CONTROL $P=0.070$, $r=0.539$; $PM_{2.5}$ $P=0.459$, $r=0.237$; HFD $P=0.484$, $r=0.213$).

Moreover, groups that received HFD showed more than 6.000 min.mg/dl in the IAUC-GTT and 2% of body fat, and only the HFD+$PM_{2.5}$ group showed more than 8.000 min.mg/dl in the IAUC-GTT. The association of HFD and $PM_{2.5}$ induces metabolic impairment, characterized with more than 2% of body fat and 30g of body weight (Figure 5H).

DISCUSSION

We hypothesized that environmental conditions could directly influence metabolic responses in pre-existent risk profile organism, as in adiposity condition. Thus, we evidenced in our study, changes in biometric profile, related to HFD consumption that promoted higher percentage of adipose tissue, which is positively correlated with the increase in body weight and obesity.

In fact, the increase in body fat was previously showed in other studies as an effect of the consumption of HFD (Matsuzawa-Nagata et al. 2008; Yan et al. 2011). However the environmental conditions in study, in this case, the air pollution, does not have the same effect on adiposity in other exposure models, other researchers have shown that in intratracheal exposure for 3 weeks (Yan et al. 2011) or exposure chamber for 12 weeks (Sun et al. 2009), it was not observed $PM_{2.5}$ effect on body fat mass, even when associated with HFD. In contrast, our model showed that intranasal treatment with $PM_{2.5}$ associated with HFD during 24 weeks, promotes higher adiposity.

In addition to these changes (increase adipose tissue), our study showed lower growth of muscle mass and pancreatic tissue in animals that received HFD and were exposed to $PM_{2.5}$. The decrease in weight of these tissues may have contributed to the lower body weight gain, as evidenced by the final body weight of the animals in the HFD+ $PM_{2.5}$ group. Considering that, the muscle tissue plays a key role in the metabolism regulation, especially in the regulation of glucose levels, the lowest mass gain may be related to diabetogenic effects, since the decrease in the muscle mass is associated to impaired glucose tolerance, a condition for T2DM (Sone and Kagawa 2005).

This condition of tissue stress could develop initial compensatory responses, such as expression of HSP70, a cytoprotection to muscle loss, oxidative stress and protein damage, as a response against insulin resistance or impaired glucose uptake by muscle cells (Geiger and Gupte 2011). However, in our study, no changes were observed in the expression of HSP70 in this tissue.

In the same way, the lowest development of pancreatic tissue, also suggests tissue stress, which was induced by HFD associated to $PM_{2.5}$. Moreover, the lower lipid peroxidation in pancreas possibly was a result of lower mass and increase in antioxidant capacity (CAT activity), indicate a response of tissue injured (Sone and Kagawa 2005; Buettner et al. 2007). Levels of ROS production may increase and overwhelm antioxidant defenses, leading to

mitochondrial dysfunction, DNA oxidation, lipid peroxidation and β-cell death. The reduction of pancreatic β-cells, thus, causing a reduction in insulin secretion and in cell mass is a common feature associated with both types of diabetes (T1DM or T2DM) (Newsholme et al. 2013).

In our study, HFD treated animals exposed to $PM_{2.5}$ presented lower capacity of glycemic control. This effect may be related to the tissue stress, and consequently a depletion of HSP70 expression in the pancreas, in animals that received HFD. As HSP70 is required for protection of pancreatic islets, a decrease of intracellular HSP70 content (or its deficiency) might represent a way for the development of T2DM (Bittencourt and Newsholme 2011), induced in this case by HFD. The damage in pancreas, induced by HFD consumption, is evident in ineffective response to the glucose tolerance test.

Beyond the effects caused by the consumption of HFD, we observed that at the 24th week, after long-term exposures to low doses of $PM_{2.5}$, animals that received HFD and have been exposed to $PM_{2.5}$ had a worse response to the glucose overload. Previous studies showed that long-term exposure to $PM_{2.5}$ and HFD causes glucose intolerance in experimental models with mice (Xu et al. 2010; Yan et al. 2011), by the exposure chamber or intratracheally. We used intranasal instillation of low doses of $PM_{2.5}$ (5μg/10μL), and only at the 12th week of HFD intervention that was possible to observe the effects of HFD under the glucose intolerance. According to Liu et al. (2013a), $PM_{2.5}$ exposure attenuated whole-body insulin sensitivity and glucose homeostasis after a substantial latency period (> 8 weeks) (Liu et al. 2013a). The pollution introduced at the 12^{th} week, showed no effect on the GTT IAUC up to 20 weeks of the study, however, we found that low doses of $PM_{2.5}$ impaired GTT response at the 24^{th} week.

Stressful conditions established in NMRT, like pancreas, leads to changes in glucose profile in the animals of this study, marked by the expression of HSP70. Moreover, higher HSP70 expression in liver may represent a stressed tissue, interfering in lipid profile response to the association of HFD and $PM_{2.5}$. This association impairs the metabolic levels throughout the 24-week, and we propose that the modulation of HSP70 in the pancreas and liver may be related to impaired blood glucose and interfering in triglycerides levels.

The challenge imposed by HFD+$PM_{2.5}$ in liver is evident by higher SOD activity and HSP70 expression, which characterizes a cytoprotection response to stress. However, the increase in antioxidant activity and overexpression of HSP70, that occurs in response to stress in attempt to avoid liver damage, wasn´t enough to prevent metabolic disorders (increased triglyceride

levels). We observed no modifications in cholesterol levels, because HFD-induced hypercholesterolemia appears only with cholesterol added to the diet (Buettner et al. 2007; Yan et al. 2011), not the case of our experiment model.

We emphasize that HFD+$PM_{2.5}$ exerts systemic effects in target organs promoting metabolic challenges. However, the key organ in this process is still an enigma that research has sought to elucidate. According to Sun et al. (2009), metabolic changes may be due to a dysfunction in adipose tissue, causing systemic alterations. This tissue is becoming recognized as an important organ in the regulation of glucose metabolism, and changes in fatty acid homeostasis has been considered as a cause of IR and T2DM (Herman 2008; Mendez et al. 2013; Schuster 2010).

Studies have shown that the adipose tissue is active in chronic inflammatory processes, such as obesity (Schuster 2010). Xu et al. (2010), discuss in their work the term "metainflammation", related to metabolic dysfunction derived from inflammation, suggesting that metabolic changes are related to an up-regulation of pro-inflammatory cytokines and ROS production by nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), in response to the consumption of HFD and enhanced by exposure to $PM_{2.5}$.

The induction of ROS generation, in the EWAT (Matsuwa-Nagata et al. 2008), requires an increased SOD activity in this tissue, as observed in our study. Additionally, the increase in HSP70 expression is remarkably evidenced as a stress response to $PM_{2.5}$, such as HFD. This cytoprotection mediated by HSP70 and SOD may have contributed to adipocytes defense against dangerous extracellular signaling as HSP70 extracellular (eHSP70) and cytokines, presents at high levels in the circulation in both obesity (Schuster 2010), exposure to PM (van Eeden et al. 2001; Liu et al. 2013b), and finally, in T2DM individually (Rodrigues-Krause et al. 2012)

We consider the adipose tissue as the central organ of metabolic dysfunction developed by effect of both HFD and pollution. In this way, the expansion of this tissue mass, showed by increased adiposity, leads to recruitment and activation of immune cells in metabolic tissues (such as liver, pancreas and muscle) (Ferrante Jr 2013). To summarize our data we propose that adiposity promote systemic metabolic effect induced by HFD diet and exposure to $PM_{2.5}$, showed in Graphical Abstract (Fig. 6).

Considering the response of oxidative stress and HSP70 expression to the challenges imposed by the association of HFD and $PM_{2.5}$ in NMRT, we suggest that air pollution can be an

important factor that potentiates the development of diabetes, particularly in individuals with a pre-existing risk factor, as adiposity.

CONCLUSION

In conclusion, our study demonstrates that long-term exposure to $PM_{2.5}$ markedly worsens the glycemic response to glucose overload in animals previously treated with HFD, and promoted relevant alterations in oxidative profile and in HSP70 expression in nutrient metabolism related tissues, providing evidence for an important interaction between environmental and dietary challenges.

REFERENCES

Aebi H. 1984 Catalase in vitro. Methods Enzymol 105:121.

Bae S, Pan Xc, Kim Sy, Park K, Kim Yh, Kim H, Hong Yc. 2010. Exposure to particulate matter and polycyclic aromatic hydrocarbons qnd oxidative stress in school children. Environ Health Perspect 118(4):579-83.

Bittencourt Jr PIH, Newsholme P. A Novel L-Arginine/L-Glutamine Coupling Hypothesis: Implications for Type 1 Diabetes. 2011. InTech Open, November.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Buege JA, Aust, SD. 1978. Microssomal lipid peroxidation. Methods Enzymol. 52:302-309.

Buettner R, Schölmerich J, Bollhheimer LC. 2007. High fat diets: modeling the metaboic disorders of human obesity in rodents. Obesity 15(4):798-808.

Chen JC, Schwartz J. 2008.Metabolic syndrome and inflammatory responses to long-term particulate air pollutants. Environ Health Perspect 116(5):612-7.

De Maio A. 2011. Extracellular heat shock proteins, cellular export vesicles, and the Stress observation System: A form of communication during injury, infection, and cell damage. Cell Stress and Chaperones 16(3): 235-249.

Emmerechts J; Hoylaerts MF. 2012. The effect of air pollution on haemostasis. Hamostaseologie 32(1):5-13.

Fajersztajn L, Veras M, Barrozo LV, Saldiva P. 2013. Air pollution: a potentially modifiable risk factor for lung cancer. Nat Rev Cancer 13(9):674-8.

Ferrante Jr W. 2013. The immune cells in adipose tissue. Diabetes, Obesity and Metabolism 15(Suppl.3):34–38.

Geiger PC, Gupte AA. 2011. Heat shock proteins are important mediators of skeletal muscle insulin sensitivity. Exerc Sport Sci Rev 39(1):34-42.

Heck TG, Pizzato S, Ludwig MS, De Bittencourt Jr. PIH. 2012. Role of Heat Shock Proteins in Skeletal Muscle. In: Julianna Cseri. (Org.). Skeletal Muscle, InTech Open Access Company 1ed.:1-18.

Heck TG, Scholer CM, de Bittencourt PI. 2011. HSP70 expression: does it a novel fatigue signaling factor from immune system to the brain? Cell Biochem Funct 29:215–26.

Herman MA, Kahn BB. 2006. Glucose transport and sensing in maintenance of glucose homeostasis and metabolic harmony. J Clin Invest 116(7):1767-75.

Hooper PL; Hooper PL. 2009. Inflammation, heat shock proteins, and type 2 diabetes. Cell Stress Chaperones 1(2):113-5.

Jacobs L, Emmerechts J, Mathieu C, Hoylaerts MF, Fierens F, Hoet PH, et al. 2010. Air Pollution–Related Prothrombotic Changes in Persons with Diabetes. Environmental Health Perspectives 118:191-196.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee MO. 1928. Determination of the surface area of the white rat with its application to the expression of metabolic results. Am J Physiol 89:24–31.

Liu C, Xu X, Bai Y, Wang T, Rao X, Wang A, Sun L. 2013a. Pollution–Mediated Susceptibility to Inflammation and Insulin Resistance: Influence of CCR2 Pathways in Mice. Environmental Health Perspectives 122(1): 17-26.

Liu C, Ying Z, Harkema J, Sun Q, Rajagopalan S. 2013b. Epidemiological and Experimental Links between Air Pollution and Type 2 Diabetes. Toxicol Pathol 41:361.

Marklund S, Marklung G. 1974 Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469-474.

Matsuzawa-Nagata N, Takamura T, Ando H, Nakamura S, Kurita S, Misu H, et al. 2008. Increased oxidative stress precedes the onset of high-fat-diet induce insulin resistance and obesity. Metabolism 57(8):1071-7.

Mendez R, Zheng Z, Fan Z, Rajagopalan S, Sun Q, Zhang K. 2013. Exposure to fine airborne particulate matter induces macrophage infiltration, unfolded prot einresponse, and lipid deposition in white adipose tissue. Am J Transl Res 5(2):224-34.

Mukhopadhyay I, Nazir A, Saxena DK, Chowdhuri DK. 2003. Heat shock response: hsp70 in environmental monitoring. J Biochem Mol Toxicol 17(5):249-54.

Pearson JF, Bachireddy C, Shyamprasad S, Goldfine AB, Rownstein JS. 2010. Association Between Fine Particulate Matter and Diabetes Prevalence in the U.S. Diabetes Care 33(10):2196-2201.

Rajagopalan S, Brook RD. 2012. Air pollution and type 2 diabetes: mechanistic insights. Diabetes 61(12):3037-45.

Rhoden Cr, Lawrence J, Godleski JJ, González-Flecha B. 2004. N-Acetylcysteine prevents lun inflammation after short-term ihalation to concentrated ambient particles. Toxicol Sci 79(2):296-303.

Rodrigues-Krause J, Krause M, O'hagan C, De Vito G, Boreham C, Murphy C, et al. 2012. Divergence of intracellular and extracellular HSP72 in type 2 diabetes: does fat matter? Cell Stress Chaperones 17:293–302.

Schuster D. 2010. Obesity and the development of type 2 diabetes: the effects of fatty tissue inflammation Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. Diabetes Metab Syndr Obes 3:253–262.

Sone H, Kagawa Y. 2005. Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat-diet-induce diabetic mice. Diabetologia 48(1):58-67.

Sun Q, Yue P, Deiuliis JA, Lumeng CN, Kampfrath T, Mikolaj MB, et al. 2009. Ambient air pollution exaggerates adipose inflammation and insulin resistance in a mouse model of diet-induced obesity. Circulation 119(4):538-46.

Torres-Leal FL, Fonseca-Alaniz MH, Rogero MM, Tirapegui J. 2010. The role of inflamed adipose tissue in the insulin resistance. Cell Biochem Funct 28(8):623-31.

USA-FAO – Food and Agriculture Organization. Carbohydrates is Human Nutrition. 1997. Available in: http://www.fao.org/docrep/w8079e/w8079e00.htm#Contents.

Van Eeden SF, Tan WC, Suwa T, Mukae H, Terashima T, Fujii T, et al. 2001. Cytokines involved in the systemic inflammatory response, induced by exposure to particulate matter air pollutants (PM_{10}). Am J Respir Crit Care Med 164(5):826-30.

Xu X, Yavar Z, Verdin M, Ying Z, Mihai G, Kampfrath T, et al. 2010. Effect of early particulate air pollution exposure on obesity in mice: role of p47phox. Arterioscler Thromb Vasc Biol 30(12):2518-27.

Xu Z, Xu X, Zhong M, Hotchkiss IP, Lewandowski RP, Wagner JG, et al. 2011. Ambient particulate air pollution induces oxidative stress and alterations of mitochondria and gene expression in brown and white adipose tissues. Particle and Fibre Toxicology 8(20):1-14.

Yan YH; Chou CC; Lee CT; Liu JY; Cheng TJ. 2011. Enhanced insulin resistance in diet induced obese rats exposed to fine particles by instillation. Inhal Toxicol 23(9):507-19.

TABLES

Table 1. Experimental groups.

Groups	***Diet (24 weeks)***	***Instillation (after the first 12 weeks)***
Control (CONTROL, n=15)	standard diet (4.0 % fat)	*10µL saline solution*
Polluted ($PM_{2.5}$*, n=15)*	standard diet (4.0 % fat)	*10µL* $PM_{2.5}$ (5µg/10µL)
High Fat Diet (HFD, n=14)	HFD (60.0 % fat)	*10µL saline solution*
*High Fat Diet Polluted (*HFD+$PM_{2.5}$*, n=15)*	HFD (60.0 % fat)	*10µL* $PM_{2.5}$ (5µg/10µL)

FIGURES

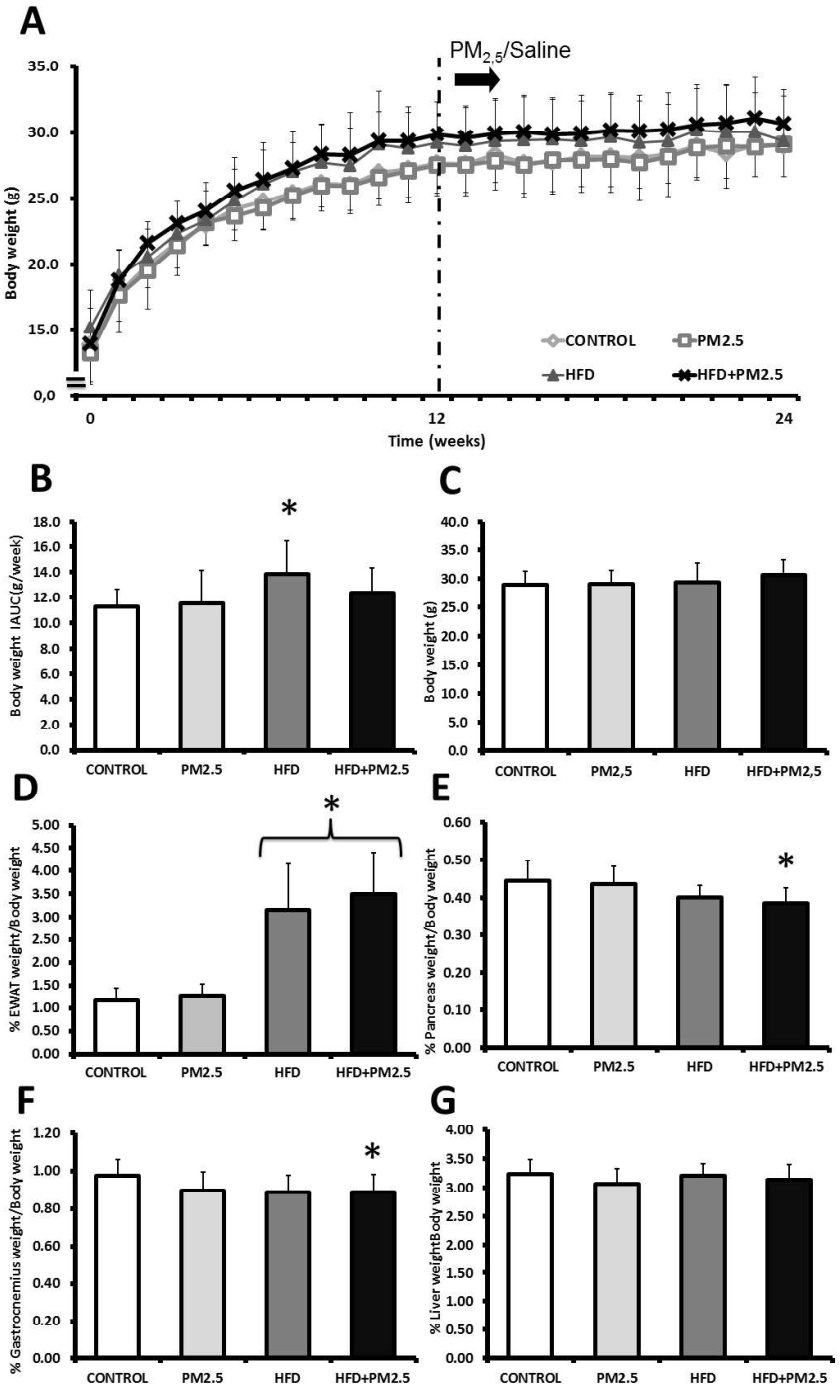


Figure 1. Effect of $PM_{2.5}$ exposure on body weight and percentage weight of tissues in mice fed with standard or HFD for 24 weeks. (mean ± SD) **(A)** Body weight over the 24 weeks of study. **(B)** Incremental area under the curve of the body weight over the 24 week study. *Difference between HFD *vs* CONTROL and $PM_{2.5.}$ P=0.009 $F_{3,53}$=4.291. **(C)** Body weight of the animals at 24th week of study. P=0.371 $F_{3,55}$=1.066. **(D)** Percentage of EWAT weight/Body weight at 24th week of study. *Difference between HFD and HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5}$. P<0.001 $F_{3,51}$=40.82. **(E)** Percentage of Pancreas weight/Body weight at 24th week of study. *Difference between HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5.}$ P=0.008 $F_{3,54}$=5.604. **(F)** Percentage of Gastrocnemius weight/Body weight at 24th week of study. *Difference between HFD+$PM_{2.5}$ *vs* CONTROL. P=0.028 $F_{3,55}$=3.275. **(G)** Percentage of Liver weight/Body weight at 24th week of study. P=0.270 $F_{3,57}$=1.343. *n=13-15* per group.

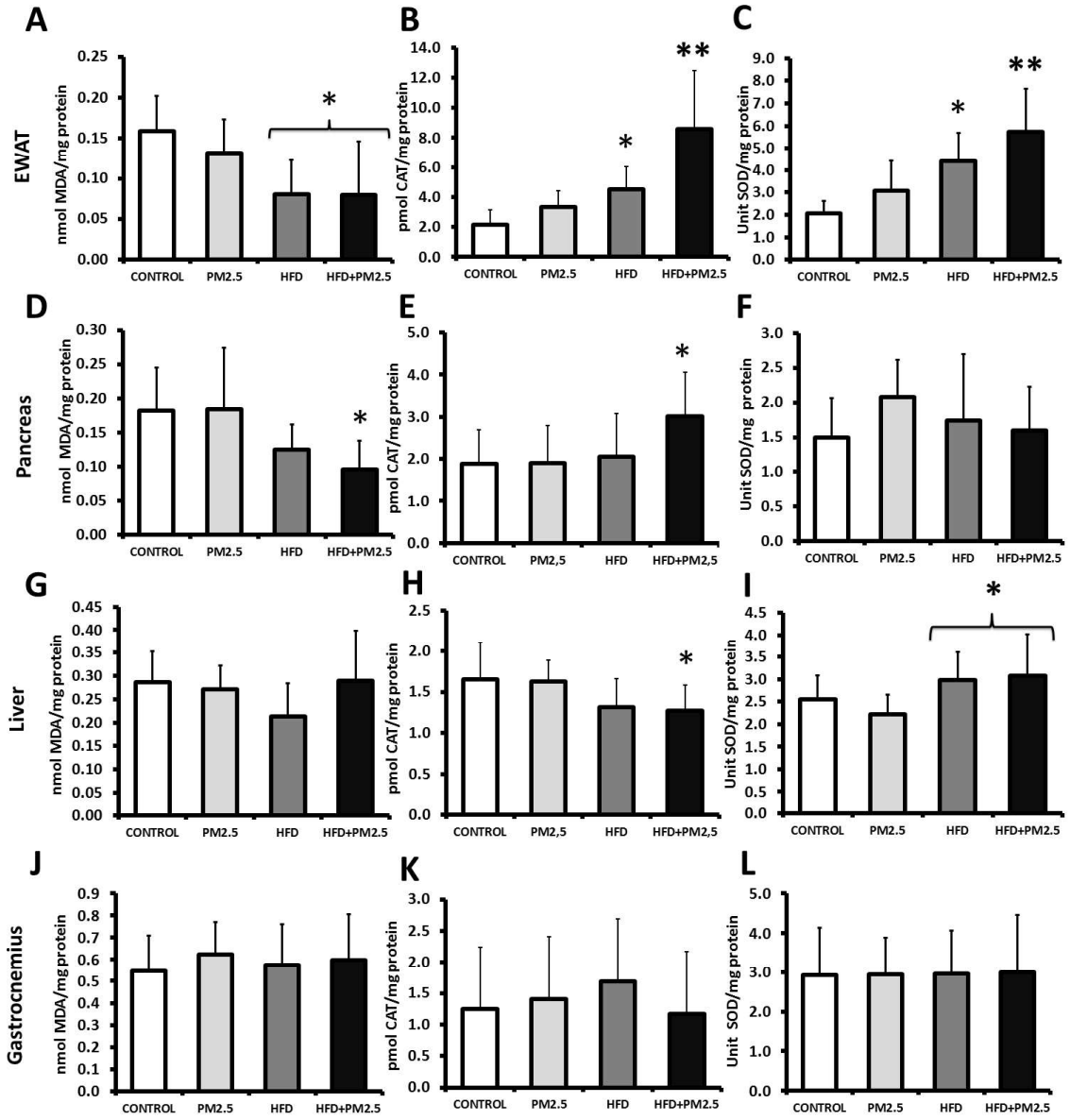


Figure 2. Effect of $PM_{2.5}$ exposure on Oxidative Stress (MDA content, activity of CAT and SOD) in mice fed with standard or HFD for 24 weeks. (mean ± SD) **(A)** Lipid peroxidation (MDA) in EWAT at 24th week of study. *Difference between HFD and HFD+$PM_{2.5}$ vs CONTROL and $PM_{2.5.}$ P<0.001 $F_{3,48}$=8.303. **(B)** CAT activity in EWAT at 24th week of study. *Difference between HFD vs CONTROL **Difference between HFD+$PM_{2.5}$ vs CONTROL, $PM_{2.5}$ and HFD. P<0.001 $F_{3,43}$=18.904. **(C)** SOD activity in EWAT at 24th week of study. *Difference between HFD vs CONTROL. **Difference between HFD+$PM_{2.5}$ vs CONTROL and $PM_{2.5}$. P<0.001 $F_{3,38}$=14.085. **(D)** Lipid peroxidation (MDA) in pancreas at 24th week of study. *Difference between HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5.}$ P=0.005 $F_{3,40}$=5.096. **(E)** CAT activity in pancreas at 24th week of study. *Difference between HFD+$PM_{2.5}$ vs CONTROL and $PM_{2.5.}$ P=0.0105 $F_{3,53}$=4.157. **(F)** SOD activity in pancreas at 24th week of study. P=0.140 $F_{3,47}$=1.921. **(G)** Lipid peroxidation (MDA) in liver at 24th week of study. P=0.174 $F_{3,39}$=1.751. **(H)** CAT activity in liver at 24th week of study. *Difference between HFD+$PM_{2.5}$ *vs* CONTROL. P=0.0081 $F_{3,53}$=4.384. **(I)** SOD activity in liver at 24th week of study. *Difference between HFD and HFD+$PM_{2.5}$ *vs* $PM_{2.5.}$ P=0.0081 $F_{3,47}$=4.451**. (J)** Lipid peroxidation (MDA) in gastrocnemius muscle at 24th week of study. P=0.796 $F_{3,45}$=0.341. **(K)** CAT activity in gastrocnemius muscle at 24th week of study. P=0.279 $F_{3,49}$=1.320. **(L)** SOD activity in gastrocnemius muscle at 24th week of study. P=1.00 $F_{3,44}$=0.005. *n=8-15* per group.

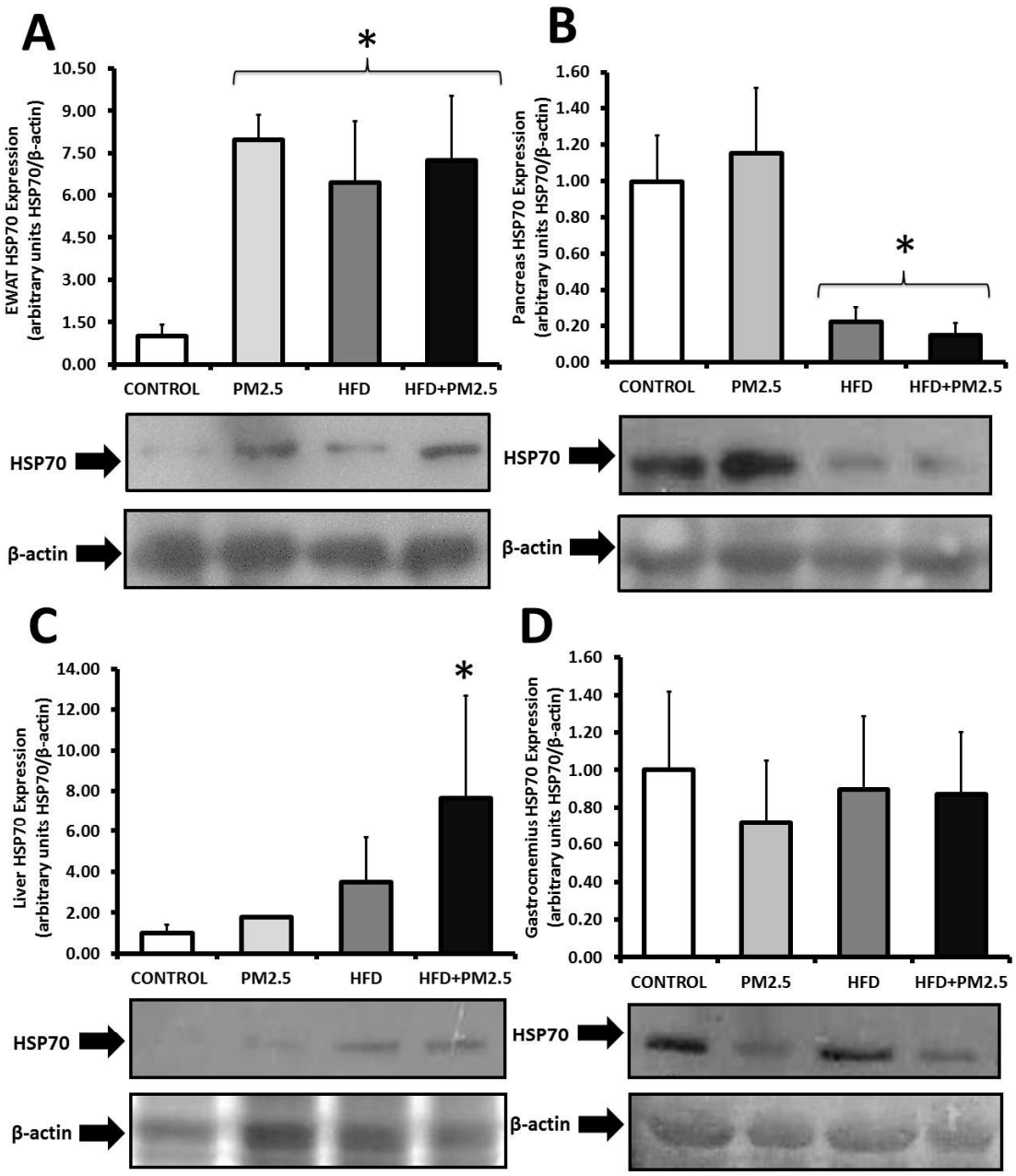


Figure 3. Effect of $PM_{2.5}$ exposure on HSP70 expression in mice fed with standard or HFD for 24 weeks. (mean ± SD) **(A)** HSP70 expression in EWAT at the 24th week. *Difference between $PM_{2.5}$, HFD and HFD+$PM_{2.5}$ *vs* CONTROL. P=0.004 $F_{3,12}$=9.070. **(B)** HSP70 expression in pancreas at the 24th week. *Difference between HFD and HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5}$. P=0.0009 $F_{3,20}$= 8.947. **(C)** HSP70 expression in liver at the 24th week *Difference between HFD+$PM_{2.5}$ *vs* CONTROL. P=0.454 $F_{3,12}$ =4.022. **(D)** HSP70 expression in gastrocnemius muscle at the 24th week P=0.5207 $F_{3,34}$= 0.7680. *n=3-10* per group.

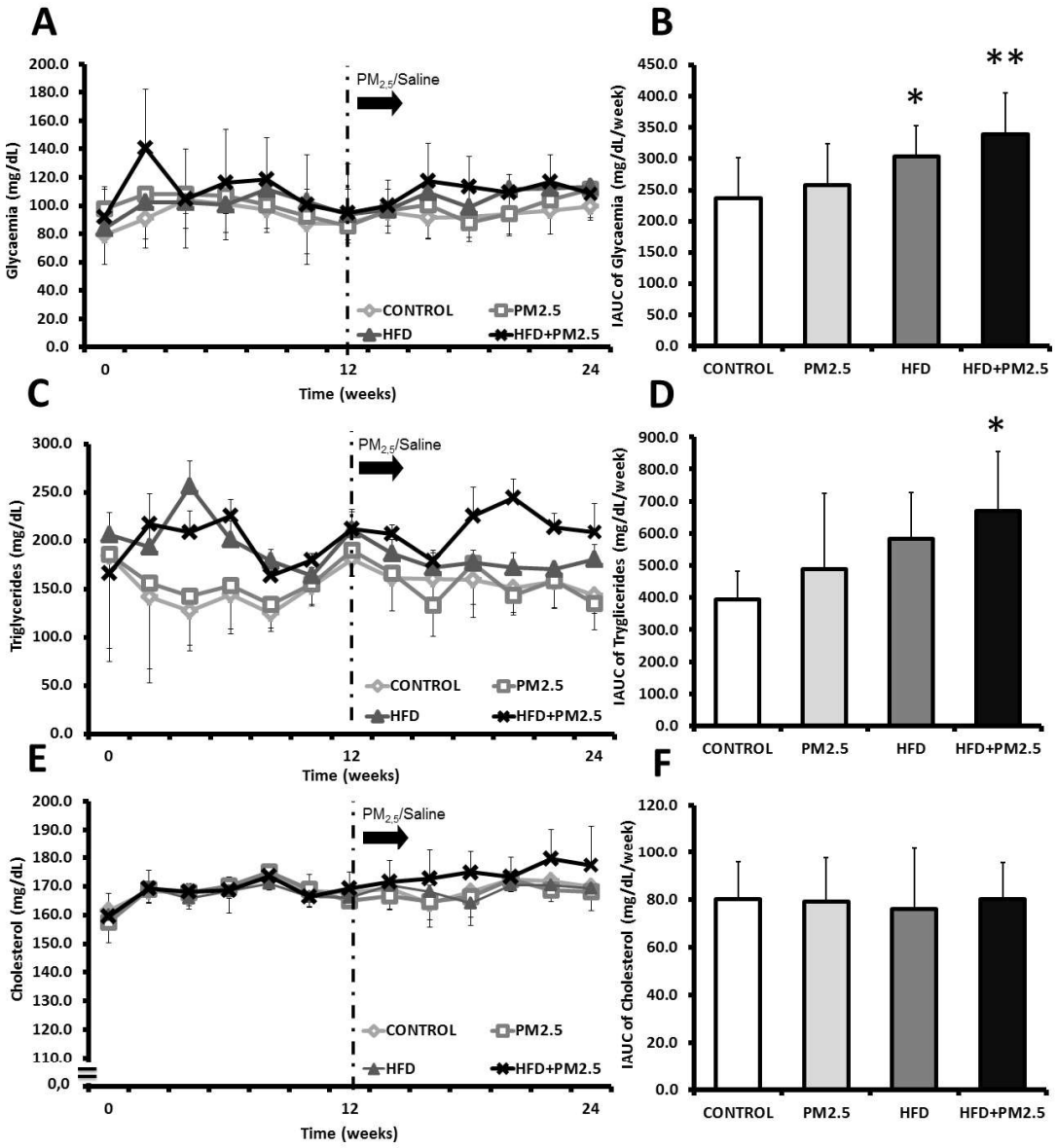


Figure 4. Effect of $PM_{2.5}$ exposure on glycemia, triglycerides and cholesterol; the response in the IAUC over 24 weeks; and glycemia, triglycerides, cholesterol levels at the 24th week of the study in mice fed with standard or HFD. (mean ± SD) **(A)** Glycemia levels over 24 weeks of study. **(B)** IAUC of Glycemia levels over 24 weeks of study. *Difference between HFD *vs* CONTROL; **Difference between HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5.}$ P<0.001, $F_{3,32}$=8.052. **(C)** Triglycerides levels of the animals over 24 weeks of study. **(D)** IAUC of Triglycerides levels over 24 weeks of study. *Difference between HFD+$PM_{2.5}$ *vs* CONTROL. P<0.001 $F_{3,21}$=9.828. **(E)** Cholesterol levels over 24 weeks of study. **(F)** IAUC of Cholesterol levels over 24 weeks of study. P=0.264, $F_{3,19}$=1.454. *n=6-9* per group.

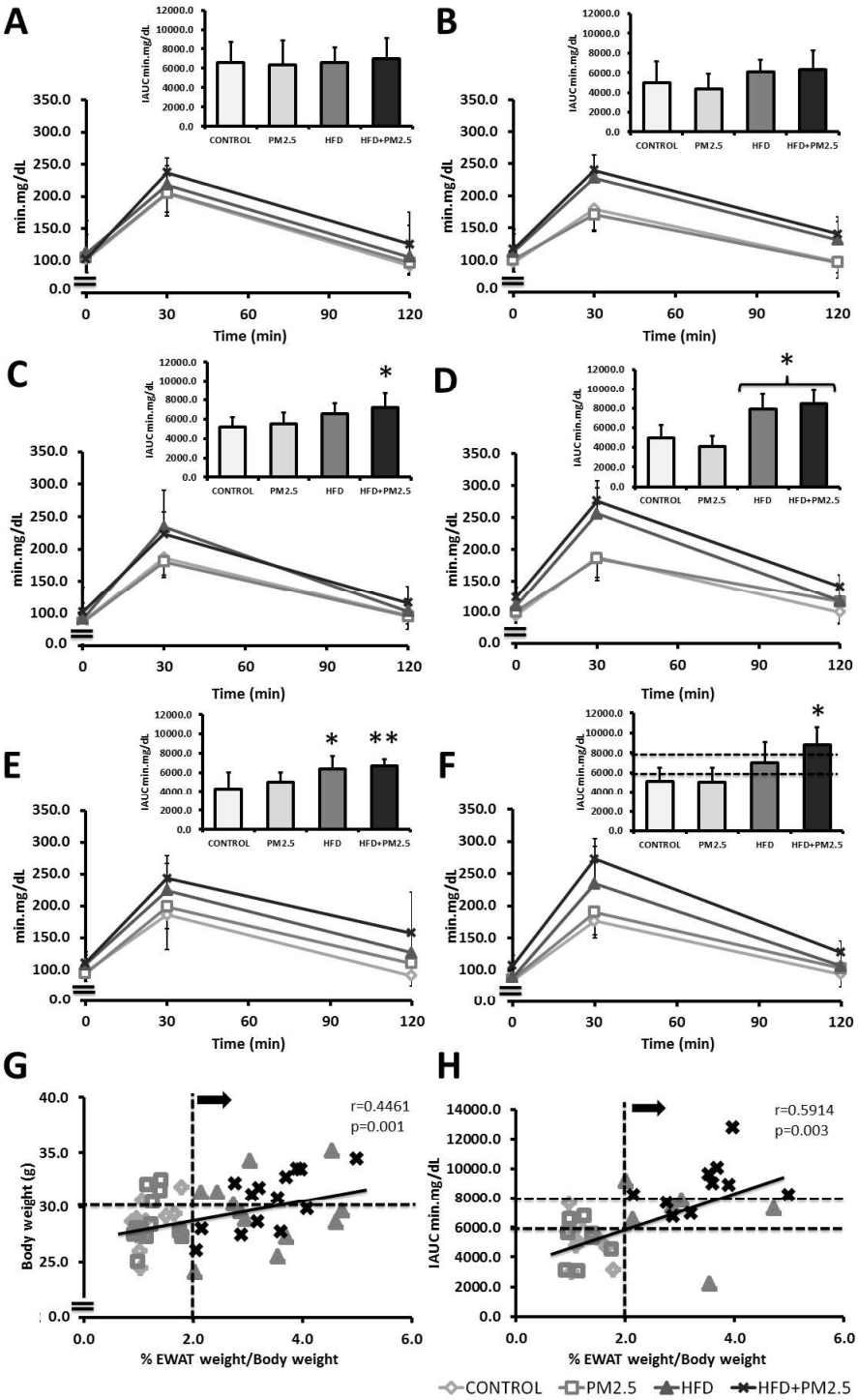


Figure 5. Effect of $PM_{2.5}$ exposure on the response to Glucose Tolerance Test; on correlations of body weight, % EWAT weight/body weight and IAUC of response to GTT in mice fed with standard or HFD for 24 weeks. (mean ± SD) **(A)** Response at the 4th week. P=0.954 $F_{3,28}$=0.109. **(B)** Response at the 8th week. P=0.071 $F_{3,38}$=2.554. **(C)** Response at the 12th week. *Difference between HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5.}$ P=0.004 $F_{3,32}$=5.455. **(D)** Response at the 16th week. *Difference between HFD and HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5.}$ P<0.001 $F_{3,29}$=20.665. **(E)** Response at the 20th week. *Difference between HFD *vs* CONTROL. **Difference between HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5.}$ P=0.002 $F_{3,28}$=6.748. **(F)** Response at the 24th week. *Difference between HFD+$PM_{2.5}$ *vs* CONTROL and $PM_{2.5}$. P<0.001 $F_{3,35}$=11.106. **(G)** Percentage of EWAT weight/body weight versus Body weight. Pearson correlation test. **(H)** Percentage of EWAT weight/body weight versus IAUC response to GTT at the 24th week. Pearson correlation test. *n=5-14* per group.

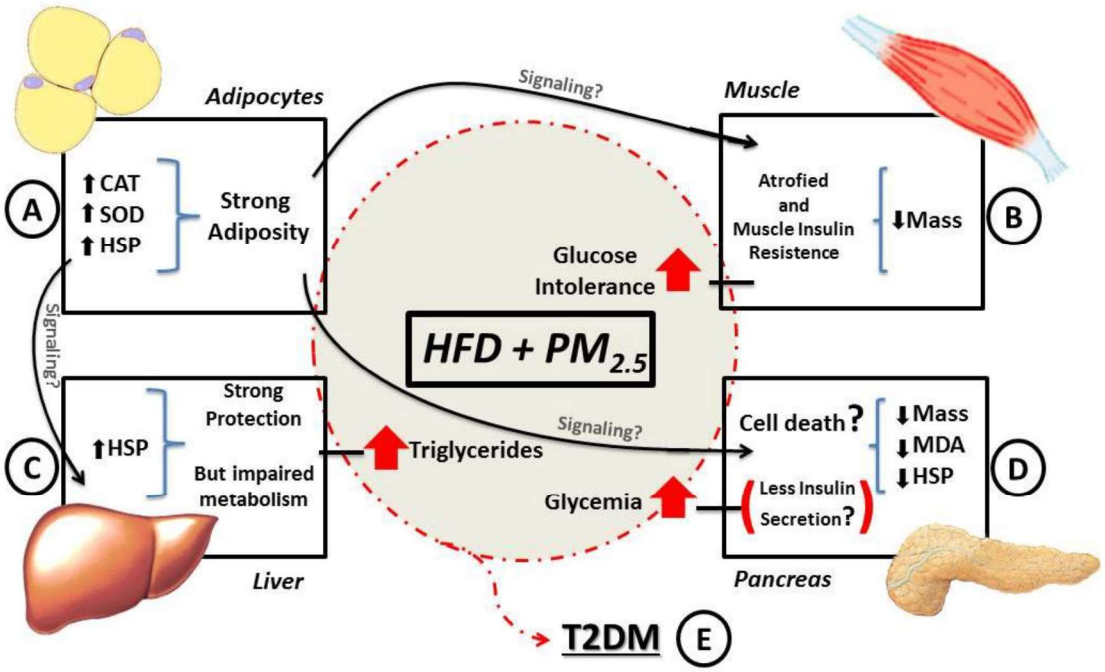
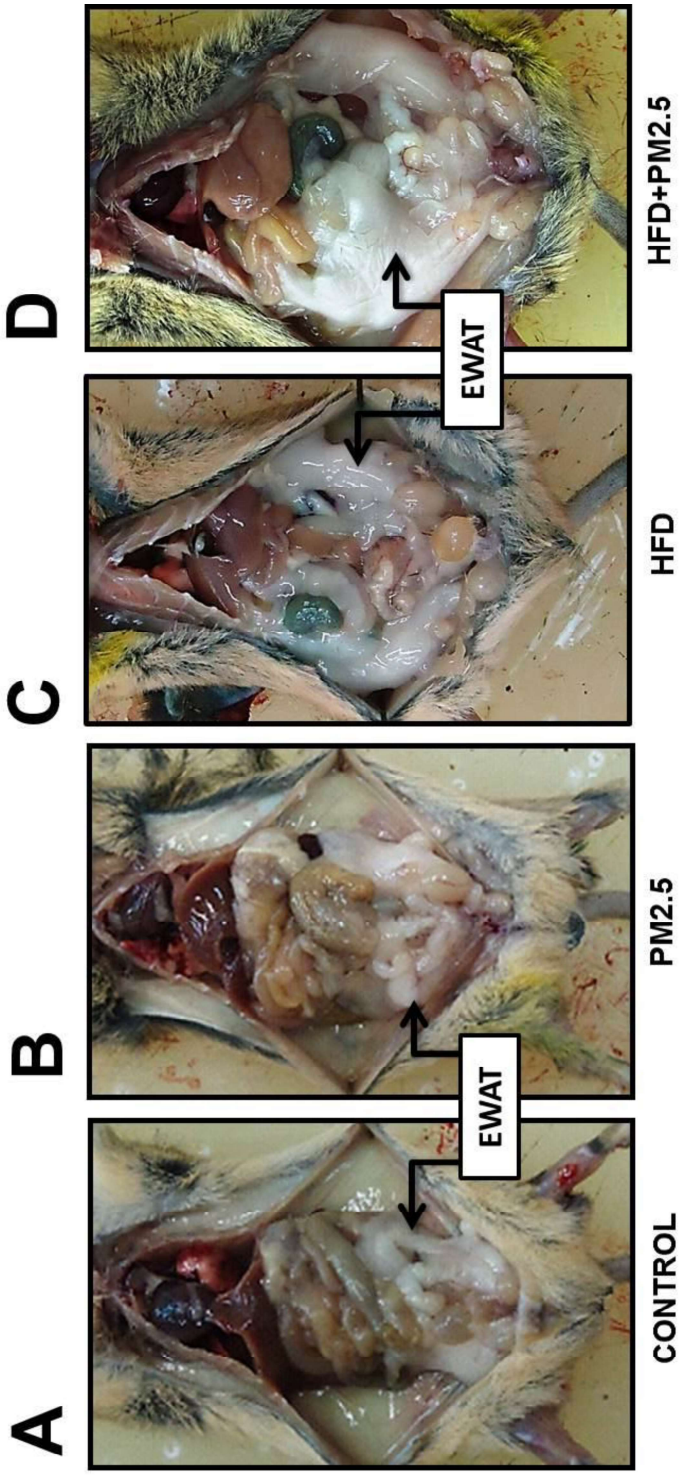


Figure 6. Graphical Abstract demonstration of the effects of the association between dietary fat diet and long-term exposure to inhaled air pollution ($PM_{2.5}$). In our study **(A)** HFD+$PM_{2.5}$ promoted an increase in adipocytes (EWAT mass), which showed high protection capacity, showing higher HSP70 expression and SOD activity. **(B)** The expansion of this tissue, led to lower muscle mass. **(C)** HFD intake and signaling from adipose tissue requires adequate response of the liver, which showed a strong protection against oxidative cellular damage (higher SOD activity and expression of HSP70), but was not able to prevent the increase in circulating triglycerides, when associated with $PM_{2.5}$ exposure. **(D)** This systemic metabolic modification was also promoted by the pancreas, which showed to be insufficient to produce cellular defenses. Lower expression of HSP70 in this tissue is connected as a marker of cellular dysfunction, also showed lower mass and MDA content, thus increasing the levels of glucose in the blood. **(E)** Therefore, the results presented in this study (increased triglycerides, glycemia and glucose intolerance) showed that the association of HFD and $PM_{2.5}$ exposure is a risk for the development of metabolic diseases, such as type 2 diabetes mellitus.

SUPPLEMENTAL MATERIAL



Supplemental Material, Figure S1. Effect of $PM_{2.5}$ exposure on anatomic adiposity (EWAT gain) in mice fed with standard or HFD for 24 weeks. **(A)** CONTROL, **(B)** $PM_{2.5,}$ **(C)** HFD and **(D)** HFD+$PM_{2.5}$.

CONCLUSÃO

Nosso estudo demonstrou que a associação entre consumo de dieta hiperlipídica e exposição crônica à poluição atmosférica por material particulado fino promove:

a) Aumento na adiposidade, com consequente desenvolvimento de obesidade.
b) Redução de massa muscular e pancreática, sugerindo dano tecidual.
c) Diferentes respostas no perfil oxidativo e na expressão de HSP70 nos tecidos relacionados ao metabolismo, evidenciando prejuízo aos tecidos metabólicos.
d) Aumento nos níveis de Glicemia e Triglicerídeos, demostrando agravo no metabolismo glicêmico e lipídico.
e) Prejuízo na resposta a uma sobrecarga de glicose, sugerindo desenvolvimento de intolerância a glicose.

Desta forma, nosso estudo sugere que os desafios impostos pelo consumo de dietas ricas em gordura, desenvolve um fator de risco pré-existente, como a obesidade, aumentando a susceptibilidade aos efeitos da exposição à poluição atmosférica, que agrava condições metabólicas, sendo potencial fator de risco para o desenvolvimento de diabetes. Entretanto, mais estudos serão necessários para investigar qual o tecido chave neste processo, e quais os efeitos da via inflamatória no prejuízo destas condições.

ANEXOS

1. **Normas do periódico Environmental Health Perspectives**

INSTRUCTIONS TO AUTHORS

Who We Are

What We Publish

About Your Manuscript

Manuscript Preparation

EHP Style

Manuscript Submission

Publication Sequence

Types of References (complete list)

Abbreviations

EHP Instructions to Authors PDF (264 KB)

WHO WE ARE

Environmental Health Perspectives (*EHP*) is a monthly open-access journal that publishes peer-reviewed research and news concerning human health and the environment. One of the overarching principles of the journal is to provide a forum for the objective and balanced presentation of scientifically credible information. Although *EHP* is sponsored by the National Institute of Environmental Health Sciences (NIEHS), its editorial policies are independent of the institute.

In 2004 *EHP* became an open-access journal. All content published since the beginning of the journal in 1972 is available free online. *EHP* is committed to promoting the discussion and exchange of information internationally.

WHAT WE PUBLISH

The environmental health sciences include many fields of study and increasingly comprise multidisciplinary research areas. *EHP* publishes articles from a wide range of scientific disciplines encompassing mechanistic research, experimental and observational human studies, and *in vitro* and *in vivo* animal research with a clear relationship to human health effects. Studies involving exposure science, climate change, ecologic issues, or effects on wildlife populations are welcome, but the relevance of the findings to human health should be made clear. *EHP* also addresses ethical, legal, social, and policy issues related to environmental public health. Because children are uniquely sensitive to their

environments, *EHP* devotes a research section specifically to issues surrounding children's environmental health.

EHP provides additional information on environmental health issues through its News and Editorials. Although *EHP* welcomes ideas for News and Editorials, the journal does not accept unsolicited manuscripts of these types. Please contact the Editor-in-Chief for further information.

ABOUT YOUR MANUSCRIPT

All papers submitted to *EHP* are evaluated by a group of consulting editors to determine whether the topic is within the scope of the journal and to evaluate adherence to word limits and journal format. Papers also are assessed for originality, scientific quality, environmental health significance, clarity of presentation, and conciseness. Before papers are sent for peer review, they are screened for possible plagiarism (seeScientific Integrity below), and authors must submit a Competing Financial Interests Declaration form on behalf of all authors (see Competing Financial Interests below). Papers selected for review are assigned to an Associate Editor, who identifies reviewers and makes recommendations to the Editor-in-Chief. Members of the Editorial Review Board serve as a pool of potential reviewers of papers. Both the Board of Associate Editors and the Editorial Review Board are composed of leading scientists from all segments of the environmental health sciences. The overall acceptance rate of papers submitted to the journal in 2011 was 15%.

a) Types of Manuscripts

Manuscripts in the categories below are considered for publication. All manuscripts are peer reviewed except Correspondence. See Article Lengthbelow for details concerning word limits.

Correspondence (≤ 750 words) should address specific scientific issues or questions raised by Research or News Articles published in the journal within the previous 6 months. Authors of papers cited in Correspondence will be given the opportunity to respond. Letters addressing issues raised in previously published letters are discouraged. Correspondence may include a brief table or small figure if it is critical to the discussion. New data must not be included. Authors may include data from or redrawing of previously published materials as long as the work is cited and written permission from the original authors and/or publishers has been granted for republication in both printed and electronic form. Each figure is considered equivalent to 250 words toward the total word count. Correspondence that cites abstracts or

unpublished observations is not acceptable and will not be published. Letters that are highly polemic or personal in nature will not be published. Correspondence is not peer reviewed and is published at the discretion of the *EHP* editors. Conclusions and opinions expressed by the authors do not necessarily reflect the policies of *EHP*.

Commentaries (≤ 5,000 words) present information and personal insight on a particular topic. Commentaries should not be extended critiques of single articles appearing in *EHP* or elsewhere. Factual data should be included to substantiate arguments. *EHP* reserves the right to reject Commentaries without review if they are perceived as being too polemic or personal in nature. *EHP* also reserves the right to propose that Commentaries be reviewed as one side of a point/counterpoint debate. Assuming the original author agrees, *EHP* will ask another author to address the opposite side of an argument. If both papers are accepted, *EHP* will publish them together. Manuscripts on ethical, legal, social, or policy issues may also be accepted in this category.

Research Articles (≤ 7,000 words) report original scientific research and discovery. Research Articles may come from any field of scientific research relevant to the study of human health and the environment.

Emerging Issue Reviews (≤ 5,000 words) identify emerging ideas, concepts, or trends in the area of environmental health sciences. These papers have a highly focused narrative and a limited set of references. Because the intent of the Emerging Issue Review is to get novel ideas into the literature in a timely fashion, the review of these manuscripts will be expedited.

Substantive Reviews (≤ 10,000 words) provide an overview, integration of information, and critical analysis of a particular field of research or theme related to environmental health sciences. Previous research should be comprehensively reviewed regardless of whether the findings are consistent with expectations or the review authors' hypotheses. It is appropriate for authors to discuss the strengths and weaknesses of individual studies, focus on high-quality studies that add to the weight of the evidence on the topic under review, identify information gaps, and make recommendations for future research. Lengthy historical perspectives generally are not appropriate.

Quantitative Reviews and Meta-Analyses (≤ 10,000 words) present, contrast, and (when appropriate) combine data across studies to address a specific study question related to environmental health. Inclusion criteria and strategies used to search the literature should be explicitly described, along with analytic methods used to evaluate or combine data. The

potential for publication bias and heterogeneity among studies should be investigated, and graphical displays of data contributed by individual studies are encouraged. The strengths and weaknesses of individual studies and potential causes of discordant findings among studies also should be discussed. As with Substantive Reviews, authors should integrate and critically analyze information from previous research, identify information gaps, and make recommendations for future research.

Reviews Based on Meetings or Conferences (≤ 10,000 words) should review the state of the science for a particular area, identify research gaps and needs, and explain how the outcome of the meeting or conference addresses those gaps and needs. These reviews should focus on the science or theme but not on the conference or meeting itself. *De novo* data, participant lists, dialogue of workgroups or committees, and discussion of the internal organization of the meeting are not allowed. These papers should be submitted to *EHP* no more than 1 year after the meeting or conference takes place. Prospective authors should consult with the Editor-in-Chief before submitting a review based on a meeting or conference.

*b) **Originality of Submission***

Contributions submitted to *EHP* must be original works of the author(s) and must not have been previously published in print or online or simultaneously submitted to another publication. Previously published material (e.g., figures, tables) may be included in Commentaries and Reviews, assuming the original authors have given permission to reproduce the material and all copyright issues have been resolved. For original Research Articles, previously published schemata or illustrative figures are acceptable with the proper attribution. Text or narrative from guidance documents, technical reports, and position papers by various government and nongovernmental organizations may be considered if they include new information. *EHP* will consider papers from dissertations that have been published in their entirety by a university in partial fulfillment of a degree. Manuscripts presented at a scientific meeting but not published in full or under review for publication elsewhere also will be considered. Previously published material may be included in the Supplemental Material of the paper. As indicated in *Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication* (International Committee of Medical Journal Editors), it is the responsibility of the author to make a full statement to the editor concerning materials in a manuscript that might be considered redundant or duplicative. For additional clarification, please contact the Editor-in-Chief.

c) *Scientific Integrity*

EHP requires assurances that animals used in a study have been treated humanely and with regard for the alleviation of suffering. Research involving humans must have been conducted according to the Common Rule. Research involving humans also must be approved by an appropriate institutional review board and comply with all relevant national, state, and local regulations. For research conducted outside the United States and thus exempt from U.S. federal regulations, authors must perform the research in accordance with principles of the Declaration of Helsinki. Approval and compliance with research requirements regarding human subjects must be noted, and information regarding informed consent procedures must be described in the "Methods" section of manuscripts concerning human subjects research.

EHP is sometimes confronted with issues regarding potential research misconduct, such as plagiarism or data fabrication. Authors should be aware that all papers submitted to *EHP* are screened routinely for plagiarism, defined as "the appropriation of another person's ideas, processes, results, or words without giving appropriate credit" (American Medical Association. 2007. AMA Manual of Style: A Guide for Authors and Editors, 10th edition. New York:Oxford University Press). Instances of documented plagiarism and allegations of data fabrication will be brought to the attention of the authors' host institutions. Documented cases of plagiarism or data fabrication could lead to a 3-year ban on future publication in*EHP* by the authors, a published Expression of Concern, and/or retraction of the paper.

d) *Dual-Use Research*

EHP anticipates receiving submissions on research that, based on current understanding, can be reasonably anticipated to provide knowledge, products, or technologies that could be directly misapplied by others to pose a threat to public health and safety, agriculture, plants, animals, or the environment (also known as dual-use research). Papers flagged for dual-use issues by *EHP* editors will undergo an additional level of review concerning the implications to society of publishing such a paper, and *EHP* reserves the right to seek expert advice in such cases. Authors should be aware that *EHP* could determine that the risks to public health and safety of publishing the paper outweigh the benefits of publishing, even if the paper has otherwise been deemed acceptable for publication.

e) *Competing Financial Interests*

EHP has a policy of full disclosure. Authors must declare all actual or potential competing financial interests involving people or organizations that might reasonably be perceived as

relevant. Disclosure of competing interests does not imply that the information in the article is questionable or that conclusions are biased. Decisions to publish or reject an article will not be based solely on a declaration of a competing interest.

For each manuscript, authors must submit a Competing Financial Interests Declaration (CFI) form. Papers will not be processed for peer review unless a CFID form has been submitted. Authors of Correspondence and Editorials also are required to submit a CFID form.

Authors must disclose all actual or potential competing financial interests occurring within the last 3 years, including but not limited to

- Grant support
- Employment (past, present, or firm offer of future)
- Patents (pending or applied)
- Payment for expert witness or testimony
- Personal financial interests by the authors, immediate family members, or institutional affiliations that may gain or lose financially through publication of the article
- Forms of compensation, including travel funding, consultancies, board positions, patent and royalty arrangements, stock shares, or bonds. Diversified mutual funds or investment trusts do not constitute a competing financial interest. Authors should carefully examine the wording of documents such as grants and contracts to determine whether there might be an actual or potential competing interest.

  Employment of any author by a for-profit or nonprofit foundation or advocacy group or work as a consultant also must be indicated on the CFID form.

  As a condition of review and publication, authors must further certify that their freedom to design, conduct, interpret, and publish research is not compromised by any controlling sponsor.

  A statement of disclosure consistent with the information contained in the CFID form must be included in the Acknowledgments section of the manuscript submitted to the journal. If there are no actual or potential competing financial interests, a declaration of “no competing financial interests” must be included in the Acknowledgments of the manuscript.

  Editors and reviewers also must disclose to the Editor-in-Chief any actual or potential competing interests, both financial and nonfinancial, that have occurred within the last 3 years

and could reasonably be perceived as relevant. Competing nonfinancial interests include former or current mentor–student relationships, faculty appointments in the same department or organization, familial relationships, service on advisory boards that oversee the research under review, collaborations, or membership in organizations that hold ideological views that are contradictory to the theme or topic under review.

EHP relies on the integrity of all authors to provide accurate disclosure statements. However, authors can expect scrutiny of their statements by the editors, reviewers, and readership. Alleged inaccuracies of declared competing interests should be addressed to the Editor-in-Chief. *EHP* will impose a 3-year ban on publication in *EHP* by any authors found to have willfully failed to disclose a competing financial interest. A paper may also be retracted or an Expression of Concern published and appended to the article.

MANUSCRIPT PREPARATION

f) Article Length

All words in the main text, title pages, abstract, tables, and references count toward *EHP* word limits. In addition, each figure is counted as 250 additional words. Manuscripts that do not conform to the word limits may be returned to the author(s) for revision before the review process is initiated. Depending on the topic and potential impact of a paper, the Editor-in-Chief reserves the right to waive word limits. Authors should consider placing some types of information such as lengthy descriptions of previously published methods into Supplemental Material; however, these methods must be summarized briefly in the text of the paper. Information included in Supplemental Material does not count toward the word limit. The judicious use of references also may help meet the following word limits:

- Correspondence: ≤ 750 words
- Commentaries: ≤ 5,000 words
- Research Articles: ≤ 7,000 words
- Emerging Issue Reviews: ≤ 5,000 words
- Substantive Reviews: ≤ 10,000 words
- Quantitative Reviews and Meta-Analyses: ≤ 10,000 words
- Reviews Based on Meetings or Conferences: ≤ 10,000 words

g) Parts of a Manuscript

Title Pages

The title pages should include the following items in the order shown, beginning on the first page of the manuscript:

- Manuscript title, not to exceed 20 words [titles generally should not contain abbreviations or numerical values, with the exception of abbreviated study names (e.g., NHANES)]
- Names of the authors spelled out in full
- Affiliations of all authors (department, institution, city, state/province, and country)
- Name of and contact information for corresponding author to whom page proofs should be sent, including complete address for express mail service, telephone number, and e-mail address
- A short running title, not to exceed 50 characters and spaces
- 5–10 key words, listed in alphabetical order, for indexing purposes
- Acknowledgments, including grant information
- A competing financial interests declaration
- A list of relevant abbreviations and definitions used in the manuscript.

Abstract

All papers must include a structured abstract of ≤ 250 words, which should not contain references. No information should be reported in the abstract that does not appear in the text of the manuscript. In general we recommend that authors indicate study names or sources of data that are integral to the study in the title or abstract. Conclusions should mention the relevance of the work to environmental health science. Headings to be used in the structured abstracts vary by article type as described below:

- Commentaries: Background, Objectives, Discussion, Conclusions
- Research Articles, Quantitative Reviews, and Meta-Analyses: Background, Objectives, Methods, Results, Conclusions
- Substantive Reviews, Emerging Issue Reviews, and Reviews Based on Meetings or Conferences: Background, Objectives, Methods, Discussion, Conclusions

Main Text

The organization of the text will vary by article type and roughly reflects the structure of the abstract with some exceptions as described below:

- Commentaries: Introduction (comprising the Background and Objectives stated in the abstract), Discussion, Conclusions
- Research Articles: Introduction (comprising the Background and Objectives stated in the abstract), Methods, Results, Discussion, Conclusions. Concise subheadings (≤ 8 words each) may be used to designate major topics within each of these sections; do not include tables and figures in these headings.
- Reviews: Introduction (comprising the Background and Objectives stated in the abstract), Methods (including data sources), Results (as appropriate), Discussion, Conclusions

References, Tables, Figures, and Supplemental Material

The following items should be provided after the main text of the paper in this order: References, Tables, Figure Legends, Figures, Supplemental Material. The References, Tables, and Figure Legends must each begin on a new page of the manuscript. Figures and Supplemental Material should be provided as separate files. Additional information concerning each of these sections is provided in *EHP* Style below.

h) Conformance to EHP *Style Guidelines*

Manuscripts submitted to *EHP* must conform to all *EHP* style requirements as described in *EHP* Style below. Authors should take special note of requirements for citations and references, figures, and tables. Manuscripts that do not conform to style requirements may be returned to the authors for modification before the initiation of the peer-review process. This step will cause a significant delay in the review and possible acceptance of the manuscript. All manuscripts must be submitted to *EHP* in English.

i) Manuscript Formatting

Manuscript pages must be numbered consecutively, beginning with the title page, and lines should be numbered in the original submission and all subsequent revisions. The manuscript must be prepared using Times New Roman font at 12-point size. The manuscript must be double-spaced, with all margins set at 1 inch.

For additional information, see the *AMA Manual of Style: A Guide for Authors and Editors*, 10th edition (American Medical Association 2007). A basic source for spelling is *Merriam-Webster's Collegiate Dictionary*, 11th edition.

Resources for assistance with research, presentation, and language are available from the following organizations:

- International Committee of Medical Journal Editors (*Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication*)
- AuthorAID.

EHP **STYLE**

j) ***Plain Language***

EHP covers all disciplines engaged in the broad field of environmental health sciences. Therefore, authors should write in a clear and simple manner, in the active voice, and avoid unnecessary jargon, so the article is understandable to readers in other disciplines and to those whose first language is not English. In deference to the breadth of the journal's readership, please define terms that may not be universally recognized among all environmental health scientists.

Clearly define all outcomes, exposures, predictors, confounders, and covariates, and describe the methods or assays used to characterize study data. Results should be presented in a clear and unambiguous manner. Comparison groups or reference conditions should be clearly indicated when reporting measures of association or effect and when reporting *p*-values for statistical tests comparing outcomes or effects between groups.

We recommend against the use of "-fold" terminology because it can be difficult to determine whether it is being used to describe relative versus absolute differences or changes between groups or conditions.

Whenever possible, provide an estimate of variability or precision when reporting measures of association or central tendency (e.g., confidence intervals, standard deviations, interquartile ranges), regardless of whether *p*-values are also reported for these estimates.

k) Abbreviations

All abbreviations, including abbreviations for elements (e.g., Fe, Cu) and chemical compounds [e.g., polychlorinated biphenyls (PCBs), carbon dioxide (CO_2)], should be defined in the text on first use with abbreviations used thereafter.

Units of measure should be abbreviated only when a specific amount is given (e.g., "concentration of 10 ng/mL" versus "units of nanograms per milliliter").

l) In-Text Citations and Reference Lists

References and citations must be formatted according to *EHP* style as described below. This will reduce copyediting time and the number of author queries included in page proofs. Authors should double-check all references for accuracy and completeness of information, spelling, diacritical marks, symbols, subscripts/superscripts, and italics. Authors are fully responsible for the accuracy of their references.

In-Text Citations

All in-text citations must be in name/date form. Place the citation immediately after the textual information cited, placing name and date within parentheses without a comma. EndNote is a useful source for *EHP* reference style.

- Single author: (Wing 2002)
- Two authors: (Wing and Wolf 2000)
- Three or more authors: Use first author's last name plus "et al." (Wing et al. 2008)
- Multiple sources cited at one time: List publications alphabetically by author in the citation. Separate publications by the same author(s) with commas and those by different authors with semicolons: (Aldridge et al. 2005; Jameson et al. 2006; Levin et al. 2007; Slotkin 2004a, 2004b; Slotkin et al. 2008)
- Multiple sources cited at one time with different first authors but same last name and date: Use first author's last name plus initial(s) (Smith A 2000; Smith J 2000).

Provide references for any quotations used in the text. For example:

According to Rubin et al. (2001), "it is only with a multidisciplinary and collaborative approach that the environmental and public health significance of *Pfiesteria* will be fully understood."

All manuscripts submitted but not yet accepted, unpublished data, and personal communications—any items that must be cited but are not accessible to the public—must appear in the text in parentheses but should not be listed in the references: (Ramsdell JS, Moeller PDR, personal communication); (Reeves MK, unpublished data).

Reference List

Authors are fully responsible for the accuracy of their references. The list of references should begin on a new page after the Conclusions of the manuscript. All references must include

- Author/editor last name plus initials (for six or fewer authors; if there are more than six authors, use "et al." after the sixth) or authoring agency
- Year of publication
- Full title of article or chapter (lower case)
- Title of journal (abbreviated according to BIOSIS, *Index Medicus*, or PubMed) or book/proceedings in title case
- For books and meeting reports, city/state/country of publication and name of publisher
- Volume and inclusive page numbers
- DOI number, if available, with online publication date; this information is required for articles published online only.

If you are uncertain what to include, please include all information.

List references alphabetically by the last name of the first author. If the first author has more than one publication, list references in alphabetical order (letter by letter) of subsequent authors. If the first author shares the last name with another first author (Smith JM vs. Smith RB), alphabetize by initials. If you list more than one publication by the same author/group of authors, arrange publications by date, early to late. If you list more than one publication published in the same year by the same author/group of authors, use a, b, c, and so on to distinguish the publications.

Sample Alphabetical List

Slotkin TA. 2004a. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol 198:132–151.

Slotkin TA. 2004b. Guidelines for developmental neurotoxicity and their impact on organophosphate pesticides: a personal view from an academic perspective. Neurotoxicology 25:631–640.

Slotkin TA. 2005. Developmental neurotoxicity of organophosphates: a case study of chlorpyrifos. In: Toxicity of Organophosphate and Carbamate Pesticides (Gupta RC, ed). San Diego:Elsevier Academic Press, 293–314.

Slotkin TA, MacKillop EA, Ryde IT, Tate CA, Seidler FJ. 2007. Screening for developmental neurotoxicity using PC12 cells: comparisons of organophosphates with a carbamate, an organochlorine and divalent nickel. Environ Health Perspect 115:93–101.

Slotkin TA, Persons D, Slepetis RJ, Taylor D, Bartolome J. 1984. Control of nucleic acid and protein synthesis in developing brain, kidney, and heart of the neonatal rat: effects of a difluoromethylornithine, a specific, irreversible inhibitor of ornithine decarboxylase. Teratology 30:211–224.

Slotkin TA, Seidler FJ. 2007. Comparative developmental neurotoxicity of organophosphates *in vivo*: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. Brain Res Bull 72:232–274.

*m) **Types of References***

Journal article—conventional reference

Lewin SW, Arthur JR, Riemersma RA, Nicol F, Walker SW, Millar EM, et al. 2002. Selenium supplementation acting through the induction of thioredoxin reductase and glutathione peroxidase protects the human endothelial cell. Biochim Biophys Acta 1593:85–92.

Journal article—DOI reference

Fanshawe TR, Diggle PJ, Rushton S, Sanderson R, Lurz PWW, Glinianaia SV, et al. 2007. Modelling spatio-temporal variation in exposure to particulate matter: a two-stage approach. Environmetrics; doi:10.1002/env.889 [Online 17 December 2007].

Journal article—conventional reference and DOI reference

Berglund M, Lind B, Björnberg KA, Palm B, Einarsson Ö, Vahter M. 2005. Inter-individual variations of human mercury exposure biomarkers: a cross-sectional assessment. Environ Health 4:20; doi:10.1186/1476-069X-4-20 [Online 3 October 2005].

Journal article, "in press"

Theppeang K, Glass TA, Bandeen-Roche K, Todd AC, Rohde CA, Schwartz BS. In press. Sex and race/ethnicity differences in lead dose biomarkers: predictors of lead in blood, tibia, and patella in older, community-dwelling adults in an urban setting. Am J Public Health.

Chapter in edited book

Clark K, Cousins I, MacKay D, Yamada K. 2003. Observed concentrations in the environment. In: The Handbook of Environmental Chemistry, Vol 3, Part Q: Phthalate Esters (Staples CA, ed). New York:Springer, 125–177.

Agency as author

Institute of Laboratory Animal Resources. 1996. Guide for the Care and Use of Laboratory Animals. 7th ed. Washington, DC:National Academy Press.

Proceedings

Zaslavsky I, Pezzoli K, Valentine D, Lin A, Sarabia H, Ellisman MH, et al. 2006. Integrating GIS and portal technologies for assessing environmental health impacts of Hurricane Katrina. In: Proceedings from the Second International Conference on Environmental Science and Technology, 19–22 August 2006, Houston, TX, Vol 2 (Starrett SK, Hong J, Lyon WG, eds). Houston, TX:American Science Press, 385–390.

Website

NTP (National Toxicology Program). 2008. NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A. NIH Publication no. 08-5994.

Available: http://cerhr.niehs.nih.gov/evaluations/chemicals/bisphenol/bisphenol.pdf [accessed 24 June 2010].

(Additional reference samples are available below.)

n) Footnotes

Do not use footnotes. Place all textual information within the manuscript and all references in the proper form both in text and in the reference list.

o) Preparing Tables and Figures

Tables

Each table must begin on a new page after the References. Tables must be numbered with

Arabic numerals, followed by a brief title (not to exceed 25 words). Tables should contain no more than three layers of column headings, and the entire table should fit on one journal page or less. Large tables may be published online as Supplemental Material. A column heading must be provided for each column. Rather than placing additional column heads in the middle of a table, a new table should be created. Tables must be created using the Table feature in Microsoft Word. List abbreviations and definitions under each table. Type footnotes directly after the abbreviations, beginning on the next line. General footnotes to tables must be indicated by lowercase superscript letters beginning with "a" for each table. Footnotes indicating statistical significance must be identified in the following order: asterisks (*, **), number signs ($^{\#}$, $^{\#\#}$), and daggers (†, ††). The comparison to which the *p*-value applies must be clearly indicated (e.g., "compared with untreated controls"). For presentation of data in tables, please use the "±" symbol for arithmetic mean and standard deviation or standard error (e.g., "mean ± SE") and parentheses for the standard error when presented with the geometric mean [e.g., "GM (SE)"]. Please present number and percent as "*n* (%)" (i.e., in one column separated with one space). Confidence intervals should be presented in parentheses in the same column as the point estimate, with the upper and lower bounds separated by a comma [e.g., (0.1, 2.3)].

Figure Legends

Figure legends should be provided on a new page after tables. Each figure legend should include a title for the entire figure and descriptors for each panel [e.g., "Figure 1. Incidence of hepatocellular adenomas (*A*) and carcinomas (*B*) in mice exposed to DEHP"]. Define error bars and any abbreviations not defined in the text. Footnotes indicating statistical significance must be identified in the following order: asterisks (*, **), number signs (#, ##), and daggers (†, ††). The comparison to which the *p*-value applies must be clearly indicated (e.g., "compared with controls from the corresponding age group"). Type footnotes directly after the abbreviations beginning on the next line.

Figures

Each figure must be provided as a separate file in one of the following formats: TIFF, JPG, EPS, or PDF. Do not embed figures in the main text (Microsoft Word) file. Each figure must be labeled with the figure number. For TIFF or JPG format, the resolution should be 300 dpi for color images, 600 dpi for grayscale images, and 1200 dpi for line art (black-and-white art). JPG files should be saved on the "highest quality" setting. Color images should be RGB and

saved at a minimum of 8 bits per channel. Because figures may be reduced or enlarged to fit our layouts, sufficient resolution is essential. Vector images should be saved as editable EPS files. Any images embedded in the EPS should also be included in a separate file. Do not convert text to path outlines before submission.

Graphics must fit standard letter-size paper (8.5 × 11 inches, portrait orientation). Multiple panels within a figure also must fit on a single page. All letters, numbers, and lines must be clearly legible and easy to differentiate. Provide a key defining representational elements (e.g., dotted/dashed lines, symbols) for each figure. All axes must be clearly labeled, giving both the measure and the unit of measurement where applicable. Consistency among terms and styles (including symbols and colors) used in figures is desirable. For example, if a black circle represents the control in Figure 1, a black circle (or a black bar) should be used for controls in all other figures. Photomicrographs should include a scale bar in each image, and the length should be specified in the typed figure legend (e.g., "bar = 10 μm").

EHP editors reserve the right to request that complex figures (e.g., figures with multiple panels showing information in a variety of formats, or that include panels related to different experiments) be divided into separate figures for publication. Questions concerning figures should be directed to EHPmanuscripts@niehs.nih.gov.

Image Integrity

Adjusting an image for brightness and contrast is acceptable if it is applied to the entire image. Background data of gels and blots must not be removed. The final image must accurately represent the original data.

*p) **Supplemental Material***

EHP welcomes reasonable amounts of material suitable for inclusion as online documentation for submitted manuscripts. Examples are bioinformatic data, formulae, statistical derivations, full gene data and analysis, additional high-resolution microscopic data, kinetic analyses, and other supporting tables, figures, or videos. The submitted manuscripts must be able to stand alone in the absence of Supplemental Material. All information included as Supplemental Material should be directly relevant to the article and cited in the main body of the paper. The principal methodological approach must be clearly described in the main body of the paper and not relegated to Supplemental Material.

Supplemental Material must not exceed 2,000 words, including text, tables, references, and figure legends plus an additional 250 words per figure. If the Supplemental Material exceeds this limit, the author must request a waiver from the Editor-in-Chief before the paper is submitted to the journal. Authors may provide a separate (ideally permanent) web repository for information that is not included in the Supplemental Material file if they believe it would be of interest to readers. This material should be clearly identified as not peer reviewed. This information should be cited in the text and included in the reference list (formatted as a website).

Information included as Supplemental Material does not count toward the word count for the paper. Supplemental Material must be uploaded as a separate single PDF file and labeled as such. Supplemental Material will be peer reviewed along with the manuscript and thus must meet the same rigorous standards.

Supplemental Material PDF files are linked to papers through a common DOI number. We use Supplemental Material files "as is" (i.e., *EHP* will not copyedit or reformat the file). Therefore, please carefully check files to confirm that they are complete, accurate, and ready for publication.

- Provide a single Supplemental Material file in PDF format.
- Begin the file with a title page that indicates "Supplemental Material" followed by the title of the paper and the author list.
- Include page numbers, but remove all line numbers before generating the PDF file.
- Provide a Table of Contents (on or after the title page) if the Supplemental Material comprises multiple tables, figures, and/or sections of text.
- Place figure legends below corresponding figures. Landscape (versus portrait) layout may be used when needed.
- Tables or figures included in the Supplemental Material should be labeled as Supplemental Material, Table S1; Supplemental Material, Figure S1; and so on.
- When referring to Supplemental Material in the main manuscript, indicate the table, figure, or section as follows: See Supplemental Material; see Supplemental Material, Table S1; see Supplemental Material, p. 6; see Supplemental Material, Part 2.

- A separate reference list must be included in the Supplemental Material file for any sources cited in the Supplemental Material, even if they are cited in the main paper.

q) Public Databases

Manuscripts using microarrays must follow the Minimum Information About a Microarray Experiment (MIAME) guidelines developed by the Microarray Gene Expression Data (MGED) Society. On acceptance, all integral data supporting the article's conclusions should be submitted to either the ArrayExpress or GEO database.

MANUSCRIPT SUBMISSION

r) Manuscript Central

Manuscripts submitted to *EHP* will be processed using Manuscript Central, an online manuscript submission and tracking program.

s) Initial Submission of a Manuscript

Authors should either log in or select the "Create a New Account" icon to create a new account. To determine if an account exists, e-mailEHPmanuscripts@niehs.nih.gov. Once logged in to the Manuscript Central site, authors must select the "Author Center" link. From this point, the system will guide the user through the submission process. Online help is available at all times during the process via the "Get Help Now" button in the upper right corner of the screen. Users may also exit and reenter the submission process at any time before completing a manuscript submission.

After completing an online submission, authors must submit a CFID form as soon as possible. The assigned manuscript number should be noted on the form. Authors should complete and sign the form, then submit a scanned document by e-mail to EHPmanuscripts@niehs.nih.gov. Completed forms may also be faxed to (301) 480-2956.

Authors can monitor the progress of submissions at any time by logging in to the Author Center using their ID and password. Forgotten passwords may be obtained by entering your e-mail address in the "Password Help" section of *EHP*'s Manuscript Central homepage. If an account exists, instructions for resetting the password will be e-mailed to the user.

Manuscripts may be submitted only via the online system. Manuscripts submitted by other methods (e.g., hard copy, e-mail) will not be processed.

t) Required Cover Letter

A cover letter must accompany the manuscript and include the following points:

- Assurances that the manuscript *a*) is an original work, *b*) has not been previously published whole or in part, and *c*) is not under consideration for publication elsewhere
- A statement that animals used in research have been treated humanely according to institutional guidelines, with due consideration to the alleviation of distress and discomfort. The source of those guidelines must be provided
- A statement that participation of human subjects did not occur until after informed consent was obtained
- Confirmation via the required CFID form that all authors have disclosed any actual or potential competing interests regarding the submitted article and the nature of those interests
- If applicable, written permission from any copyright holder (usually the publisher) to reproduce figures, tables, questionnaires, or a substantial block of text in both print and electronic forms
- A statement indicating that all authors *a*) have read the manuscript, *b*) agree the work is ready for submission to a journal, and *c*) accept responsibility for the manuscript's contents
- The names and e-mail addresses of up to six possible preferred reviewers, as well as up to six nonpreferred reviewers for the manuscript
- If applicable, a statement concerning previous publication of a manuscript or materials that might be considered redundant or duplicative.

u) Peer Review

Manuscripts are assessed for originality, scientific quality, environmental health significance, clarity of presentation, and conciseness. Scientific quality and environmental significance have a higher weight than the other criteria.

All new submissions undergo an initial review by a group of consulting editors. Authors will be notified within 1-3 weeks if their paper is not selected for peer review based on the evaluation. Papers selected for peer review (~ 20% of new submissions) are assigned to an Associate Editor, who identifies at least two peer reviewers and makes recommendations to the Editor-in-Chief based on their reviews. Reviewer names are not provided to authors, but author names are available to reviewers. Reviewers are asked to complete their review within

2 weeks. Authors may nominate up to six preferred reviewers for the manuscript. Providing the names and contact information, including e-mail addresses, is strongly encouraged. Authors may also identify up to six nonpreferred reviewers.

After editorial consideration, a decision letter and reviewers' comments will be e-mailed to authors. If a revision of the manuscript is required, authors must submit the revised manuscript to *EHP* within six weeks of the request. If authors fail to meet this deadline, the submission will be canceled unless the authors have obtained prior permission for an extension from the Editor-in-Chief. Authors must submit both the revised manuscript and a letter responding to reviewers' comments.

*v) **Resubmission of a Revised Manuscript***

If *EHP* requests revisions or accepts the manuscript, authors will need to submit all of the following through Manuscript Central:

- All text, tables, and figure legends must be in one Microsoft Word document. Please ensure that any symbols and equations appear correctly on printed copies and that all figures and tables are cited in the body text in numerical order.
- Each revised figure must be submitted as a separate file in one of the following formats: TIFF, JPG, EPS, or PDF. Each figure must be labeled with the correct figure number for the revised manuscript. Additional information on formatting and content requirements is provided in "Preparing Tables and Figures" above.
- Authors must submit a cover letter with point-by-point responses to the reviewers' comments, a copy of the revised manuscript with changes tracked in Microsoft Word, and a clean version of the revised manuscript with all changes accepted. To expedite the processing of revised manuscripts, it is important to be as specific as possible in responding to reviewers' comments. Authors should copy the editors' and/or reviewers' comments into the response letter and respond to each comment individually, including the specific changes made in response to each comment (if any) and where the changes are located in the revised draft. As in the original submission, the revised manuscripts must have numbered lines to facilitate locating specific text or changes.
- Revised manuscripts must conform to *EHP* length requirements, even if additional material is added to the manuscript in response to reviewer requests. Authors may want to consider moving text, tables, and figures to Supplemental Material to reduce the length of the

manuscript, provided such material is not necessary for most readers to follow or interpret the findings. Authors should consult with the Associate Editor for their paper for additional guidance if needed. Papers that substantially exceed *EHP* word limits may be returned to authors for additional revisions to reduce their length before acceptance.

PUBLICATION SEQUENCE

w) Advance Publication

Authors will be notified their paper has been accepted provisionally, at which point they may be asked to respond to additional post-review requests from the *EHP* Editor-in-Chief or Science Editor. Authors also are asked at this point to conduct their own final review of their paper to confirm it is ready for Advance Publication. Final acceptance will occur after papers have undergone in-house editorial review for scientific content and accuracy and compliance with *EHP* formatting and CFI requirements.

Papers are not copyedited until they are prepared for print publication. *EHP* publishes unedited PDF versions of articles online as Advance Publication articles within 3 working days of final acceptance unless a prepublication embargo period is agreed upon in advance (for more information on embargoes, see Press Releases and Embargo Policy below). Additionally, unedited abstracts are published online in PubMed and on the *EHP* website.

Advance Publication articles are citable using the assigned DOI (Digital Object Identifier) number for the article. The DOI number enables the article to be immediately referenced and establishes publication priority. The PDF version of Advance Publication articles will be replaced with the copyedited, formatted version as soon as possible, but the DOI number will remain with the copyedited article. In addition to the DOI number, the copyedited article will include assigned volume and page numbers that will allow full conventional citation.

x) Copyediting/Page Proofs

To prepare each paper for final publication, *EHP* staff will convert electronic material to a desktop publishing format and copyedit the manuscript. The copyedited version, with embedded author queries, will be converted to PDF page proofs and sent to authors by e-mail. The authors can use free Acrobat Reader software to review the proofs. There are two methods of correcting and returning proofs:

- Authors may use Comment and Markup Tools in Acrobat and e-mail the corrected proof.

- Authors may print the proof and write corrections directly on the printed copy; then scan the marked proof and return it by e-mail. Authors using this method should also include with the page proofs a list of itemized changes (including their locations).

  The copyedited proofs of an article may be slightly different from the Advance Publication version as a result of the editing process, but no substantive changes will be allowed. Any significant changes at this stage of processing will require a correction to be published at the end of the article. Extensive changes cannot be made at the proof stage; only minor changes, such as spelling, grammar, clarification, and referencing, should be requested. If new information has become available after acceptance of the manuscript, an addendum in proof can be included with the permission of the Editor-in-Chief.

y) ***Publication Date of Record***

Manuscripts accepted for publication in *EHP* will appear online within 3 working days of final acceptance unless they have been embargoed. The date the article is posted on the website will be considered the publication date of record.

z) ***Copyright, Reproduction, and Citation***

EHP is a publication of the U.S. Federal Government. Publication of *EHP* lies in the public domain and is therefore without copyright. All text from*EHP* may be reprinted freely. Use of materials published in *EHP* should be acknowledged (for example, "Reproduced with permission from*Environmental Health Perspectives*"); pertinent reference information should be provided for the article from which the material was reproduced. Articles from *EHP*, especially the News section, may contain photographs or illustrations copyrighted by other commercial organizations or individuals that may not be used without obtaining prior approval from the holder of the copyright.

aa) ***Press Releases and Embargo Policy***

Authors are responsible for arranging media outreach with their own press offices in conjunction with *EHP*. *EHP* will tailor publication dates to suit the needs of authors and their press officers. We recommend an embargo period of at least two full working days for any paper that receives a press release. This gives members of the media time to prepare stories and contact corresponding authors for additional information.

Authors whose papers have been provisionally accepted for publication should contact Susan Booker, *EHP* News Editor, to coordinate embargo and publication dates. Authors or press officers should also provide *EHP* a copy of their final press release.

Upon final acceptance *EHP* will send press officers a PDF copy of the Advance Publication article to be distributed to media who request it. All pre-press materials will be clearly identified as embargoed and will include the embargo date and time established by *EHP* in conjunction with the authors. Authors must adhere to *EHP*'s embargo policy, and authors and media alike are responsible for ensuring that all third parties with whom they share pre-press materials honor the embargo.

TYPES OF REFERENCES

Journal article—conventional reference

Waalkes MP, Liu J, Diwan BA. 2007. Transplacental arsenic carcinogenesis in mice. Toxicol Appl Pharmacol 222:271–280.

Journal article—DOI reference

Latendresse JR, Bucci TJ, Olson G, Mellick P, Weiss C, Thorn B, et al. 2009. Genistein and ethinyl estradiol dietary exposures in multigenerational and chronic studies induce similar poliferative lesions in mammary gland of male Sprague-Dawley rats. Reprod Toxicol; doi:10.1016/j.reprotox.2009.04.006 [Online 19 April 2009].

Journal article—conventional reference and DOI reference

Glas AM, Floore A, Delahaye LJ, Witteveen AT, Pover RC, Bakx N, et al. 2006. Converting a breast cancer microarray signature into a high-throughput diagnostic test. BMC Genomics 7:278; doi:10.1186/1471-2164-7-278 [Online 30 October 2006].

Journal article, "in press"

Holmes AK, Maisonet M, Rubin C, Kieszak S, Barr DB, Calafat AM, et al. In press. A pilot study of exposures to endocrine-disrupting compounds in pregnant women and children from the United Kingdom. Int J Child Adolesc Health.

Article in non-English language

Rateau JG, Broillard M, Morgant G, Aymard P. 1986. Etude experimental chez le lapin de l'effet de la cholestyramine dans le traitement des diarrhees infectieuses d'orgine cholerique [in French]. Actualite Therapeut 22:289–296.

Magazine article

Grant M. 1997. The cell from hell. People, 19 May:101–103.

Newspaper article

Clabby C. 2001. Study details how centuries of fishing depleted sea life. News and Observer (Raleigh, NC) 27 July: B1.

Book

Luna LG. 1968. Manual of Histopathologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. New York:McGraw-Hill.

Book, edited

Gross TL, Ihrke PJ, Walder EJ, eds. 1992. Veterinary Dermatopathology. St. Louis, MO:Mosby Year Book.

Chapter in edited book

Gurevitch J, Hedges LV. 1993. Meta-analysis: combining the results of independent experiments. In: The Design and Analysis of Ecological Experiments (Scheiner SM, Gurevitch J, eds). New York:Chapman e Hall, 378–398.

Book chapter, "in press"

McCoy KA, Guillette LJ. In press. Endocrine disruptors. In: Amphibian Biology. Vol 8. Conservation and Decline of Amphibians (Heatwole HF, ed). Chipping Norton, New South Wales, Australia:Surrey Beatty e Sons.

Agency monograph

IARC (International Agency for Research on Cancer). 1993. Cadmium and cadmium compounds. IARC Monogr Eval Carcinog Risk Hum 58:119–237.

Agency as author

CDC (Centers for Disease Control and Prevention). 2005. Fourth National Report on Human Exposure to Environmental Chemicals. Atlanta, GA:Centers for Disease Control and Prevention. Available:http://www.cdc.gov/exposurereport/ [accessed 14 January 2010].

Proceedings

Ibrahim K. 1994. The status of marine turtle conservation in Peninsular Malaysia. In: Proceedings of the first ASEAN Symposium Workshop on Marine Turtle Conservation, 6–10

December 1993, Manila, Philippines (Nacu A, Trono R, Palma JA, Torres D, Agas F Jr, eds). Manila, Philippines:ASEAN, 87–103.

Technical paper

NTP. 2006. Toxicology and Carcinogenesis Studies of Bromodichloromethane (CAS No. 75-27-4) in Male F344/N Rats and Female B6C3F$_1$ Mice (Drinking Water Studies). TR 532. Research Triangle Park, NC:National Toxicology Program.

Dissertation/thesis

Gelobter M. 1993. Race, Class, and Outdoor Air Pollution: The Dynamics of Environmental Discrimination from 1970 to 1990 [PhD Dissertation]. Berkeley, CA:University of California, Berkeley.

Software manual

SAS Institute Inc. 2001. SAS/STAT Guide for Personal Computers, Version 8. Cary, NC:SAS Institute, Inc.

Website

CDC (Centers for Disease Control and Prevention). 2003. National Health and Nutrition Examination Survey Homepage. Available:http://www.cdc.gov/nchs/nhanes.htm [accessed 6 August 2008].

Online database

National Center for Biotechnology Information. 2011. PubMed. Available: http://www.ncbi.nlm.nih.gov/pubmed/ [accessed 14 July 2011].

Abstract

Barbeito AG, Guelfi N, Varga MR, Pehar M, Beckman J, Barbeito L, et al. 2005. Chronic low-level lead exposure increases survival of G93A SOD-1 transgenic mice [Abstract]. In: Amyotrophic Lateral Sclerosis: Beyond the Motor Neuron. Available: http://iibce.edu.uy/ALSmeeting/abstract.htm[accessed 14 April 2008].

Federal regulation

U.S. Environmental Protection Agency. 2001. National primary drinking water regulations. Arsenic and clarifications to compliance and new source contaminants monitoring. Final rule. Fed Reg 66:6076–7066.

Executive order; federal regulation

Clinton WJ. 2000. Executive Order 13148. Greening of the government through leadership in environmental management. Fed Reg 65:24595–24606.

U.S. Government document

U.S. Environmental Protection Agency. 2004. Air Quality Criteria for Particulate Matter. EPA/600/P-99/002aF. Research Triangle Park, NC:U.S. Environmental Protection Agency.

State document

State of Maryland. 1998. Water Quality Improvement Act of 1998. Annapolis, MD:General Assembly.

Law

Food Quality Protection Act of 1996. 1996. Public Law 104-170.

Court case

Leach *v.* E.I. du Pont de Nemours e Co. 2002. Civil Action No. 01-C-608, 2002 WL 1270121. Circuit Court of Wood County, West Virginia, 10 April 2002.

ABBREVIATIONS

All nonstandard abbreviations [e.g., organochlorine (OC) pesticides, limit of detection (LOD), polymerase chain reaction (PCR)] and abbreviations for elements (e.g., Fe, Cu, Ag) and chemical compounds [e.g., polychlorinated biphenyls (PCBs), carbon dioxide (CO_2)] should be defined in the text on first use and abbreviated thereafter.

Standard abbreviations, which do not need to be defined, are shown below. Units of measure should be abbreviated only when a specific amount is given (e.g., "concentration of 10 ng/mL" versus "units of nanograms per milliliter").

Abbreviation	Description
Å	angstrom
amu	atomic mass unit
ATP	adenosine 5′- triphosphate
BW	body weight
°C	degrees Celsius
cm	centimeter
cm^2	square centimeter
cm^3	cubic centimeter
Da	dalton
df	degrees of freedom

DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
ELISA	enzyme-linked immunoadsorbent assay
ft	foot
g	gram
g	gravity (10,000 x *g*)
gal	gallon
Gy	gray (unit of absorbed dose of ionizing radiation)
ha	hectare
HEPES	*N*-2-hydroxyethylpiperazine-*N′*-2-ethane sulfonic acid
HPLC	high-performance liquid chromatography
hr	hour
Hz	hertz
i.d.	inside diameter
IM	intramuscular
in.	inch
IU	international unit
J	joule
kDa	kilodalton
kg	kilogram
km	kilometer
K_m	Michaelis constant
L	liter
lb	pound
ln	natural logarithm
M	molar
m	meter
m^2	square meter
m^3	cubic meter
mCi	millicurie
µg	microgram
mg	milligram
mi	mile
µL	microliter
min	minute
mL	milliliter
mM	millimolar
mm	millimeter
mol	mole
mRNA	messenger RNA
n	number
ng	nanogram
nL	nanoliter
nmol	nanomole
o.d.	outside diameter

pg	picogram
ppb	parts per billion
ppm	parts per million
ppt	parts per trillion
RNA	ribonucleic acid
RNase	ribonuclease
SD	standard deviation
SDS/PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error, standard error of the mean
sec	second
U	unit
V	volt
vol/vol	volume/volume
W	watt
wt	weight
wt/vol	weight/volume
yd	yard

2. Parecer da Comissão de Ética no Uso de Animais (CEUA)

PARECER DO COMITÊ

Assim, mediante a importância social e científica que o projeto apresenta a sua aplicabilidade e conformidade com os requisitos éticos, somos de parecer favorável à realização do projeto classificando-o como **APROVADO**, pois o mesmo atende aos Requisitos Fundamentais das Normas de Conduta para a Utilização de Animais no Ensino, Pesquisa e Extensão da UNIJUI, assim como as responsabilidades do pessoal envolvido no uso de animais da Resolução Normativa Nº1 do CONCEA, de 09 de julho de 2010, descreve no seu artigo 9º Aos pesquisadores, docentes e responsáveis técnicos por atividades experimentais, pedagógicas ou de criação de animais compete:

I – assegurar o cumprimento das normas de criação e uso ético de animais;

II – submeter à CEUA proposta de atividade, especificando os protocolos a serem adotados;

III – apresentar à CEUA, antes do início de qualquer atividade, as informações e a respectiva documentação, na forma e conteúdo definidos nas Resoluções Normativas do CONCEA;

IV – assegurar que as atividades serão iniciadas somente após decisão técnica favorável da CEUA e, quando for o caso, da autorização do CONCEA;

V – solicitar a autorização prévia à CEUA para efetuar qualquer mudança nos protocolos anteriormente aprovados;

VI – assegurar que as equipes técnicas e de apoio envolvidas nas atividades com animais recebam treinamento apropriado e estejam cientes da responsabilidade no trato dos mesmos;

VII – notificar à CEUA as mudanças na equipe técnica;

VIII – comunicar à CEUA, imediatamente, todos os acidentes com animais, relatando as ações saneadoras porventura adotadas;

IX – estabelecer junto à instituição responsável mecanismos para a disponibilidade e a manutenção dos equipamentos e da infra-estrutura de criação e utilização de animais para ensino e pesquisa científica;

X – fornecer à CEUA informações adicionais, quando solicitadas, e atender a eventuais auditorias realizadas.

Solicita-se ao (à) pesquisador (a) o envio a esta CEUA, de relatórios parciais sempre quando houver alguma alteração no projeto, bem como o relatório final.

Ijuí, 05 de novembro de 2013.

Prof. Daniel Curvello de Mendonça Müller
Coordenador da CEUA/UNIJUÍ

Comissão de Ética no uso de Animais CEUA UNIJUÍ