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***Drosophila melanogaster:* UM MODELO ALTERNATIVO PARA O ESTUDO DOS EFEITOS BIOQUÍMICOS E COMPORTAMENTAIS CAUSADOS PELA EXPOSIÇÃO AO MANGANÊS DURANTE O DESENVOLVIMENTO EMBRIONÁRIO**

São Gabriel
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Dissertação apresentada ao programa de Pós-Graduação _Stricto Sensu_ em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do título de Mestre em Ciências Biológicas.

Orientador: Profª. Drª. Thaís Posser

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AVALIAÇÃO DOS EFEITOS DA EXPOSIÇÃO *IN VIVO* AO MANGANÊS DURANTE O DESENVOLVIMENTO EMBRIONÁRIO DE *Drosophila melanogaster* SOBRE O SISTEMA ANTIOXIDANTE E VIAS DE SINALIZAÇÃO CELULAR
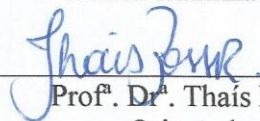
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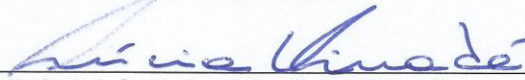Dissertação defendida e aprovada em: 30/07/2014.
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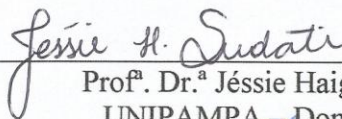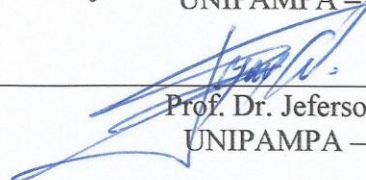Profª. Drª. Thaís Posser
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RESUMO

O Manganês (Mn) é um elemento essencial para o ser humano. Sua absorção ocorre através da dieta, onde os níveis absorvidos são homeostaticamente controlados pelo sistema de excreção biliar; ou pela inalação. A exposição ao metal durante o período de desenvolvimento embrionário ou primeiros estágios de vida neonatal está associada a déficits cognitivos e comportamentos hiperativos. A exposição ao Mn nesse período é particularmente crítica uma vez que a aborção do metal é maior. Nesse sentido, o presente estudo buscou identificar as alterações bioquímicas e comportamentais geradas em *Drosophila melanogaster* pela exposição ao Mn durante o período de desenvolvimento embrionário. Para a pesquisa, *D. melanogaster* foram tratadas com cloreto de manganês ($MnCl_2$) nas concentrações de 0,1mM, 0,5mM e 1mM ao longo de todo o ciclo de desenvolvimento. Ao eclodirem, as moscas foram utilizadas nos ensaios bioquímicos, comportamentais e de dosagem de metais. Nas concentrações de 0,5mM e 1mM as moscas foram significativamente mais rápidas que o grupo controle ($p < 0,005$), avaliado pelo teste de Geotaxia Negativa, além de apresentarem níveis aumentados de Mn ($p < 0,0001$) à medida em que houve uma diminuição nos níveis de Ca, Fe, Cu, Zn e S. A viabilidade celular avaliada no grupo 1mM ($p < 0,05$) esteve diminuída, enquanto que observou-se um aumento na produção de espécies reativas de oxigênio através do teste de DCF-DA, nos grupos 0,5mM e 1mM ($p < 0,05$). Com relação à expressão de genes responsivos ao estresse celular, 1mM de Mn aumentou significativamente a expressão de RNAm de CAT, SOD ($p < 0,005$) e HSP83 ($p < 0,0001$), conforme a técnica de qPCR-RT. Em contrapartida, a atividade das enzimas catalase e superóxido dismutase não foram alteradas nos grupos 0,5mM e 1mM. Significativo aumento na atividade das enzimas tioredoxina redutase ($p < 0,05$) e glutationa-S-transferase ($p < 0,005$ e $p < 0,0001$, respectivamente) foram observados. Com relação à modulação da fosforilação da via de transdução de MAPKs, Mn não alterou a fosforilação de ERK e JNK, entretanto, em 1mM o Mn inibiu a fosforilação de $p38^{MAPK}$. Nossos dados indicam um possível processo adaptativo à intoxicação com Mn em *D. melanogaster* levando a aumento de defesas antioxidantes e inibição de via de sinalização associadas com o processo apoptótico. Um desequilibrio na homeostasia de metais essenciais, associado a aumentada atividade locomotora também foi observado, fato este que sugere estar associado à atividade hiperativa em crianças.

Palavras-Chave: estresse oxidativo, neurotoxicidade, manganês, MAPK, *Drosophila melanogaster*

ABSTRACT

Manganese (Mn) is an essential element for humans. It is absorbed through the diet, and the rate of absorption is homeostaticaly controlled by biliary excretion; or inhalation. Exposure to the metal during embryonic development and childhood phases are associated with cognitive deficits and hyperactive behaviors. These periods are particularly critical once occurs a higher metal absorption. The present study aimed to identify biochemical and behavioral alterations in *Drosophila melanogaster* caused by Mn-exposure during the period of embryonic development. Fruit flies *D. Melanogaster* were treated with manganese chloride ($MnCl_2$) at concentrations 0.1mM, 0.5mM and 1mM over the developmental period. After eclosion, the flies were used in biochemical and behavior assays and for metal content determination. At 0.5mM and 1mM concentrations, the flies were significantly faster than the control group ($p<0.005$), evaluated by Negative Geotaxis assay, besides, they exhibited an improvement in Mn levels ($p<0.0001$) while than a decreasing in Ca, Fe, Cu, Zn and S levels. Cell viability decreased at 1mM group whereas there was an improvement in reactive oxygen species production evaluated by DCF-DA assay, in 0.5mM and 1mM groups ($p<0.05$). Expression of cellular stress responsive genes was investigated by the real time PCR assay. At 1mM Mn increased the mRNA CAT, SOD ($p<0.005$) and HSP83 ($p<0.0001$) expression. On the other hand, catalase and superoxide dismutase enzyme activity was not altered at 0.5 and 1mM groups, while that a significant increasing in thioredoxin reductase ($p<0.05$) and glutatione-S-transferase ($p<0.005$ and 0.0001, respectively) enzymes activity were observed. Concerning the phosphorylation of MAPK signaling pathway, Mn treatment did not alter ERK and JNK phosphorylation, but at 1mM an inhibition on $p38^{MAPK}$ phosphorylation occurred. Our results indicate a possible adaptive response to Mn intoxication in *D. melanogaster* represented by an enhancement in antioxidant defenses and inhibition of signaling pathways associated with apoptotic process. An unbalance of essential metals homeostasis, associated with an improved locomotor activity was observed which could be associated with hyperactive activity reported for children.

Keywords: oxidative stress, neurotoxicity, manganese, MAPK, *Drosophila melanogaster*

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MANUSCRITO

LISTA DE ABREVIATURAS

ASK1	Quinase sinalizadora de apoptose 1
CAT	Catalase
DA	Dopamina
DCF	2'-7'-diclorofluoresceína
DCF-DA	2'-7'-diclorofluoresceína-diacetato
DMT1	Proteína transportadora de metal divalente
ERK	Quinase regulada por sinal extracelular
ERO	Espécies reativas de oxigênio
GST	Glutationa-S-transferase
H_2O_2	Peróxido de hidrogênio
JNK	Quinase-c-Jun-amina-terminal
MAPK	Proteínas quinases ativadas por mitógeno
mg	Miligrama
MKKK	Quinase da proteína quinase ativada por mitógeno
MKP	Fosfatase da proteína quinase
mL	Mililitro
mM	Milimolar
MMT	metil ciclopentadienil manganês tricarbonil
Mn	Manganês
Mn_3O_4	Tetróxido de manganês
$MnCl_2$	Cloreto de manganês
MnSOD	Manganês-superóxido dismutase
MTT	3-(4,5-dimetiltiazol-il)-2,5-difenil brometo de tetrazolina
$OH^{\cdot}$	Radical hidroxila
$P38^{MAPK}$	Proteína quinase ativada por mitógeno de 38Kda
Pb	Chumbo
PCR	Reação em cadeia de polimerase
SDS	Dodecil Sulfato de Sódio
SDS-PAGE	Eletroforese unidimensional em gel de poliacrilamida e SDS
SLC39	Família carreadora de soluto SLC39
SOD	Superóxido dismutase

TH	Tirosina hidroxilase
TNF-α	Fator de necrose tumoral α
TrxR	Tioredoxina redutase
V-ATPase	Enzima ATPase do tipo V
Zn	Zinco

SUMÁRIO

1 INTRODUÇÃO

1.1 Manganês: essencialidade e toxicidade

O manganês (Mn) é um metal de transição situado na família 7 da Tabela Periódica. É o quinto metal e o décimo segundo elemento mais abundante da crosta terrestre. Está naturalmente presente em rochas, solo, água e alimentos. Possui número atômico 25 e massa atômica da ordem de 54,94 unidades de massa atômica. Apresenta números de oxidação bastante variáveis que vão desde -3 até +7, porém as principais formas de absorção através da dieta se dá nas formas de cátions bivalente e trivalente (revisado por SANTAMARIA, 2008; ROCHA; AFONSO, 2012; IUPAC, 2013).

É um elemento essencial para os seres humanos, sendo encontrado em todos os tecidos. É necessário para o metabolismo normal de aminoácidos, lipídios, proteínas e carboidratos. Além disso, é constituinte de enzimas, tais como as metaloproteínas Mn-superóxido dismutase (MnSOD), encontrada nas mitocôndrias, responsável por catalisar a geração de peróxido de hidrogênio (H_2O_2) a partir de radicais superóxido; e glutamina sintetase, enzima ATP-dependente expressa por astrócitos e oligodentrócitos, que catalisa a reação de síntese de glutamina a partir do glutamato recaptado da fenda sináptica e amônia (revisado por TAKEDA, 2003; revisado por ASCHNER; ASCHNER, 2005; SIEGEL et al., 2006; revisado por BARBOSA et al., 2010).

A dieta é a maior fonte de absorção do Mn nos seres humanos. Aproximadamente 3 a 5% do Mn ingerido é absorvido pelo intestino, o restante é excretado através das fezes. A absorção elevada de Mn através do trato gastrointestinal, de forma que possa ser tóxico, é rara, já que se for ingerido em excesso é metabolizado pelo fígado e excretado por meio da bile (MERGLER, 1999; revisado por AU et al., 2008).

Um fator que interfere na absorção através do trato gastrointestinal é a idade do indivíduo. É reconhecido que durante o período neonatal os níveis de Mn absorvidos são maiores, já que o sistema de excreção biliar ainda não está completamente desenvolvido. Isso faz com que mais Mn se acumule no cérebro ou em outros tecidos (revisado por ASCHNER; ASCHNER, 2005).

No entanto, a absorção de Mn através do trato respiratório é mais eficiente, no sentido de contribuir para a acumulação do metal no cérebro, dependendo do tamanho das partículas. Essas partículas inaladas são absorvidas nos alvéolos pulmonares e vão direto para o cérebro

através da circulação sanguínea, não passando pelo controle homeostático do fígado (revisado por DOBSON et al., 2004; ROELS et al., 2012).

A exposição crônica a altos níveis de Mn é associada a distúrbios no sistema nervoso central que levam a alterações cognitivas e comportamentais (MERGLER; BALDWIN, 1997; revisado por ASCHNER; ASCHNER, 2005; revisado por AU et al., 2008; revisado por SANTAMARIA, 2008). Dentre os sintomas do Manganismo, como é chamada a patologia associada à intoxicação com Mn, citam-se: tremor, rigidez muscular, dificuldade para manter o equilíbrio, fadiga excessiva, perda de memória, comportamento impulsivo ou violento e instabilidade emocional (MERGLER et al., 1994; revisado por DOBSON et al., 2004; revisado por ASCHNER et al., 2007). Esses sintomas estão relacionados com danos nos neurônios dopaminérgicos, associados com o controle motor (ERIKSON et al., 2005).

A super-exposição ao Mn pode ocorrer através da ingestão de água com altas concentrações do metal, através da exposição ocupacional em minerações, fabricação de ligas metálicas, produção de baterias e contato com fungicidas como o Maneb e o Mancozeb. Além disso, alguns países utilizam o metil ciclopentadienil manganês tricarbonil (MMT) como um aditivo à gasolina, fazendo com que a liberação desse elemento no ar seja aumentada (MERGLER; BALDWIN, 1997; MERGLER, 1999).

A exposição crônica a fungicidas contendo etileno-bis-ditiocarbamato tem sido associada a perdas neurocognitivas e Doença de Parkinson em humanos (DEBBARH et al., 2002). A exposição ao Maneb, um dos fungicidas desse grupo que contém Mn em sua estrutura, cujo estado de oxidação se dá na forma de cátion bivalente, tem sido relacionada com o desenvolvimento de sintomas semelhantes aos da Doença de Parkinson em agricultores (MECO et al., 1994). Já o Mancozeb, outro fungicida desse grupo, cuja estrutura é semelhante ao do Maneb, mas que além do Mn possui zinco (Zn) em sua constituição, mostrou ser tóxico a neurônios dopaminérgicos e gabaérgicos após a exposição *in vitro*, sendo possível relacionar essa toxicidade à danos na cadeia respiratória (DOMICO et al., 2006).

O MMT ($C_9H_7MnO_3$) é um derivado orgânico do Mn, usado como um anti-detonante adicionado à gasolina (revisado por COOPER, 1984). É usado no Canadá desde 1976. Sua combustão leva à formação de óxidos de Mn, principalmente o tetróxido de manganês (Mn_3O_4) (ENVIRONMET CANADA, 1987). Partículas de Mn_3O_4 emitidas na atmosfera tem um diâmetro menor que 0,4μm, tendo sido sugerido que uma das principais fontes de contaminação ambiental e exposição ao Mn inorgânico em áreas urbanas se dá pela combustão do MMT da gasolina (MENA, 1980; ENVIRONMET CANADA, 1987; DAVIS et

al., 1988). No entanto, pouco é conhecido sobre os potenciais riscos à população humana relacionados a adição do MMT à gasolina (LORANGER; ZAYED, 1995).

Há alguns anos o MMT também é utilizado como aditivo à gasolina nos Estados Unidos, sendo estimado que 8% dos níveis de Mn_3O_4 presentes no ar das cidades são derivados da combustão do MMT (WHO, 1999).

Uma vez absorvido, o Mn tende a se acumular nas regiões cerebrais ricas em dopamina, como corpo estriado, globo pálido e substância nigra (revisado por MARTINEZ-FINLEY et al., 2013). Vários são os mecanismos envolvidos com o transporte do Mn através da barreira hematoencefálica, dentre estes: proteína transportadora de metal divalente (DMT1), receptores de transferina, receptor ionotrópico de glutamato, canais de Ca^{2+} voltagem ou peptídeos-dependentes e através da proteína ZIP8, membro da família carreadora de soluto SCL39 (SLC39) (ASCHNER; GANON, 1994; revisado por DOBSON et al., 2004; revisado por ASCHNER et al, 2007; revisado por AU et al, 2008; revisado por MARTINEZ-FINLEY et al., 2013). Estes mecanismos estão ilustrados na figura 1.

Do ponto de vista bioquímico, a toxicidade do Mn é mediada pela auto-oxidação de dopamina, produção de radicais livres, diminuição dos mecanismos de defesa antioxidante e alterações na função mitocondrial (revisado por MARTINEZ-FINLEY et al., 2013).

Pouco é conhecido sobre qual concentração de Mn pode ser considerada tóxica aos seres humanos, uma vez que a maior parte dos estudos epidemiológicos avalia indicadores tais como concentração de Mn no cabelo (MENEZES-FILHO et al., 2011; BOUCHARD et al., 2007; TORRES-AGUSTÍN et al., 2013), no sangue (MERGLER et al., 1994; WASSERMAN et al., 2006; MENEZES-FILHO et al., 2011; TORRES-AGUSTÍN et al., 2013) e na urina (MERGLER et al., 1994) e não os níveis de Mn aos quais esses sujeitos estão expostos. No entanto, a National Academy of Sciences (2001) recomenda que a ingestão diária adequada seria de 2,3 e 1,8 mg/dia para homens e mulheres adultos, respectivamente. Considerando prévios estudos, esta mesma agência indica que um nível máximo de ingestão de Mn tolerável seria de 11 mg/dia para adultos, tendo em vista que não são conhecidos efeitos tóxicos relacionados a ingestão de quantidades inferiores a esse valor.

Figura 1 - Esquema representado os diversos mecanismos de transporte de Mn através da barreira hematoencefálica
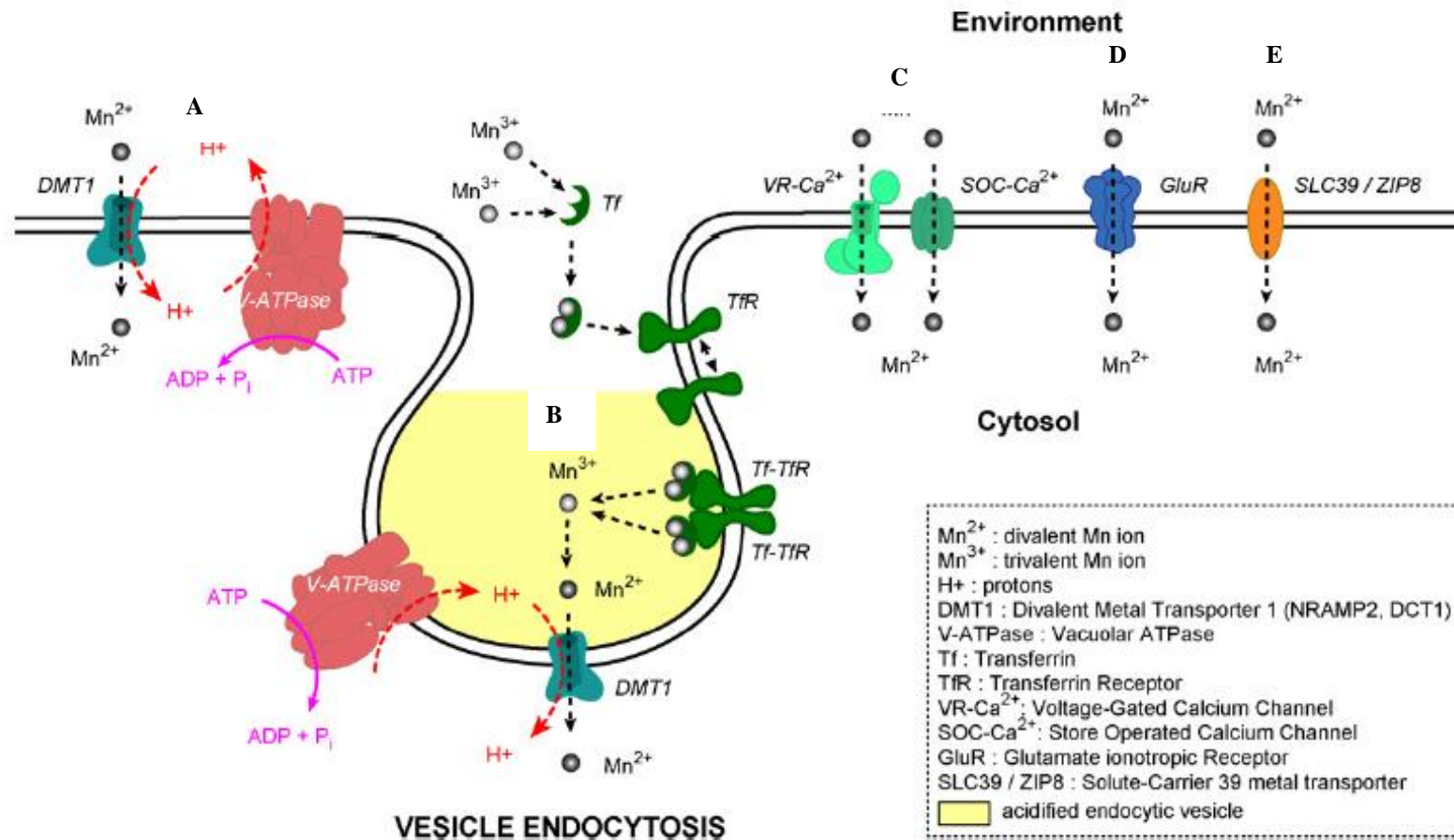
DMT1 é um transportador do Mn^{2+}, é ativado por um próton produzido pela hidrolização de ATP pela enzima ATPase do tipo V (V-ATPase) **(A)**. O transporte de prótons gerados via V-ATPase é responsável pela acidificação de vesículas endocíticas, isso leva à liberação dos íons Mn^{3+} que foram carreados via transferina e ligaram-se aos receptores de transferina. Uma vez liberados são convertidos em íons Mn^{2+} que podem ser transportados via DMT1 **(B)**. Outros mecanismos pelos quais o Mn^{2+} pode atravessar a membrana hematoencefálica são os canais de Ca^{2+} voltagem ou peptídeo-dependentes **(C)**, os receptores ionotrópicos do glutamato **(D)** ou proteínas ZIP8, membros da família carreadora de soluto 39 (SLC39) **(E)**.

Fonte: adaptado de Au et al, 2008.

1.2 Exposição ao manganês durante o período de desenvolvimento embrionário

Durante o período de desenvolvimento embrionário, o sistema nervoso é mais suscetível a insultos causados por metais. Assim como o chumbo (Pb), é sabido que o Mn é capaz de atravessar a placenta ou ser excretado através do leite materno (BETHARIA; MAHER, 2012). Dentre os efeitos adversos causados pela exposição de crianças ao Mn, o déficit cognitivo e comportamentos hiperativos são bem reportados pela literatura (revisado por MENEZES-FILHO et al., 2009; MENEZES-FILHO et al., 2011; ROELS et al., 2012; TORRES-AGUSTÍN et al., 2013).

Estudo epidemiológico desenvolvido por Takser et al. (2003), que acompanhou mulheres grávidas e os seus filhos até completarem seis anos de idade, sugeriu que altos níveis de retenção de Mn durante o período embrionário podem afetar o desenvolvimento psicomotor das crianças. Outro estudo que avaliou a exposição pré-natal ao Mn e os efeitos causados em crianças com testes até 54 meses após o nascimento, indicou que crianças com altos níveis de exposição pré-natal ao Mn apresentaram comportamentos mais impulsivos, agressivos, hiperativos, além de desatenção e desobediência (ERICSON et al., 2007).

Em estudos com animais, Pappas et al. (1997) expuseram fêmeas de ratos e seus filhotes ao Mn diluído na água, desde a concepção até 30 dias após o nascimento. O grupo tratado com uma concentração de 10mg/mL aumentou em 2,5 vezes a concentração de Mn no córtex cerebral e apresentaram comportamento hiperativo nos testes realizados nos animais com 17 dias de vida.

1.3 Relação do Manganês com o Estresse Oxidativo

Radicais livres são átomos ou moléculas que contêm um número ímpar de elétrons na sua última camada. São formados através de reações de óxido-redução. Eles são altamente reativos e estão relacionados a várias doenças como arterosclerose, câncer, doenças neurodegenerativas e também envelhecimento (revisado por ANDERSON, 1996; revisado por FERREIRA; MATSUBARA, 1997).

A mitocôndria, por meio da cadeia transportadora de elétrons, é a principal fonte geradora de radicais livres. Além disso, fatores exógenos como xenobióticos, radiações ionizantes, metais pesados, tabagismo e ingestão de álcool, também contribuem para a geração dos mesmos. O sistema antioxidante, composto por enzimas ou outras substâncias de

origem dietética como vitamina A, vitamina C, vitamina E e licopeno, tem a função de inibir ou reduzir os danos causados pelos radicais livres (revisado por BARBOSA et al., 2010).

O estresse oxidativo acontece quando existe um desequilíbrio entre os fatores pró-oxidantes e os antioxidantes. Dentre as consequências relacionadas ao estresse oxidativo citam-se: danos no DNA, proteínas, membranas lipídicas ou morte celular, por necrose ou apoptose (revisado por ANDERSON, 1996; HALLIWELL; WHITEMAN, 2004; revisado por BARBOSA et al., 2010).

O Mn^{2+}, assim como outros cátions bivalentes (Fe^{2+}, Zn^{2+} e Cu^{2+}), é conhecido pela sua capacidade de produzir H_2O_2 e radicais hidroxila ($OH^\bullet$) através da Reação de Fenton. Essas espécies reativas de oxigênio (ERO) estão envolvidas com o processo de disfunção mitocondrial, que leva à liberação de fatores pró-apoptóticos, levando à morte celular (RÍO; VÉLEZ-PARDO, 2004).

O Mn é capaz de inibir complexos da cadeia respiratória, levando a uma diminuição no potencial de membrana mitocondrial e consequentemente aumento na produção de ERO. Neste sentido, pode-se considerar que o estresse oxidativo é um dos reconhecidos mecanismos de toxicidade do Mn (ZHANG et al., 2004; GIORDANO et al., 2009; revisado por KARKI et al., 2013; revisado por FARINA et al., 2013).

1.4 O sistema de sinalização celular (MAPK)

As proteínas quinases ativadas por mitógenos (MAPK) são importantes proteinas evolutivamente conservadas nos eucariotos. As mais estudadas proteínas dessa família são a quinase regulada por sinal extracelular (ERK), quinase-c-Jun-amina-terminal (JNK) e proteína quinase ativada por mitógeno de 38 KDa ($p38^{MAPK}$). Elas conectam os receptores de membrana com mecanismos regulatórios do interior da célula. Respondem ao estresse físico e químico, controlando a sobrevivência e adaptação da célula. Em resposta a estímulos extracelulares elas regulam cascatas de sinalização celular que incluem a regulação da expressão de alguns genes, diferenciação, proliferação, sobreviência e morte celular (CHANG; KARIN, 2001, revisado por CHEN et al., 2001; revisado por BOGOYEVITCH; COURT, 2004; revisado por AOUADI et al., 2006). Uma variedade de fatores, tais como exposição à metais, estresse hídrico e térmico, ERO ou espécies reativas de nitrogênio podem contribuir para a ativação das MAPKs (revisado por CHU et al., 2004).

JNK ½ são proteínas envolvidas em diversas doenças como a diabetes, arterosclesose, doença de Parkinson e doença de Alzheimer. Têm sido muito estudadas por relacionarem-se

ao estresse celular e a morte celular apoptótica. Já ativação da proteína p38^{MAPK} no sistema nervoso é considerada uma etapa na resposta ao estresse neuronal, essa proteína está envolvida com a resposta inflamatória, apoptose e ciclo celular (revisado por MIELKE; HEDERGEN, 2000; revisado por AOUADI et al., 2006).

Estudos prévios realizados com ratos que receberam injeções intraperitoneais de MnCl$_2$ por cinco dias após o 8º dia de nascimento, mostraram que o Mn é responsável por modular a fosforilação de ERK ½, mas não altera a fosforilação de JNK ½ e p38^{MAPK} (CORDOVA et al., 2012). É postulado que a ativação das MAPKs pelo Mn se dá pela ativação de quinases das proteínas quinases ativadas por mitógenos (MKKKs), que estão acima na cascata de sinalização das MAPKs, além da inativação de fosfateses das proteínas quinases (MPKs) (CRITTENDEN; FILIPOV, 2011). Logo, a cascata de MAPKs pode ser considerada importante alvo associado à toxicidade deste metal.

1.5 A *Drosophila melanogaster* como modelo de estudo em toxicologia

A *Drosophila melanogaster,* também conhecida como mosca da fruta teve um papel central no desenvolvimento da biologia ao longo do século XX. Foi usada inicialmente como organismo conveniente para estudo de teorias evolutivas. Posteriormente, os estudos realizados por Morgan com este organismo permitiram estabelecer as bases do conhecimento da genética moderna (ARIAS, 2008).

A *Drosophila melanogaster* é um artrópode pertencente a sub-espécie de insetos dípteros *Drosophilidae.* Foi o primeiro organismo modelo utilizado em estudos experimentais com células eucarióticas. Sua anatomia característica, tais como presença de olhos compostos, cerdas e asas, permite o estudo de fenótipos de neurodegeneração sem afetar a sobrevivência do organismo (revisado por HIRTH, 2010). O gênero *Drosophila* possui aproximadamente 1500 espécies diferentes com características variadas quanto à aparência, comportamento e hábitat. Dentre estas, a espécie mais utilizada em estudos como organismo modelo é a *Drosophila melanogaster,* um pequeno inseto com aproximadamente 3mm de comprimento. Esta mosca tende se acumular em torno de frutas que estão em processo de putrefação (revisado por PARVATHI et al., 2009).

O desenvolvimento embrionário da *Drosophila* é dependente da temperatura ambiente, durando de 10 a 15 dias. O ciclo de vida da mosca, representado na figura 2, compreende o ovo, estágio larval, pupa e o estágio adulto (revisado por PARVATHI et al., 2009).

Figura 2 - Ciclo de vida da *Drosophila melanogaster*
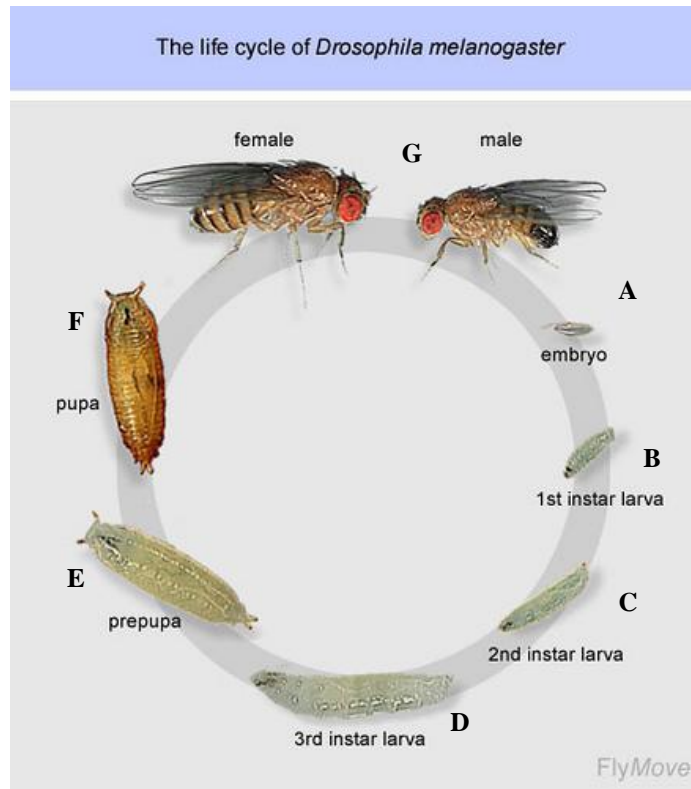
O ovo possui aproximadamente 0,5 milímetro. Pode ser posto pela fêmea logo após a fecundação ou pode ficar retido no útero durante os primeiros estágios do desenvolvimento embrionário (**A**). O estágio larval compreende três ínstares. A larva é muito ativa e alimenta-se vorazmente do meio em que se encontra. Ao final do terceiro estágio a larva tem aproximadamente 4,5 milímetros (**B, C** e **D**). Durante a fase de pupa ocorre a destruição de órgãos e tecidos larvas e a organização das estruturas adultas (**E** e **F**). Quando a metamorfose está completa a mosca emerge da pupa, inicialmente tem a coloração clara, mas com o passar de algumas horas adquirem a coloração mais escura típica do estágio adulto. A *Drosophila melanogaster* apresenta dimorfismo sexual, a fêmea é maior que o macho; seu abdômen é mais alongado. Outra forma de diferenciação são marcas no abdômen, enquanto a fêmea apresenta bandas escuras e claras intercaladas, o macho tem as últimas bandas fusionadas (**G**).
Fonte: adaptado de Parvathi et al., 2009.

Durante as duas últimas décadas, a *Drosophila melanogaster* tem sido utilizada com modelo de estudo para doenças humanas neurodegenerativas, tais como doença de Parkinson, Alzheimer, doença de Huntington, esclerose lateral amiotrófica, dentre outras doenças neuromotoras (revisado por CHAN; BONINI, 2000; revisado por SANG; JACKSON, 2005; revisado por HIRTH, 2010). Dentre as razões que justificam o uso desse modelo animal citam-se:

- A grande homologia entre os genes humanos e os genes das Drosophilas que permitem estudos para compreender o papel de determinadas proteínas em patologias específicas (revisado por PARVATHI et al., 2009; revisado por PANDEY; NICHOLS, 2011). Alguns desses genes estão sumariados na tabela 1.

- A *Drosophila* apresenta um complexo sistema nervoso e comportamentos complexos como aprendizagem e memória (revisado por CHAN; BONINI, 2000).

- Possui um rápido ciclo de vida, sua manipulação é relativamente fácil, tem baixo custo de manutenção e exigência de infraestrutura, sua alimentação é simples, além de estar livre de imposições éticas (revisado por PARVATHI et al., 2009; revisado por HIRTH, 2010).

Tabela 1 - Genes relacionados a doenças em humanos que possuem homólogos em *Drosophila melanogaster*

Proteína codificada pelo gene em humanos	Doença relacionada
Proteína precursora amilóide (APP)	Doença de Alzheimer
Presenilina 1 e 2 (PSN-1/2)	Doença de Alzheimer
Tau	Doença de Alzheimer
Apolipoproteína (APOe4)	Doença de Alzheimer
α-sinucleína	Doença de Parkinson
Parkin	Doença de Parkinson
Glicocerebrosidase (GBA)	Doença de Parkinson
Gama polimerase (POLG)	Doença de Parkinson
Cu/ Zn Superóxido Dismutase 1	Esclerose Lateral Amiotrófica
Senataxina (SETX)	Esclerose Lateral Amiotrófica
Proteína de membrana vesícula-associada (VAPB)	Esclerose Lateral Amiotrófica
Proteína de sobrevivência de neurônios motores ½ (SMN-½)	Atrofia Muscular Espinhal

Fonte: Chan, Bonini (2000); Sang, Jackson (2005); Hirth (2010)

1.6 Contextualização da pesquisa

Embora os efeitos neurotóxicos do Mn *in vivo* tenham sido bem demonstrados, utilizando em sua maioria, mamíferos como animais modelo, (KEEN et al.*,* 1999; ZHANG et al., 2004; revisado por AOUADI et al.*,* 2006; GUILARTE et al., 2006; revisado por SANTAMARIA, 2008; MORELLO et al.*,* 2008; GIORDANO et al.*,* 2009) há uma lacuna no que tange ao conhecimento dos efeitos de sua exposição ao longo do período de desenvolvimento embrionário. Estudos prévios demonstraram que a exposição ao Mn na vida adulta de *Drosophila melanogaster* causou déficits comportamentais e diminuição da viabilidade, sendo este modelo portanto sensível e representativo de efeitos neurotóxicos associados à exposição ao Mn (BONILLA-RAMIREZ et al., 2011).

Em geral, estudos que avaliam os efeitos neurotóxicos do Mn abordam a exposição ocupacional como um dos principais fatores de risco (MERGLER; BALDWIN, 1997; MERGLER, 1999; revisado por ROTH, 2006). Outros estudos relatam que o déficit cognitivo e alterações comportamentais, como comportamento hiperativo estão relacionados à exposição ao Mn durante a infância (WASSERMAN et al., 2006; BOUCHARD et al., 2007; ERICSON et al., 2007; FARIAS et al., 2010; HENN et al., 2010; MENEZES-FILHO et al., 2011; ROELS et al., 2012; MARTINEZ-FINLEY et al., 2013; TORRES-AGUSTÍN et al., 2013). Porém, até o momento não estão completamente elucidados os mecanismos relacionados a essa neurotoxicidade gerada pela exposição ao metal durante o período neonatal e da infância.

Assim sendo, o presente trabalho foi desenvolvido a fim de contribuir com a elucidação dos mecanismos bioquímicos relacionados à neurotoxicidade deste metal, absorção de Mn e outros metais essenciais, além de demonstrar de forma inédita possíveis consequências bioquímicas e comportamentais causadas na *Drosophila melanogaster* após a exposição embrionária ao metal.

2 OBJETIVOS

2.1 Objetivo Geral

Elucidar os mecanismos de ação do Mn em *Drosophila melanogaster* em consequência à exposição ao metal durante o período de desenvolvimento embrionário e propor um novo modelo animal para o estudo da toxicidade associada à exposição ao Mn.

2.2 Objetivos Específicos

- Determinar as alterações locomotoras apresentadas pela *Drosophila melanogaster* como consequência à exposição ao Mn durante o período de desenvolvimento embrionário.

- Investigar variações nos níveis de Mn e outros elementos essenciais no corpo de moscas expostas ao metal.

- Analisar mudanças ocorridas no sistema de defesa antioxidante da *Drosophila melanogaster* avaliando a influência da exposição ao Mn na geração de ERO e alterações na viabilidade celular.

- Avaliar a modulação de genes responsivos ao estresse celular.

- Avaliar a modulação da fosforilação de MAPKs após a exposição ao Mn.

3 MANUSCRITO

Este manuscrito está disposto na forma como foi submetido e aprovado para publicação no periódico Experimental and Clinical Sciences International Online Journal for Advances in Science – EXCLI Journal (ISSN: 1611-2156). O mesmo contempla os materiais e métodos utilizados no decorrer dessa pesquisa, assim como apresenta os resultados e discussões pertinentes a esta dissertação.

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Drosophila melanogaster – an alternative model for studying behavioral and biochemical effects of Manganese exposure during embryonic development

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Abstract

Embryonic and neonatal periods are especially susceptible to metal exposure. Manganese (Mn) is an essential element but in excess it can induce toxicity. In this study we used *Drosophila melanogaster* as a model to investigate biochemical and behavioral alterations upon Mn exposure during embryonic development. Flies were treated with standard medium supplemented with manganese chloride at 0.1 mM, 0.5 mM and 1mM from egg to adult stage. At 0.5 mM and 1 mM Mn concentrations, newly ecloded flies showed a significant enhance in locomotor activity, assayed by negative geotaxis behavior, than the control group ($p<0.005$). In addition, a significant increase in Mn levels ($p<0.0001$) was observed, while Ca, Fe, Cu, Zn and S levels were significantly decreased. A significant drop in cell viability occurred in flies exposed to 1mM Mn, whereas there was an induction in reactive oxygen species production at 0.5mM and 1mM Mn concentrations ($p<0.05$). At 1mM, Mn increased the mRNA of catalase, superoxide dismutase ($p<0.005$) and Hsp83 ($p<0.0001$) expression, without altering Catalase and Superoxide Dismutase activity. The activity of Thioredoxin reductase and Glutatione-S-transferase enzymes was augmented. Mn treatment did not alter ERK and JNK1/2 phosphorylation, but at 1mM an inhibition on p38^{MAPK} phosphorylation occurred. Together these data suggest a mechanism of adaptation in response to presence of Mn in the embryonic life of fruit flies.

Introduction

Manganese (Mn) is an essential element to living organisms. It is the twelfth most abundant element in earth´s crust and is present in rocks, water, soil and food, normally associated with other elements (Santamaria 2008; Farina et al. 2013). Environmental and occupational exposure to Mn may occur by contact with fungicides such as Maneb, Manconzeb, methylcyclopentadienyl manganese tricarbonyl (MMT), an anti-knock agent in gasoline, Mn-ore mining, Mn alloy production and dry alkaline battery manufacturing (Mergler and Baldwin 1997; Mergler 1999).

Dietary ingestion is the main source of Mn for humans and Mn absorption takes place mostly in the gastrointestinal tract where it is controlled homeostatically in the intestinal wall (Au et al. 2008). The developmental stage influences Mn absorption which is improved during the neonatal period. Furthermore during neonatal period biliary excretion is poorly developed, such fact may result in elevated concentrations of Mn in the brain or other tissues (Aschner and Aschner 2005).

Exposure to high levels of Mn may lead to pathological conditions, including neurodegeneration (Mergler et al. 1994). The mechanisms mediating Mn toxicity are complex and not completely understood. Some of them include: (1) Mn accumulation in astrocytes leading to disruption of their ability to promote neuronal differentiation and decreasing glutamate uptake by astrocytes (Erikson and Aschner 2003; Giordano et al. 2009); (2) Mn induced- loss of dopaminergic neurons (Stanwood et al. 2009); (3) Inhibition of respiratory chain complexes and induction of reactive oxygen species (ROS) production (Zhang et al. 2004; Sriram et al. 2010). In children, Mn exposure is associated with alterations on psychomotor and cognitive development; furthermore it has been described a positive correlation between Mn exposure and hyperactivity (Menezes-Filho et al. 2011; Roels et al. 2012; Torres-Agustín et al. 2013). The brain is especially susceptible to metal intoxication over embryonic development. It is known that Mn is able to cross the placenta and to be excreted in the maternal milk (Betharia and Maher 2012).

The use of alternative models in toxicological studies has been growing over the years. The fruit fly *Drosophila melanogaster* has served as a unique and powerful model for studies on human genetics and diseases. Although humans and flies are only distantly evolutionarily related, almost 75% of disease related genes in humans have functional orthologs in the fly (Deepa et al. 2009; Pandey and Nichols 2011). Moreover, the fast and external developmental cycle of this organism enable the study of toxicological effects of compounds

during developmental period. All these advantages make this model appropriated for studies related with metal toxicity (Bonilla-Ramirez et al. 2011; Paula et al. 2012) and human neurodegeneration (Hirth 2010).

Considering the embryonic development period as particularly sensitive to Mn exposure, in this work we aimed to investigate the behavior and biochemical alterations caused by Mn exposure during the embryonic development of *Drosophila melanogaster* focusing on antioxidant system and MAPK signaling pathways. The levels of Mn and major essential elements were determined as well.

Materials and methods

Reagents

Anti-phospho-p38 [MAPK], anti-phospho JNK, anti-phospho ERK, anti ERK and ß-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). EDTA (CAS 60-00-4), glycine (CAS 56-40-6), tris(hydroxymethyl) aminomethane (CAS 77-86-1) and ammonium persulfate (CAS 7727-54-0) were purchased from Serva (Heidelberg, Germany). L-Glutathione reduced (CAS 70-18-8), 1-chloro-2,4-dinitrobenzene (CAS 97-00-7), sodium orthovanadate (CAS 13721-39-6), manganese (II) chloride tetrahydrate (CAS 13446-34-9), ß-mercaptoethanol (CAS 60-24-2), methanol (CAS 67-56-1), tween 20 (CAS 9005-64-5), potassium phosphate dibasic (CAS 7758-11-4), potassium phosphate monobasic (CAS 7778-77-0), potassium bicarbonate (CAS 298-14-6), anti-rabbit imunoglobulin antibody, *N,N,N′,N′*-Tetramethylethylenediamine (CAS 110-18-9), quercetin (CAS 117-39-5), protease inhibitor cocktail for use with mammalian cell and tissue extracts, 5,5´dithiobis(2-nitrobenzoic acid) (CAS 69-78-3), 2´,7´-dichlorofluorescein diacetate (CAS 2044-85-1), glycerol (CAS 56-81-5), resazurin sodium salt (CAS 62758-13-8), triton x-100 (CAS 9002-93-1), sodium chloride (CAS 7647-14-5), albumin from bovine serum (CAS 9048-46-8), HEPES (CAS 7365-45-9) and ß-nicotinamide adenine dinucleotide 2´-phosphate reduced tetrasodium salt were obtained from Sigma Aldrich (St. Louis, MO, United States). Bis-acrylamide, hybond nitrocellulose, acrylamide (CAS 79-06-1), sodium dodecyl sulfate (CAS 151-21-3), boric Acid (CAS 10043-35-3) were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other reagents were commercial products of the highest purity grade available.

Animals

Drosophila melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were maintained at 25ºC on 12h light/ dark cycle in glass bottles containing 10 mL of standard medium (mixture of 39% coarse and 32% medium corn flour, 10% wheat germ, 14% sugar, 2% milk powder, 1% salt, 1% soybean flour, 1% rye flour, a pinch of methyl Paraben (99-76-3) and lyophilized yeast. All experiments were performed with the same strain, both genders.

Animal treatment

Adults flies were placed in 10 mL of standard medium supplemented with 3mL fresh solution (0.1 mM, 0.5 mM and 1 mM) of manganese chloride ($MnCl_2$). In control group the standard medium were supplemented with 3mL of ultrapure water. After ten days laying eggs the adults flies were removed. When eggs were newly ecloded, flies with 1 to 3 days old were used for all analysis. The $MnCl_2$ concentrations were based on previous studies (Bonilla-Ramirez et al. 2011).

Locomotor Assay

Locomotor activity was determined using the negative geotaxis assay as described by Bland et al. (2009) with minor modifications. Briefly, for each assay, individual flies (1-3 days old) were immobilized on ice and placed separately in a glass tube. The cooling anesthetizing is a method of immobilization that does not affect fly neurology (Deepa et al. 2009). After 15 minutes flies were gently tapped to the bottom of the tube and the time required for flies to climb up 8cm of the tube wall was recorded. Each fly was tested 4 times at 1 minute intervals. For each experiment, the climbing mean was calculated.

Metal Content Determination

Two hundred flies per group were washed thrice in ultrapure water and then dried on a filter paper in the incubator at 37ºC for 90 minutes. Flies samples were digested in closed vessels according to the procedure described previously by Bizzi et al. (2010). Samples (~ 70 mg) were transferred to quartz vessels, and 6 mL of HNO_3 3 mol L^{-1} closing and capping the

rotor, the vessels were pressurized with 7.5 bar of oxygen using the valve originally designed for pressure release after conventional acid sample digestion. Then, the rotor was placed inside the oven. A Microwave oven (Multiwave 3000 Microwave Sample Preparation System, Anton Paar, Graz, Austria) was used. The system was equipped with eight high-pressure quartz vessels (volume of 80 mL, maximum operational temperature and pressure of 280 ºC and 80 bar, respectively). Pressure was monitored in each vessel during all the runs. Microwave heating program was started by applying (1) 1000 W, with a ramp of 5 min; (2) 1000 W for 10 min; and (3) 0 W for 20 min (cooling step). After digestion, the pressure in each vessel was carefully released. The resulting solutions were transferred to polypropylene vials and diluted up to 25 mL with water. Determination of calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), Mn, sodium (Na), phosphorus (P), sulfur (S), and zinc (Zn) was carried out using an inductively coupled plasma optical emission spectrometer (Optima 4300 DV, PerkinElmer, Shelton, USA) with axial view configuration. A concentric nebulizer and cyclonic spray chamber were used. Argon 99.996% (White Martins, São Paulo, Brazil) was used for plasma generation, nebulization and as auxiliary gas. The instrumental parameters were carried out in according with previous work (Pereira et al. 2013). Two readings were averaged to give one value per biological replicate and expressed as a mean ($\pm$) standard deviation of the mean (SD). Metals levels were expressed relative to the weight of flies used for analysis (μg metal/ μg mass).

Cellular Viability

We measured cellular viability by two different methods. Firstly the cellular viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay following Sudati et al. (2013) with minor modifications. The analysis was performed in whole body of female flies. The flies were incubated in MTT for 60 min (37ºC), after MTT was removed and the sample was incubated in DMSO for 30 min (37ºC). The absorbance from formazan dissolution by addition of DMSO was monitored in a EnsPire[R] multimode plate reader (PerkinElmer, USA) at 540nm.

The second method used was the resazurin reduction assay. The method uses the indicator resazurin to measure the metabolic capacity of cells. Viable cells reduce resazurin into resorufin, a fluorescent compound (Franco et al. 2009). Groups of 40 flies were mechanically homogenized in 1mL 20mM Tris buffer (pH 7.0) and centrifuged at 1.000 RPM for 10 min at 4ºC. The supernatant was incubated in the Elisa plates with 20mM Tris buffer

(pH 7.0) and resazurin for two hours. The absorbance was recorded using EnsPire[R] multimode plate reader (PerkinElmer, USA) at 570nm.

DCF-DA Oxidation Assay

Groups of 20 flies were mechanically homogenized in 1mL 20mM Tris buffer (pH 7.0), and centrifuged at 1.000 RPM for 10 min, 4ºC. The supernatant was used to quantify 2'-7'-dichlorofluorescein diacetate (DCF-DA) oxidation as a general index of oxidative stress following Perez-Severiano et al. (2004). The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored at regular intervals ($_{ex}$488nm and $_{em}$530nm) in a EnsPire[R] multimode plate reader (PerkinElmer, USA).

Determination of gene expression by Real-time quantitative PCR (qPCR)

Real-time quantitative PCR (qRT-PCR) was performed according to the method described by Paula et al. (2012). The primers utilized are described in Table 1. All samples were analyzed as technical and biological triplicates with a negative control. Threshold and baselines were manually determined using the StepOne Software v2.0 (Applied Biosystems, NY). SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems, NY), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The GPDH gene was used as endogenous reference presenting no alterations in response to the treatment. For each well, analyzed in quadruplicate, a ΔC_T value was obtained by subtracting the GPDH C_T value from the C_T value of the interest gene (sequences of tested genes are represented in Table 1). The ΔC_T mean value obtained from the control group of each gene was used to calculate the $\Delta\Delta CT$ of the respective gene ($2^{-\Delta\Delta CT}$).

Enzyme Assays

For enzymes activity, groups of 40 flies were mechanically homogenized in 1mL 20mM HEPES buffer (pH 7.0), and centrifuged at 14.000 RPM for 30 min at 4ºC (Franco et al. 2009). The supernatant was used for determination of glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD) and thioredoxin reductase (TrxR).

The GST activity was assayed following the procedure of Jakoby and Habig (1981) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340nm. The reaction was conducted in a mix consisting of 100mM phosphate buffer (pH 7.0), 1mM EDTA, 1mM GSH and 2.5 mM CDNB. CAT activity was assayed following the clearance of hydrogen peroxide (H_2O_2) at 240nm in a reaction media containing 50mM phosphate buffer (pH 7.0), 0.5mM EDTA, 10mM H_2O_2, 0.012% TRITON x100 according to procedure of Aebi (1984). SOD activity assay following the procedure of Kostyuk and Potapovich (1989). The assay consists in the inhibition of superoxide-driven oxidation of quercetin by SOD at 406 nm. The complete reaction system consisted of 25mM phosphate buffer (pH 10), 0.25mM EDTA, 0.8mM TEMED and 0.05μM quercetin. TrxR activity was assayed following the procedure of Holmgren and Björnstedt (1995). The test is based on the redution of oxidized thioredoxin (Trx-S_2) to reduced thioredoxin [Trx-$(SH)_2$], using NADPH at 412nm in a reaction media containing 0.1M phosphate buffer (pH 7.0), 10mM EDTA, 5mM DTNB, $0.2mg^{-ml}$ BSA, 0.2mM NADPH. All enzyme activities were performed at room teperature (25 ± 1ºC) using a Thermo Scientific Evolution 60s UV-Vis spectrophotometer. The enzyme activities were expressed in milliunits per milligram of total protein content, which was quantified following Bradford (1976).

Western blotting

Quantification as mitogen-activated protein kinases (MAPKs) phosphorylation was performed by Western blotting according with Posser et al. (2009) with minor modifications. Groups of 40 flies were mechanically homogenized at 4ºC in 200μL of buffer (pH 7.0) containing 50mM Tris, 1mM EDTA, 20mM Na_3VO_4, 100mM sodium fluoride and protease inhibitor cocktail. The homogenate were centrifuged at 4000 RPM for 10min at 4ºC and the supernatants collected. After protein determination following Bradford (1976), 4% SDS solution, ß-mercaptoethanol and glycerol was added to samples to a final concentration of 100, 8 and 25%, respectively, the samples frozen until further analysis. Proteins were separated usins SDS-PAGE with 10% gels, and then electrotransferred to nitrocellulose membranes (Paula et al. 2012). Membranes were washed in tris-buffered saline with Tween (100mM tris-HCl, 0,9% NaCl and 0,1% Tween-20, pH 7.5) and incubated overnight at 4ºC with primary antibodies namely anti-phospho-p38^{MAPK}, anti-phospho JNK, anti-phospho ERK, anti ERK and anti ß-actin. Following incubation, membranes were washed in tris-

buffered saline with Tween and incubated for 1 h at 25ºC with anti rabbit- IgG secondary specific antibodies. Antibody binding was visualized using the ECL Western Blotting substrate Kit (Promega). Band staining density was quantified using the Scion Image software (Scion Image for Windows) and expressed as the percentage (%) of the control group (mean ± standard deviation of the mean). The values were normalized using total proteins (total ERK and ß-actin) using especific antibodies.

Statistical analysis

Statistical analysis was performed using a one-way ANOVA followed by Tukey´s post hoc test. Pearson's correlation test was applied for detection of significant statistical differences among the metals. Differences were considered statistically significant at p<0,05. GraphPad Prism 5 Software was used for artwork creation.

Results

Exposure to Mn causes hyperactive-like behaviors and alters metal levels in *Drosophila melanogaster*

The evaluation of climbing behavior performance by negative geotaxis showed that flies exposed to 0.5mM and 1mM of Mn reached the limit of columns significantly (p<0.005) faster than control (Figure 1).
Levels of Mn and other essential metals were measured in in *D. melanogaster* exposed to Mn subsequently to the treatments (whole body). At concentrations of 0.5 and 1 mM, the levels of Mn in flies improved almost two and three fold respectively when comparing with control group (Table 2), whereas Ca, Cu, Na and Zn levels decreased significantly in flies treated with Mn, when comparing to the control group. Statistically, a negative significant relationship between Mn uptake and levels of Ca (r = -0,7966), Fe (r = -0,6635), Cu (r = -0,8028), S (r = - 0,6018) and Zn (r = -0,9802) occurred in response to Mn treatment (Table 3).

Mn exposure during embryonic development decreased cellular viability and increased ROS production in flies

Cell viability was evaluated through two different tests, MTT and resazurin, in both it can be observed a significant drop in cell viability at higher concentration of metal, confirming the toxicity of Mn acting at cellular level (Figure 2 A and 2 B). Many factors have been implicated in Mn-induced neurotoxicity, among them the oxidative stress caused by dopamine oxidation or its ability in interfering with cellular respiration (Aschner and Aschner 2005). In this study, exposure to Mn lead to an increase in DCF-DA oxidation, a general index of oxidative stress from 0.5 mM (Figure 3).

Mn increased the CAT and SOD mRNA expression, but not alters its enzymatic activity

Expression of the mRNA for CAT, SOD and Hsp83 (an homolog of mammalian Hsp90) (Bandura et al. 2013) was analyzed by qRT-PCR using specific primers (Table 1) in flies treated with Mn 1 mM,

CAT and SOD mRNA expression was 250 % higher in treated flies than non-treated group whereas Hsp83 expression was 1000% higher in treated group than control (Figure 4). In parallel, it was quantified the levels of antioxidant enzymes TrxR, GST, SOD and CAT. TrxR and GST activity stimulated at concentrations of 0.5mM and 1mM groups (Figure 5) while that CAT and SOD activity showed no significant differences among the groups.

Mn exposure inhibited p38^{MAPK} phosphorylation

MAPKs phosphorylation levels were investigated in flies exposed to Mn. It was observed a 40% inhibition in p38^{MAPK} phosphorylation in flies exposed to Mn at 1mM while that phosphorylation levels of extracellular signal-regulated kinases (ERK) was unaltered. C-Jun-N-terminal Kinases 2 (JNK2) phosphorylation was inhibited as well, however, this effect was not statistically significant of control (Figure 6).

Discussion

In this study, we aimed to determine biochemical and behavioral alterations in *Drosophila melanogaster* in response to Mn exposure during embryonic development, thus contributing to extend the knowledge on the use of this alternative animal model on Mn-induced toxicity studies. It was proposed a new protocol of Mn exposure during the whole

developmental cycle of flies and a wide spectrum of behavioral and biochemical analysis was conducted after eclosion.

Mn is an essential element required in key biological processes; however, high levels of Mn are associated with neurological and neuropsychiatric disorders (Mergler 1999). The risk of Mn overexposure comes from both occupational and environmental sources (Mergler and Baldwin 1997). Mn intoxication, a syndrome known as Manganism, is characterized by an extrapyramidal dysfunction and neuropsychiatric symptomatology and is associated with prolonged occupational exposure to high concentrations of this metal. Classical symptoms include irritability, intellectual deficits, compulsive behaviors, tremors and cock-like walk (Mergler 1999; Roth 2006).

In rodents, Krishna et al. (2013) showed that adult mice exposed to Mn through the drinking water presented neurobehavioral deficits and glial activation related with Mn deposition in brain. Moreover, others studies demonstrated that Mn toxicity in rats is accompanied by increased cholesterol biosynthesis and impairments in neuronal function of the hippocampus, which is involved in learning and memory (Öner and Sentürk 1995; Sentürk and Öner 1996). It has been shown that Mn supplementation during the neonatal period of rats resulted in increased Mn concentrations in tissues leading to adverse effects on motor development and behavior (Tran et al. 2002).

Mn uptake is improved during neonatal period and biliary excretion, which has been suggested as a pathway which account for Mn elimination from the body is poorly developed in this stage (Aschner and Aschner 2005). Exposure to Mn during the embryonic and early post natal periods may result in increased levels of Mn in brain and other tissues as bone, liver, pancreas and kidney (Aschner and Aschner 2005; Roels et al. 2012). Higher levels of Mn retention during the *in utero* life may affect children´s psychomotor development (Takser et al. 2003). Possible adverse effects of Mn exposure on children´s health include cognitive deficits and hyperactive behaviors (Menezes-Filho et al. 2009; Torres-Agustín et al. 2013). Children exposed to high levels of Mn during fetal period were more impulsive, inattentive, agressive, defiant, disobedient, destructive and hyperactive (Ericson et al. 2007).

In our study, flies exposed to Mn at 0.5mM and 1mM presented an increased locomotor speed in the locomotor behavior test (assayed as negative geotaxis behavior) than the control group, pointing to a hyperactive-like behavior in *Drosophila melanogaster*. Furthermore, Mn levels were substantially increased in treated flies, being this result negatively correlated with Ca, Cu, Zn, Fe and S levels. This relationship may be in part associated with a competition of metals by the same mechanism of transport through the flies

cells. Facilitated diffusion, active transport, divalent metal transport 1 (DMT1), ZIP8 and transferrin (Tf)-dependent transport mechanisms are all involved in cellular Mn transportation (Aschner et al. 2007). Among these metal transport systems, DMT1 has a very broad substrate specificity and is likely to be the major transmembrane protein responsible for the uptake of a variety of divalent cations, including Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Pb^{2+} (Gunshin et al. 1997). In the flies, many proteins involved in the metabolism of biometals such as ferritin, transferrin, iron regulatory proteins, divalent metal transporter are expressed (Bonilla-Ramirez et al. 2011). In this context, Mn uptake is less frequently studied in comparison with other metals and the mechanisms related to Mn transport are considerably more complex, occurring in most part in the divalent (II) and oxide forms (Tebo et al. 2004). Mn^{2+} has the capacity to interact and /or compete with Ca (Dittman and Buchwalter 2010). In a study performed in the aquatic insect *Hydropsyche sparna,* Mn exposure decreased cadmium (Cd) and Zn accumulation. Furthermore, increased Ca concentrations significantly reduced Mn accumulation in the insect (Poteat et al. 2012). Dittmanand and Buchwalter (2010) suggested that Mn is also absorbed by Ca transporters in aquatic insects, where increasing ambient Ca concentrations downregulate Mn accumulation.

Other important aspect of this work was the negative correlation between Mn and Fe levels. Iron deficiency has been suggested as a possible contributing cause of attention deficit and hyperactivity disorder (ADHD) in children (Konofal et al. 2008). Concomitantly, children with ADHD presented elevated serum Mn concentrations when compared with respective matched controls (Konofal et al. 2008). Recent studies have suggested that Mn accumulates in dopaminergic neurons via the presynaptic dopamine transporter (DAT) and an altered functioning of the dopaminergic system has been well established in the etiology of ADHD (Farias et al. 2010).

Our results showed a decreased cell viability evidenced by two different methodologies and increased ROS generation in flies exposed to Mn during development. Previous work by our group in PC12 cells, demonstrated that Mn leads to increased production of H_2O_2 (Posser et al. 2009). In this aspect, H_2O_2 is a highly permeable and reactive molecule being able to react with metals such as Fe, thus generating hydroxyl radicals (Río and Vélez-Pardo 2004; Barbosa et al. 2010) resulting in a propagation of oxidative damage in cells.

Tissues can respond to oxidative stress by modulating antioxidant defenses (Halliwell and Gutteridge, 2007). Herein it was evaluated the gene expression of Hsp83, CAT and SOD in *Drosophila melangaster*. Earlier studies showed that cellular stress may induce heat shock

proteins in parallel with ROS production (Kim et al. 2004; Paula et al., 2012). Our results showed a significant increase in Hsp83 mRNA levels in Mn treated flies. Previously, our group demonstrated that exposure of flies to heavy metals such as mercury causes increases in the expression of Hsp83 (Paula et al. 2012). CAT and SOD mRNA levels were significantly increased by Mn, but notably, the enzymatic activity of these proteins was unchanged. The antioxidant enzyme SOD converts superoxide radicals ($O_2^{\bullet-}$) to H_2O_2 and CAT catalyzes the conversion of H_2O_2 to oxygen (O_2) and water (Barbosa et al. 2010), thus neutralizing those reactive species. Considering that both SOD and CAT are crucial in the cell defense against oxidative stress (Halliwell and Gutteridge, 2007), one could suppose that a posttranscriptional regulation mechanism could maintain adequate levels of these proteins, however, further studies are necessary to elucidate this particular issue. Our results also showed that the activity of TrxR and GST was enhanced in Mn exposed flies. These two enzymes play an important role in protection against oxidative stress (Mustacich 2000). GST is a complex group of phase II detoxifying enzymes that participates on the metabolism of electrophilic substances including carcinogenic, mutagenic and toxic compounds (Hayes et al. 2005). TrxR is a dimeric FAD-containing enzyme that catalyzes the NADPH-dependent reduction of the active-site disulfide in Trx-S_2 to give a dithiol in Trx-$(SH)_2$ (Zhao et al. 2002). Thioredoxin consists in one of the major redox-regulating proteins displaying a number of biological activities, including cytoprotection against ROS, protein repairing and protein disulfide reduction and modulation of signaling pathways (Yan et al. 2012). Our data draw attention for an increase in the levels of Mn and TrxR activity as well, which could represent a response to oxidation of thioredoxin in response to Mn-induced oxidative stress.

MAPKs regulate the activity of a range of transcription factors thereby controlling gene expression and cellular function. The three most-studied MAPKs are ERK1/2, JNK1/2 and p38^{MAPK} (Ichijo 1999). ASK1 (Apoptosis Signaling Kinase 1) is a member of mitogen activated protein kinase kinase kinase family (MAPKK) and an upstream activator of MAPK signaling pathway (Yan et al. 2012). The redox state of thioredoxin regulates ASK1. Under normal conditions, ASK1 is bound to and inhibited by thioredoxin and when thioredoxin is oxidated, ASK1 can be activated and apoptotic signaling through the p38^{MAPK}/JNK1/2 MAPKs is initiated (Ichijo et al. 1997). Studies conducted by Yan et al. (2012) in pancreatic carcinoma cell line, showed inhibition of TrxR by indolequinones resulting in a change of Thioredoxin-1 redox state to oxidized form and activation of p38^{MAPK}/JNK1/2. Similarly, Cadmium treatment activated ASK1 and its downstream MAPK in neuronal cells (Kim et al. 2005), and inhibits components of thioredoxin system (Chrestensen et al. 2000), while that

Saben et al. 2011 demonstrated that knockdown of ASK1 as well as chemical inhibition of p38[MAPK] and JNK played protective effects against L-DOPA induced apoptosis. Herein, it is shown that Mn induced TrxR activity while than p38 [MAPK] /JNK1/2 phosphorylation were inhibited. This fact suggests an involvement of thioredoxin system in the mechanism of Mn induced toxicity. Augmented TrxR activity may represent a cellular response to high levels of ROS by Mn exposure thus preventing the oxidation of thioredoxin and its dissociation of ASK1, this fact could contribute for diminished activation of p38[MAPK] pathway upstream kinases resulting in lower levels of phosphorylation of this MAPK contributing for minimizing apoptotic cell death.

In summary, our study demonstrate for a first time that developmental exposure to Mn lead to hyperactive-like behavior and accumulation of this metal in *Drosophila melanogaster.* The observed raise in Mn levels is negatively correlated with those found for other essential metals. This result corroborates with previous studies showing that Mn accumulation and Fe deficiency is associated with hyperactive behavior in children (Ericson et al. 2007; Konofal et al. 2008). Moreover, the induction of stress responsive genes and antioxidant enzyme activity associated with inhibition of p38[MAPK] phosphorylation in higher concentrations of Mn may represent an adaptive response to oxidative stress generated by this metal, avoiding exacerbated cellular damage.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures and Tables

Ternes et al., 2014
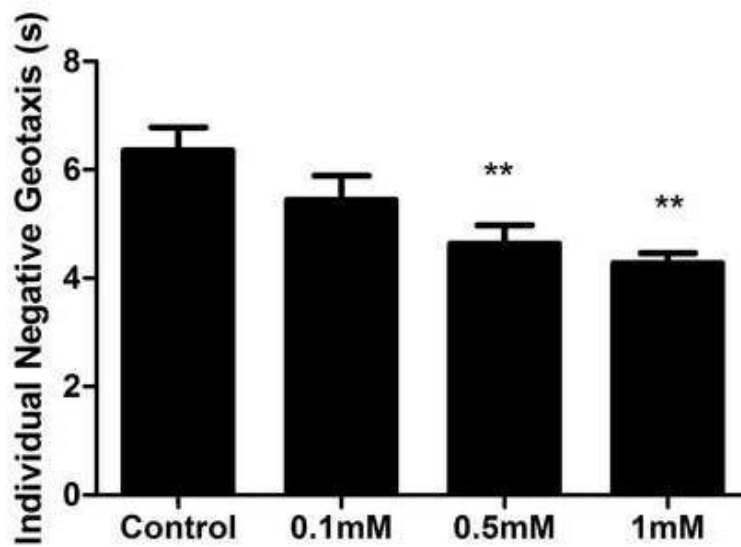
Figure 1

Figure 1. Effects of exposure to Mn during the embryonic development on locomotor performance in *Drosophila melanogaster* Results are expressed as mean (±) standard deviation (SD) and represent the time spent to climb up to 8cm in a glass tube. (n= 20-24). [**] indicates a significant effect of Mn in comparison to control group ($p<0.005$).

Ternes et al., 2014
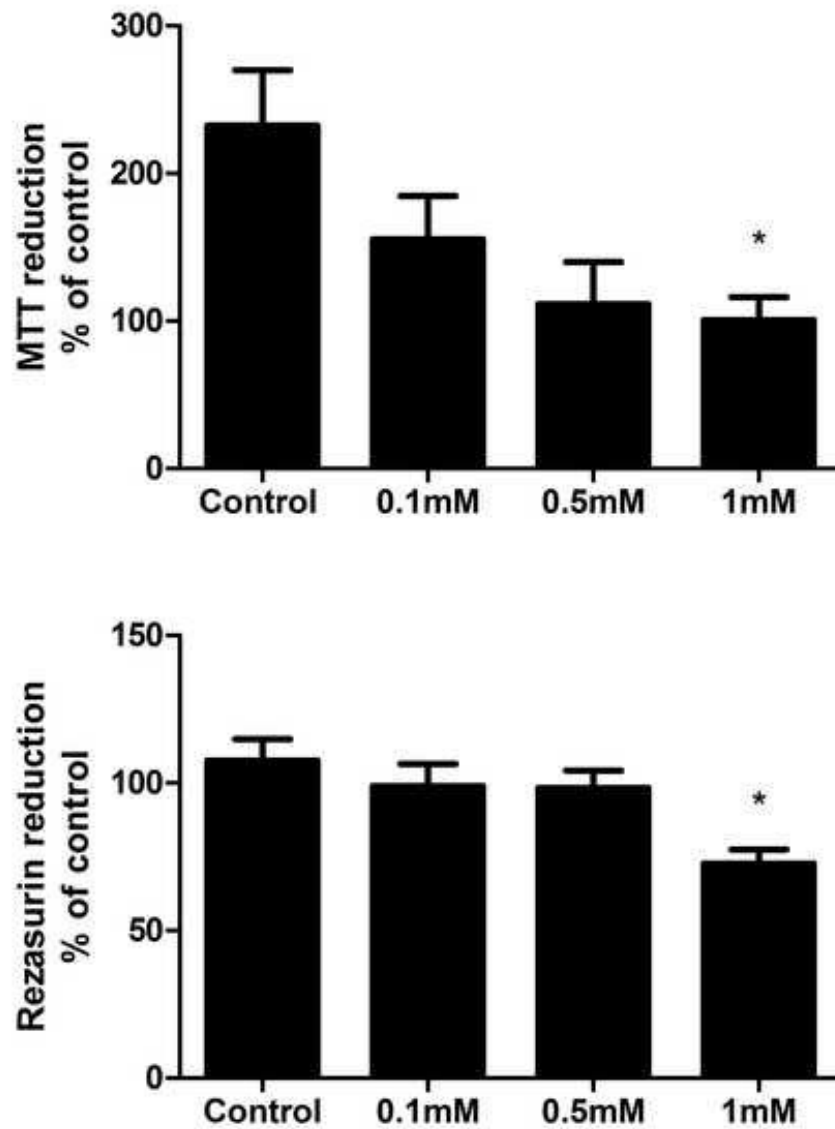
Figure 2

Figure 2. Effect of Mn exposure on cell viability in homogenate of flies treated with metal over embryonic development performed by MTT (A) and Resazurin (B) cell viability assays. For MTT, the average of absorbance in control group was 0.017 (n= 3-4); by Resazurin, absorbance in control group was 229.03 (n=4-5). Results were expressed as the percentage (%) of the control group (mean ± standard deviation) * indicates a significant effect of Mn in comparison to control group (p<0.05).
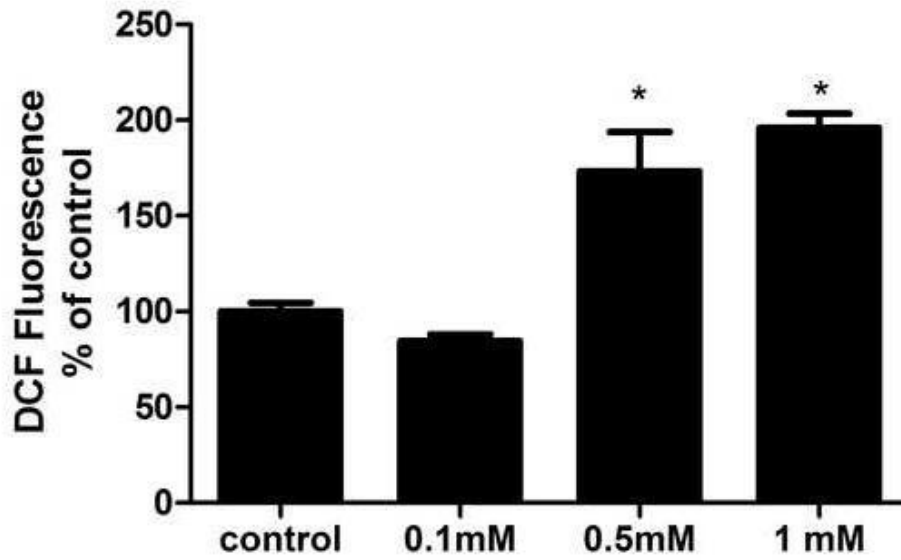
Figure 3

Figure 3. Analysis of ROS production in response to Mn-exposure during embryonic development in flies. The data showed the DCF-DA intensity of fluorescence in total flies homogenate, expressed as percentage of the control group (mean ± standard deviation) (n=3-10). * indicates a significant effect of Mn in comparison to control group ($p < 0.05$).
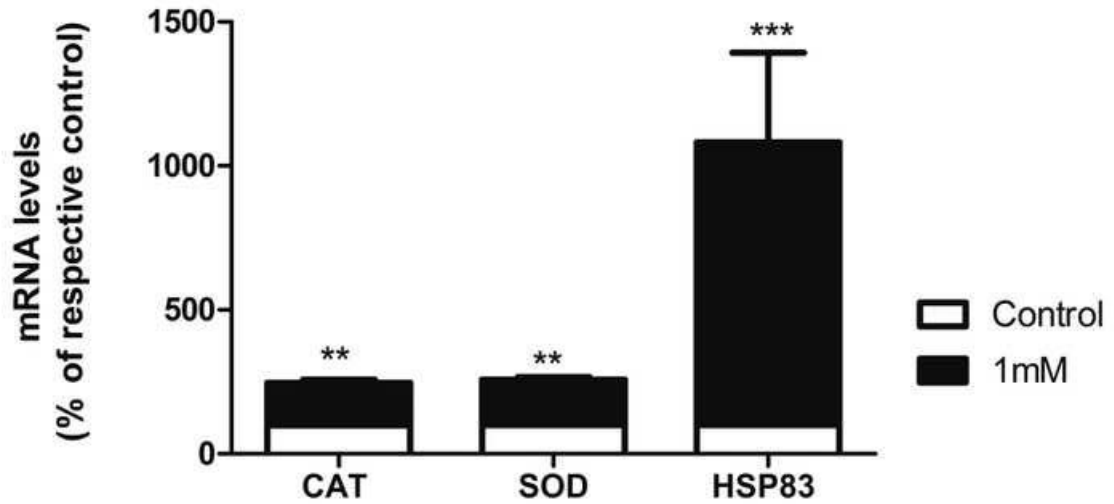
Figure 4

Figure 4. Quantitative real time PCR (qRT-PCR) analysis of CAT, SOD and HSP83 mRNA in flies Mn-exposed at 1mM. We used qRT-PCR to quantify levels of mRNA, relative to respective controls, after exposure. The data were normalized against GPDH transcript levels and each bar represents the mean ± standard deviation expressed as percent of its respective control (n=3-4) ** and *** indicates a significant effect of Mn in comparison to control group ($p < 0.005$ and $p < 0.0001$, respectively).
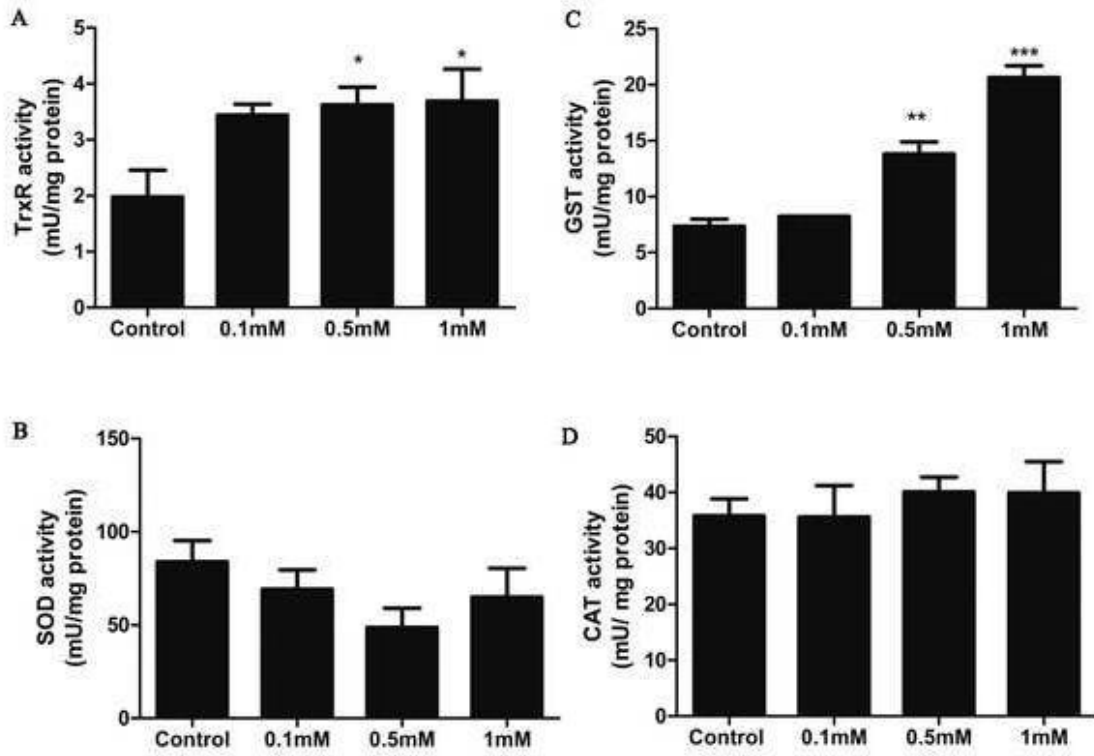
Figure 5

Figure 5. Effects observed on enzymatic activities in flies Mn-exposed during embryonic development. TrxR activity (n=5-7) (A), GST activity (n=3-4) (B), SOD activity (n=6-8) (C) and CAT activity (n=5-6) (D). The data shows the enzymatic activities in flies homogenate expressed as mean (mU/mg protein) ± standard deviation. * and *** Indicates a significant effect of Mn in comparison to control group (p<0.05 and p<0.0001, respectively).

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A   Control   0.1 mM   0.5 mM   1mM

Phospho p38^{MAPK}

Phospho ERK

Total ERK

Phospho JNK 2
Phospho JNK 1
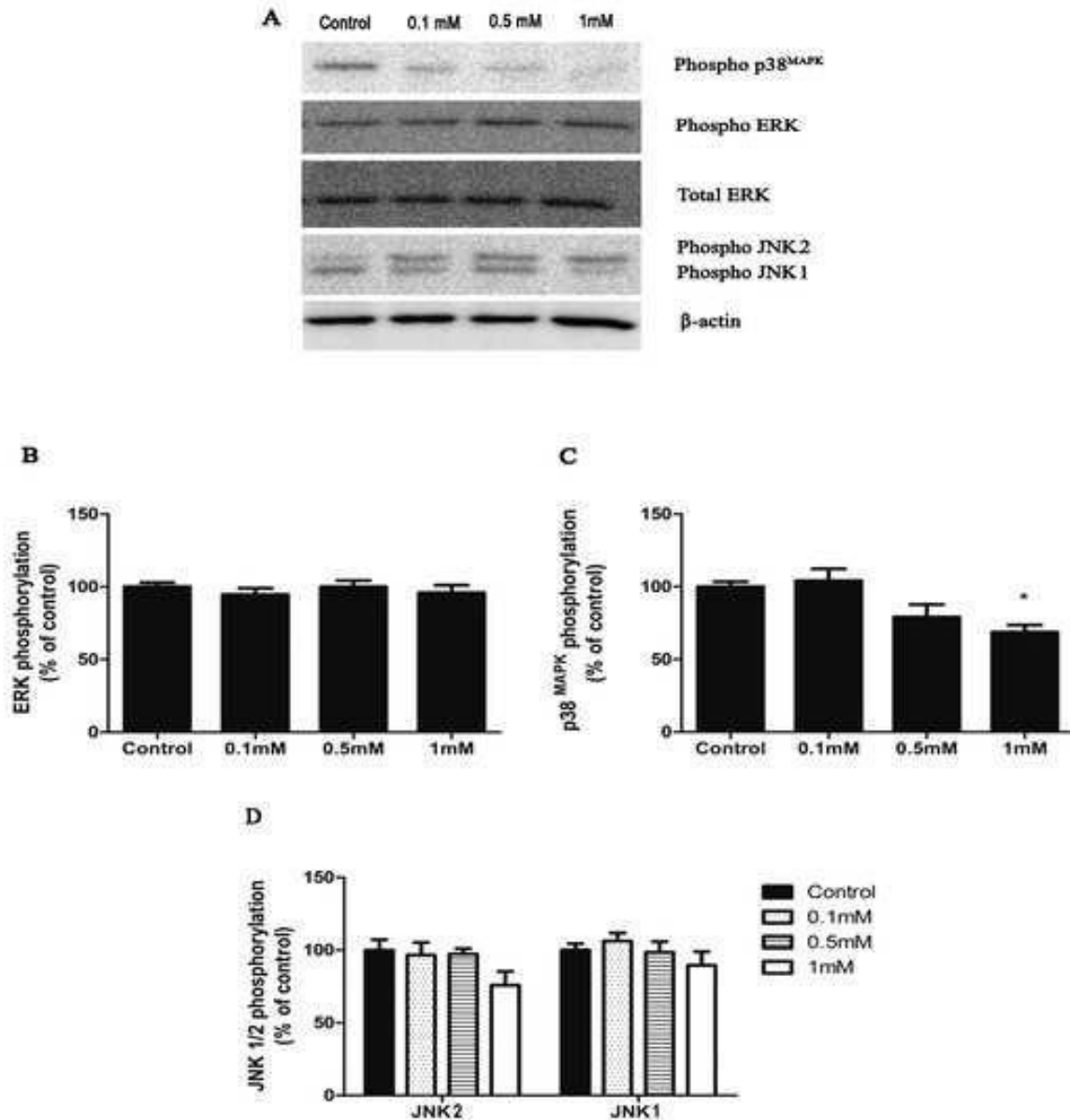
β-actin

Figure 6. Modulation of MAPKs phosphorylation in response to Mn-exposed flies during the embryonic development. The upper panel is a western blot showing phosphorylated forms of p38^{MAPK}, ERK and JNK ½ and total forms of ERK and ß-actin (A). The graphs are showing the quantification (percentage of control) of imunoreactive bands, each bar represents the mean ± standard deviation. ERK phosphorylation normalized with total ERK expression (n-5) (B). p38^{MAPK} phosphorylation normalized with ß-actin expression (n=5) (C). JNK ½ phosphorylation normalized with with ß-actin expression (n=3-5) (D). * Indicates a significant effect of Mn in comparison to control group (p<0.05).

TABLE 1. Genes tested by quantitative real-time RT-PCR analysis and used forward and reverse primers

Genes	Primer sequences
GPDH	LEFT 5' ATGGAGATGATTCGCTTCGT 3'
	RIGHT 5' GCTCCTCAATGGTTTTTCCA 3'
Catalase	LEFT 5' ACCAGGGCATCAAGAATCTG 3'
	RIGHT 5' AACTTCTTGGCCTGCTCGTA 3'
Superoxide dismutase	LEFT 5' GGAGTCGGTGATGTTGACCT 3'
	RIGHT 5' GTTCGGTGACAACACCAATG 3'
HSP83	LEFT 5' CAAATCCCTGACCAACGACT 3'
	RIGHT 5' CGCACGTACAGCTTGATGTT 3'

Table 2. Metal levels in flies Mn-exposed during the embryonic development

	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
Control	494±21	15,3±0,5	290±3	8505±83	1001±1	35±0,2	2188±3	9505±14	5049±44	286±1
0.1mM	471±13	16,3±0,5	303±7	6945±58	956±3	37±0,8	1674±5[***]	9112±28	5071±43	289±8
0.5mM	445±45	14±0,4	266±17	8190±657	947±80	63±3,2[***]	1813±136[***]	8990±638	4784±347	261±19
1mM	405±4*	13±0,3[*]	269±5	8508±126	931±4	101±1[***]	1881±10[**]	9197±100	4748±82	239±4[**]

Effect of exposure to Mn during embryonic development in metal levels of *Drosophila melanogaster*. Value were showed in $\mu g.g^{-1}$. Results were expressed as a mean (±) standard deviation (n=2). *, ** and *** indicates a significant diference in comparison to control group (p<0.05, p<0.005 and p<0.0001, respectively).

Table 3. The relationship between Mn concentration and metal levels

Variables	Correlation coeficient	P-value
Ca	-0.7960[**]	0.0020
Fe	-0.6635[*]	0.0187
Cu	-0.8028[**]	0.0017
K	0.4581	0.1342
Mg	-0.4457	0.1465
Na	-0.1368	0,6715
P	-0.1453	0.6522
S	-0.6018[*]	0.0384
Zn	-0.8902[***]	0.0001

Using pearson correlation coefficient test we evaluated the relationship between Mn concentration and other metal concentrations (n=12) *, ** and *** indicates a significant negative correlation between Mn levels and Ca, Fe, Cu, S and Zn (p<0.05, p<0.005 and p<0.0001, respectively).

4 CONCLUSÕES

Os resultados obtidos no presente trabalho indicam que a exposição ao Mn durante o período de desenvolvimento embrionário da *Drosophila melanogaster* causa alterações locomotoras e bioquímicas nesse modelo animal. Sumariamente constatou-se que:

1) As moscas expostas ao Mn apresentaram variações comportamentais, tornando-se mais rápidas no processo de escalada em comparação ao grupo controle. Sugere-se que esse aumento na velocidade de locomoção possa ser relacionado a um possível comportamento hiperativo.

2) Os níveis de Mn no corpo das moscas expostas aumentaram significantemente. Além disso, esse aumento apresentou correlação negativa com os níveis de outros metais essencias: ferro, cálcio, cobre, zinco e enxofre. Corroborando estudos já existentes que apontam uma possível competição do Mn com outros elementos nos sistemas de transporte das membranas, tais como DMT1 e canais de cálcio.

3) Houve uma diminuição na viabilidade celular, um aumento na produção de ERO e aumento na atividade das enzimas antioxidantes tioredoxina redutase (TrxR) e glutationa-S-transferase (GST) nas moscas expostas ao Mn. Esses resultados reforçam os dados apresentados pela literatura que indicam que um dos mecanismos de toxicidade desse metal está associado a alterações no funcionamento da cadeia respiratória e aumento na geração de ERO levando a uma situação de estresse oxidativo.

4) Constatou-se um aumento nos níveis de RNAm de HSP83, catalase (CAT) e superóxido dismutase (SOD). No entanto, este fato não repercutiu em um aumento nas atividades enzimáticas da CAT e da SOD.

5) A exposição ao Mn não alterou a fosforilação das MAPKs ERK e JNK, mas inibiu a fosforilação da p38^{MAPK} nas moscas expostas à maior concentração de Mn. Possivelmente por afetar a atividade de fosfatases que respondem pela modulação da fosforilação destas proteínas.

6) A inibição da fosforilação da proteína p38^{MAPK} pode também estar relacionada ao aumento da atividade da enzima TrxR, uma vez que a tioredoxina reduzida atua na modulação da quinase sinalizadora de apoptose 1 (ASK1), que faz parte da cascata de ativação das MAPKs.

7) A *Drosophila melanogaster* mostrou-se um modelo animal viável aos estudos relacionados à toxicidade do Mn.

5 PERSPECTIVAS

Diante dos resultados obtidos no presente estudo, questionamentos podem ser formulados para servirem como ponto de partida para novas investigações dos mecanismos de toxicidade do Mn. A seguir são descritos os contextos dessas indagações:

1) É estabelecido na literatura que a exposição ao Mn causa alterações cognitivas e locomotoras. O Mn leva à diminuição na quantidade de neurônios dopaminérgicos, sendo que um dos mecanismos responsáveis por essa perda neuronal é a auto-oxidação da dopamina (MERGLER et al., 1994; TRAN et al., 2002a, 2002b, revisado por MARTINEZ-FINLEY et al., 2013). Além disso, é documentado que esse metal também interfere na atividade dos astrócitos, causando uma diminuição da captação de glutamato, que fica na fenda sináptica gerando um processo de excitotoxicidade (ERIKSON; ASCHNER, 2003; revisado em DOBSON et al., 2004; GIORDANO et al., 2009). Nesse trabalho verificou-se que as moscas expostas ao Mn não apresentaram perdas locomotoras, inversamente, foram mais ágeis no teste comportamental, sugerindo um possível comportamento hiperativo. Nesse sentido surgem as seguintes perguntas:

- Esse comportamento seria apresentado apenas nos primeiros dias de vida da mosca e à medida que ela torna-se adulta esses resultados poderiam ser alterados?

- Os mecanismos relacionados a esse comportamento mais ágil não poderiam alterar o tempo de vida dessas moscas?

- Quais são os efeitos que a exposição ao Mn causa sobre comportamentos relacionados à aprendizagem e memória da *Drosophila melanogaster*?

2) É bem documento que a exposição ao Mn no período pré-natal ou durante a infância está relacionada a comportamentos hiperativos e déficit cognitivo (WASSERMAN et al., 2006; BOUCHARD et al., 2007; ERICSON et al., 2007; FARIAS et al., 2010; HENN et al., 2010; MENEZES-FILHO et al., 2011; ROELS et al., 2012; MARTINEZ-FINLEY et al., 2013; TORRES-AGUSTÍN et al., 2013). É conhecido que em estágios de toxicidade crônica de Mn ocorre uma diminuição na densidade dos receptores de dopamina do subtipo D2 (revisado por ROTH, 2003). Conforme revisado por Glickstein e Schmauss (2001), os receptores de dopamina do subtipo D3, quando inibidos, não alteraram o desenvolvimento normal de ratos, porém estes apresentaram comportamentos hiperativos. Além disso, medicamentos utilizados no tratamento de hiperatividade e déficit de atenção inibiem transportadores de dopamina, bloqueando a recaptação do neurotransmissor no neurônio pré-sináptico (revisado por BIEDERMAN,

2005). Considerando esses aspectos e o aparente comportamento hiperativo apresentado pelas moscas no presente trabalho, pergunta-se:

- A exposição ao Mn estaria inibindo algum receptor de dopamina na *Drosophila*? Em caso afirmativo, qual seria esse receptor?

3) A p38^{MAPK} é uma proteína da família das MAPKs relacionada com a resposta inflamatória, sua fosforilação estimula a produção de citocinas como o fator de necrose tumoral α (TNF- α) e também está relacionada com a ativação de mecanimos pró-apoptóticos. A inibição de fosfatases é responsável pela ativação da fosforilação dessa proteína (revisado por AOADI et al., 2006; LIU et al., 2014; LUCREZI et al., 2014). Prévios estudos indicam que o Mn induz estresse oxidativo devido à disfunção mitocondrial. Nesse contexto, o metal tem um papel pro-inflamatório ativando quinases como ERK e JNK (revisado por KARKI et al., 2013).

Uma das quinases que faz parte da cascata de sinalização que leva à ativação da p38 ^{MAPK} é a ASK1, cuja modulação relaciona-se ao estado redox do sistema tioredoxina. A tioredoxina reduzida inibe essa fosforilação da ASK1, o que repercute na não ativação da p38 ^{MAPK} (ICHIJO et al., 1997).

No presente estudo, observou-se uma inibição na fosforilação da p38^{MAPK} nas moscas expostas ao Mn durante o período de desenvolvimento embrionário, aliada a um aumento na atividade da enzima TrxR, responsável pela conversão da tioredoxina oxidada em tioredoxina reduzida. Diante disso, questiona-se:

- A exposição ao Mn estaria alterando a atividade de fosfatases?

- O aumento da atividade da TrxR estaria envolvido na inibição da fosforilação da ASK1?

- A inibição da fosforilação da p38^{MAPK} constituiu um mecanismo adaptativo da *Drosophila melanogaster* à toxicidade do Mn, visando à diminuição da morte celular por apoptose?

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ANEXO A – Demais trabalhos realizados no mestrado

Artigo aceito para publicação na revista Applied Research in Toxicology que substitui a revista Brazilian Journal of Toxicology (Revista Brasileira de Toxicologia (ISSN 1415-2983)

LONG TERM EXPOSURE TO LOW LEVELS OF MANGANESE CHLORIDE IMPROVES THE ACTIVITY AND EXPRESSION OF ANTIOXIDANT ENZYMES IN ADRENAL GLAND OF ADULT RATS

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Abstract

Human contamination with Manganese is associated with neurological symptoms similar to observed in Parkinson Disease. Cathecolaminergic system has been considered an important target of Manganese toxicity. Our study aimed to assess the possible damages induced by long term exposure to low levels of Manganese on Adrenal Gland. For this, adult male Wistar rats were treated for the period of 90 days with $MnCl_2$ diluted in drinking water (120 mg/L). Ambulatory behavior was conducted and Adrenal Gland was used for determination of biochemical parameters related to antioxidant status. Our results demonstrated that Manganese increased the activity of Glutathione Reductase and Catalase as well as the expression of Glutathione Peroxidase , and NADP(H) Quinone Oxidoreductase in Adrenal Gland. Overall, the treatment with Manganese did not induce prominent cell damage in Adrenal Gland as evaluated by poly ADP ribose polymerase cleavage, an apoptotic cell death marker. Our study provides a first time description of biochemical alterations in Adrenal Gland when submitted to long term treatment with Manganese, increasing the expression of NADP(H) Quinone Oxidoreductase and Glutathione Peroxidase 1. Taken together, results of our work may represent a biochemical adaptation of Adrenal Gland to prolonged oxidative stress induced by exposure to the metal.

Key words: catecholamine, manganse, long-term treatment, antioxidant status, glutathione peroxidase.

INTRODUCTION

Manganese (Mn) is an ubiquitous transition metal present in a range of industrial process as making steel alloys, drycell batteries, electrical coils, welding rods among others. Mn is considered an essential nutrient for humans and animals, participating as co-factor in several enzymatic reactions, such as pyruvate kinase, mitochondrial superoxide dismutase (SOD), glycosyl transferase and fatty acid synthesis (1, 2).

Long-term exposure to Mn are related with occupational exposure and is associated with neurological symptoms which may be indistinguishable from idiopathic Parkinsons Disease including fixed gaze, bradykinesia, postural difficulties, rigidity, tremor, dystonia and decreased mental status characterizing a syndrome known as Manganism (3). It has been reported that chronic consumption of drinking-water containing Mn in levels ranging from 81 to 2300 µg/l were associated with progressively higher prevalence of neurological signal in older persons (4). In this aspect, factors as source and duration of exposure as well as nutritional status can interfere in the intensity and incidence of Mn neurological symptoms.

A variety of biochemical changes are induced in response to Mn exposure, with includes glutathione (GSH) and dopamine depletion, increased oxidative stress (OS), impairment of energy metabolism and antioxidant systems

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(5-7). Mn exposure both *in vivo* an *in vitro* is associated with alteration of mitogen-activated protein kinase (MAPK) signaling transduction pathways; this family of proteins are implicated in regulation of multiple cellular events including differentiation, proliferation, cell death, adaptive and immune responses (8). Activation of these kinases may occur in response to hyperosmotic stress, cytokine exposure, and toxic injury, including OS (9, 10) and metals, such as mercury (Hg), lead (Pb), and Mn (11-13).

Previous data has evidenced the catecholaminergic system as an important target of Mn intoxication. Studies carried out in culture cells, demonstrated a depletion of dopamine in nigrostriatal neurons during Mn exposure (14,15). Other evidenced that dopaminergic mesencephalic culture cells were more susceptible to Mn toxicity than other neuron population while that in *vivo* administration of Mn caused loss of Tyrosine Hydroxylase (TH) positive cells in Substantia Nigra (16).

Concerning dopaminergic system, the adrenal medulla is the place where chromaffin cells are most commonly located. These cells secrete and collect noradrenaline, adrenaline and dopamine (DA) (17) which influences the activity of almost all tissues and organs and plays an important role in the protective response to physiological stresses such as hypoxia, hemorrhage and hypoglycemia (18, 19). Previously, our group demonstrated than pheocromacitoma cell line (PC12) was affected by Manganese Chloride (MnCl₂) exposure, inducing phosphorylation of MAPKs and cell death; such effects were mostly observed in higher concentrations and longer periods (13).

Thus, this work aimed to investigate the effects of long-term Mn exposure on enzymatic antioxidant cellular defense and modulation of expression and phosphorylation of stress responsive proteins on Adrenal Gland (AG) of adult rats.

MATERIAL AND METHODS

Materials

Manganese chloride (CAS 13446-34-9), Quercetin (Q4951) (CAS 117-39-5), 5,5´-Dithiobis(2-nitrobenzoic acid) (D8130) (CAS 69-78-3), Acetylthiocholine iodide (A5751) (CAS 1866-15-5), 1-Chloro-2,4-dinitrobenzene (237329) (CAS 97-00-7), 2´,7´-Dichlorofluorescein diacetate (DCHF-DA, 35845) (CAS 2044-85-1), 2-Mercaptoethanol (M6250) (CAS 60-24-2), and anti-rabbit immunoglobulin (alkaline phosphatase- linked antibody, A-3687) were obtained from Sigma Aldrich (St Louis, MO). Anti-phospho-p38 (Thr180/Tyr182) and anti-total-p38, anti-phospho JNK1/2 (Thr183/Tyr185) and anti-total-JNK1/2, anti-phospho ERK1/2 (Thr202/Tyr204) and anti-total-ERK1/2 and β-actin antibodies were purchased from Cell Signaling Technology. SDS (CAS 151-21-3), Acrylamide (CAS 79-06-1), Bis-acrylamide (CAS 110-26-9), hybond nitrocellulose were obtained from GE Healthcare Life Division. Anti-PARP, anti-GPx1 and GPx4,

anti-TrxR, anti-NQO1, anti-metalothioneine, anti NRF-2 and anti HSP-70 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were commercial products of the highest purity grade available.

Animal Treatment

Ten male Wistar rats (180–200 g) were obtained from Universidade Federal de Santa Maria and maintained in an air conditioned room (20-25ºC) under natural lighting conditions with water and food (Guabi-RS, Brazil) *ad libitum*. All experiments were performed in accordance with the Guiding Principles of the Committee of Animal Care of Universidade Federal de Santa Maria (0089.0.243.000-07). Animal were caged in two groups of five; after two weeks of acclimatization, one group was used as control while the second one was treated with MnCl₂ 120mg/L diluted in the drinking water for the period of 90 days. At the end of treatment the locomotor ability of animals was tested in the open field dome and subsequently, animals were decapitated and the AGs were immediately removed for biochemical analysis. The concentration of Mn selected for this study was approximately four times lower than that previously reported in the literature for similar models of exposure, and closer to that observed in epidemiological studies (20).

Open Field Test

The ambulatory behavior was assessed in an open-field test as described previously (13). The apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossing) was counted in a 6-min session.

Sample Preparation and Enzyme Assays

For enzymes activity, whole AG were mechanically homogenized with a tissue homogenizer (Marconi, Brazil) in 1 mL 0.1 M phosphate buffer, pH 7.0, and centrifuged at 1000 g for 5 min at 4ºC. The remaining supernatant was then centrifuged at 20.000 g for 30 min. The resulted supernatant was used for determination of glutathione S-transferase (GST) (21), catalase (CAT) (22), Glutathione Peroxidase (GPx) (23), glutathione reductase (GR) (24), thioredoxin reductase (TrxR) (25) and SOD (26). Protein concentration was determined by the method of Bradford et al. (27) using bovine serum albumin as the standard.

Western Blotting

Quantification of Glutathione Peroxidase 1 and 4 (GPx1/4), NADP(H) Quinone Oxidoreductase (NQO1) and nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf-2) expression, MAPKs phosphorylation and expression, metallothionein (MT), Heat shock 70 kDa protein (HSP70) and TH expression and poly (ADP)-ribose polymerase (PARP) cleavage was performed using Western blotting based on protocols previously described (13) with minor modifications. Whole AGs were mechanically homogenized

Ana Paula Lausmann Ternes; Ana Paula Zemolin; Daiane Francine Meinerz; Douglas Oscar Ceolin Mariano; Emily Pansera Wakzuk; Katiane Raquel Müller; Gustavo Felipe da Silva; Antônio Batista Pereira; João Batista Teixeira da Rocha; Jeferson Luis Franco; Thaís Posser

at 4ºC in 200 μL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and phosphatase inhibitor cocktail. The homogenates were centrifuged at 1000 g for 10 min at 4ºC and the supernatants (S1) collected. After protein determination (27) using bovine serum albumin as standard, β-mercaptoethanol and glycerol was added to samples to a final concentration of 8 and 25%, respectively, and the samples frozen until further analysis.

Proteins were separated using SDS–PAGE (10%), and then electrotransferred to nitrocellulose membranes as previously described (13). Membranes were washed in tris-buffered saline with Tween (100 mM tris – HCl, 0.9% NaCl and 0.1% Tween-20, pH 7.5) and incubated overnight (4ºC) with different primary antibodies, all produced in rabbit (anti-ERK1/2, anti-p38, anti-JNK1/2 total and phosphorylated forms, anti-β-actin and anti-PARP, anti-Nrf2, anti-TrxR, anti-HSP70 and anti-NQO1). Following incubation, membranes were washed in tris-buffered saline with Tween and incubated for 1 h at 25ºC with alkaline phosphatase-linked anti rabbit-IgG secondary specific antibodies. Antibody binding was visualized using the NBT-BCIP kit (KPL, MD, USA). Band staining density was quantified using the Scion Image (Scioncorp ver. Beta 4.0.2) software and expressed as a fold change of the mean relative to control group. The loading controls were performed by analysis of β-actin using specific antibodies.

Statistical Analysis

Statistical analysis was performed using the Student t-test. Differences were considered to be significant at the $p < 0.05$ level.

RESULTS

Mn treatment (120mg/L) did not alter the number of crossings and rearing frequency when compared with the control group (figure 1A and 1B).

Animals treated with Mn had 43%, 30% and 28% higher activity of GPx, GR and CAT respectively, than those that did not receive treatment (table 1).

Analysis of imunoblotting demonstrated an increase in expression of GPx1 and NQO1 in 36 and 30% respectively

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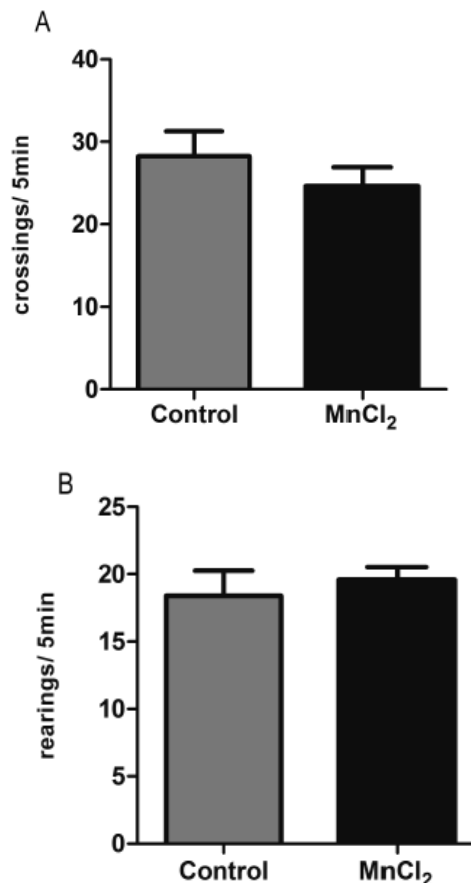
Figure 1

Figure 1. Behavioral analysis of rats exposed to 90 days with 120 mg/L with Mn in drinking water. (A) Number of crossings; (B) rearing activity. Data were analyzed by Student T test and expressed as mean ± S.E.M. N=5. No differences between groups were observed.

Table 1. Antioxidant enzymes activity.

	GPx	GR	CAT	SOD	GST	TrxR
Control	88.6±7.9	52.4±8.1	2.5±0.4	33.0±7.7	44.4±11.3	56.8±6.9
Manganese	127.4±24.2***	68.3±9.5**	3.2±0.3***	33.1±4.6	49.9±6.6	60.2±12.4

Values are expressed as mean (mU/mg protein) ± SEM, n= 5.
** p<0.01 Control *vs* Mn.
*** p<0.001 Control *vs* Mn.

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Figure 2. Modulation of GPx1 and NQO1 expression in response to long term exposure to Mn (120 mg/L) in drinking water. Adult male rats were treated for 90 days with MnCl₂ diluted in drinking water, AGs were removed and proteins separated by SDS-PAGE, and transferred to nitrocellulose membrane, which was incubated with specific primary antibodies to detection of GPx1 and NQO1. (A) GPx1 expression. (B) NQO1 expression. Expression of proteins was quantified by densitometric analysis. The data are expressed as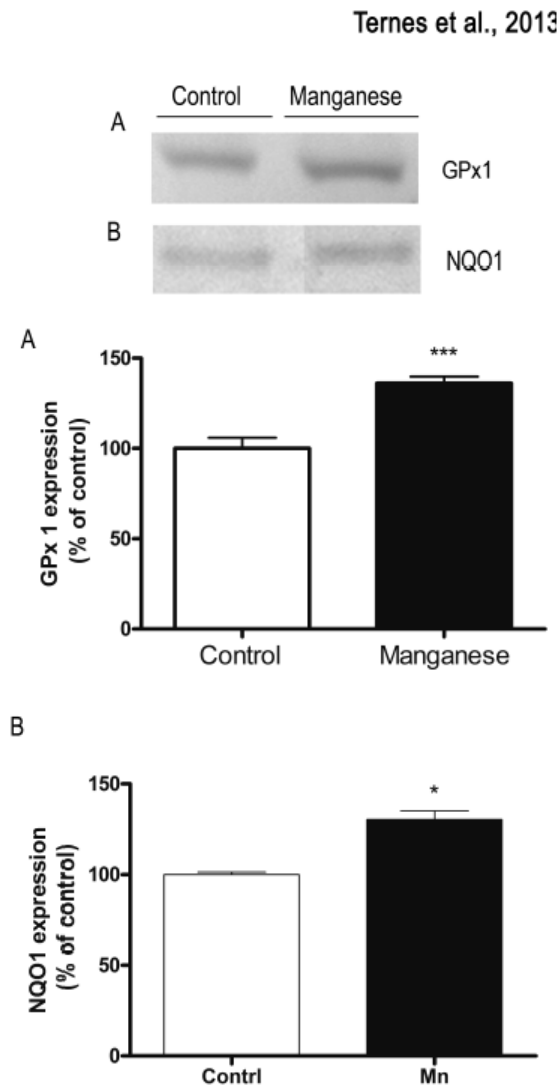 percentages of the control (considered 100%). The values are means of three to five experiments ± SD. Statistical significance: *p<0,05; **p<0.001 compared to control group.
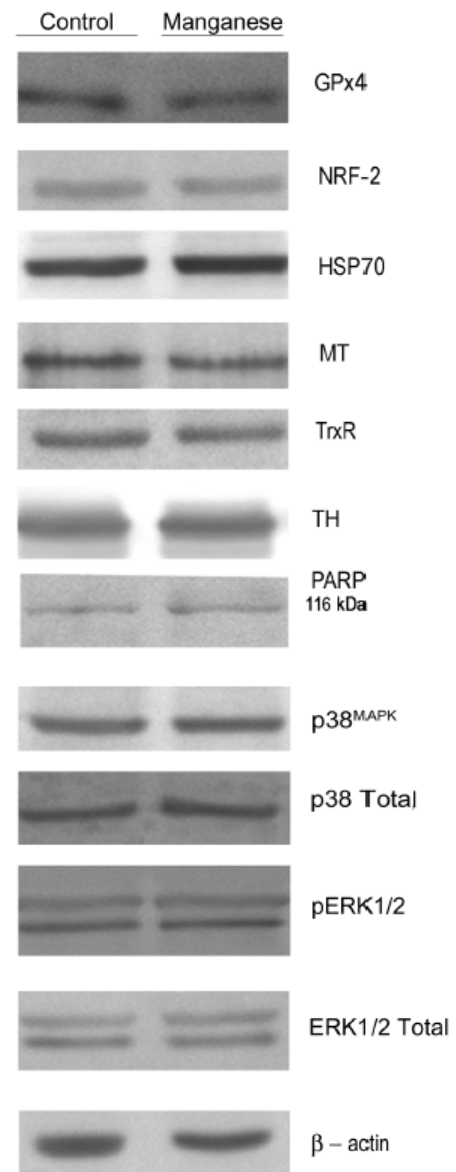
Figure 3. Analysis of proteins expression and/or phosphorylation in response to long term exposure to Mn (120 mg/L) in drink water. Adult male rats were treated for 90 days with MnCl₂ diluted in drinking water, AGs were removed and proteins separated by SDS-PAGE, and transferred to nitrocellulose membrane, which was incubated with different specific primary antibodies. Total content (expression) and phosphorylation of protein described in the picture was quantified. The panel is a Western Blotting showing expression of GPx4, Nrf2, HSP70, MT, TRx1, TH, PARP, and MAPKs (p38 and ERK1/2) total and phosphorylated forms and β-actin expression. Each blotting is representative of three independent experiments.

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(Figure 2). In counterpart, Nrf2 transcriptional factor, GPx4, HSP70, MT and TrxR expression was not altered. Proteins member of MAPK family ERK1/2, p38[MAPK] and JNK1/2 expression and phosphorylation were not modified by Mn treatment in AG. Moreover, only the full length form of PARP (116 kDa) was detected indicating that Mn long term exposure did not induce apoptotic cell death in AG. In accordance, no loss of cathecolaminergic cells was observed in response to Mn, since TH expression was unaltered. All these data are represented in the blottings of figure 3.

DISCUSSION

The present work aimed to investigate the effects caused by long term exposure to low levels of Mn through drinking water on AG taking in consideration possible alterations in antioxidant status, phosphorylation and expression of stress responsive proteins, and possible damage to catecholaminergic cells of this organ. It is important to consider that the concentration of Mn which the animals were exposed was approximately 100 times lower than that used in previous work (28), where it was observed decrease in number of crossings and oxidative stress induction in striatum following 30 days exposure.

AG is a structure important for maintenance of body homeostasis. In adrenal cortex, different steroids, as cortisone, corticosterone, 11-dehydrocorticosterone has been isolated (29). Adrenal medulla is recognized by the presence of chromaffin cells, which secret catecholamines including epinephrine and norepinephrine, important hormones in the 'fight and flight' response influencing the activity of almost every tissue (30,31). In this aspect, until now, to our knowledge, no study has been focused on toxicological effects associated to long term exposure to Mn in AG. So far, the most import finding of the present work was that even concentrations unable to cause locomotor alterations affects the biochemistry of AG leading to changes in expression of antioxidant enzymes.

Free radicals play a key role in Mn-induced neurotoxicity (32). Such aspect is related with the ability of Mn to enhance reactive oxygen species (ROS) generation through catalysis of dopamine autoxidation and quinones formation leading to oxidative damage and overcoming cell death (2). However, it is unknown if Mn can cause oxidative damage or modulating signaling proteins related with cellular stress in AG, an important site for catecholamine synthesis and release. Previous studies demonstrate that Mn enhanced autoxidation of DA increasing the generation of ROS O_2^-, H_2O_2 and OH- and cytotoxic DA-o-quinone (33, 34). Our group has previously demonstrated a considerable increase in H_2O_2 production in pheocromacitoma cell line (PC12) exposed for 24h to Mn (13), in parallel to augmented activity of TH, the step limiting enzyme in catecholamine biosynthesis. In this study, Mn stimulated the activity of enzymes GPx, GR and CAT in AG in parallel with increased

expression of NQO-1 and GPx1 isoform without altering GPx4 isoform expression. NQO1 metabolizes dopamine-derived quinones (DAQ) (35) and GPx-1 is necessary for H_2O_2 reduction, thereby the augmented expression of these enzymes by Mn contributes to the antioxidant capacity of the cell (36).

The Nrf2 mediate the induction of a set of drugmetabolizing enzymes such as GST and NQO1 (37). Nrf2 binds to Antioxidant Response Elements (ARE) transcribing multitude of antioxidant genes. Disruption of protein Keap-1- Nrf2 interaction or genetic overexpression of Nrf2 can increase the endogenous antioxidant capacity of brain thus representing protection against OS in neurodegenerative diseases (38). Nrf2 regulates the expression of antioxidant enzymes including GR, peroxiredoxin, thioredoxin and TrxR, CAT, SOD and GPx (39).

Experiments conducted by (40), showed that Mn inducted Nrf2 mRNA expression and cytosolic/nuclear migration in pheocromacitoma cell line (PC12). In the present study, long term exposure to Mn did not alter the expression of Nrf2, however, increase the expression of GPx1 isoform and NQO1. Concerning this data, it is recognized that regulation of Nrf2 that responds for its activation also involves mechanisms of phosphorylation/ dephosphorylation, acetylation/deacetylation, disrupting Nrf2/Keap1 complex and translocation of Nrf2 to the nucleus promoting transcriptional activation of Nfr2 dependent genes in response to inducing signals (37) indicating activation of this factor. Other mechanisms acting on antioxidant defense system regulated by Nrf2 includes metal-chelation by metallothioneins and induction of stress response proteins. In this aspect, we did not observed induction in expression of chaperone HSP70 and MTs, possibly these proteins were initially stimulated and returned to basal levels at the end of treatment since they are mostly responsive to initial stress response to metals (41).

MAPKs phosphorylation and expression, represented by proteins p38[MAPK], ERK and JNK1/2, as well as chaperone HSP70 and MTs expression were not affected by Mn treatment in AG. Our research group showed previously that prolonged treatment of the PC12 clone with Mn, which was developed from a pheochromocytoma tumor of the rat adrenal medulla, induced phosphorylation of JNK1/2 and p38[MAPK] and decreases cell viability only in very high concentrations of Mn (from 500 μM) (13). In this aspect this work is in accordance with data obtained from cell culture studies, since the concentration of Mn used here is four times or even lower than concentrations able to cause neurological symptoms in similar model of exposition (20, 28).

Overall, this study shows that prolonged exposure of adult rats to relatively low concentrations of Mn induces expression of at least two antioxidant enzymes (GPx1 and NQO1) in AG, without causing cell death or activation of cell stress activated proteins. Our result point out to an adaptive mechanism linked to long term exposure to Mn, inducing expression of GPx1 and NQO1 counteracting the

overproduction of ROS and protecting cells against cell damage associated with OS.
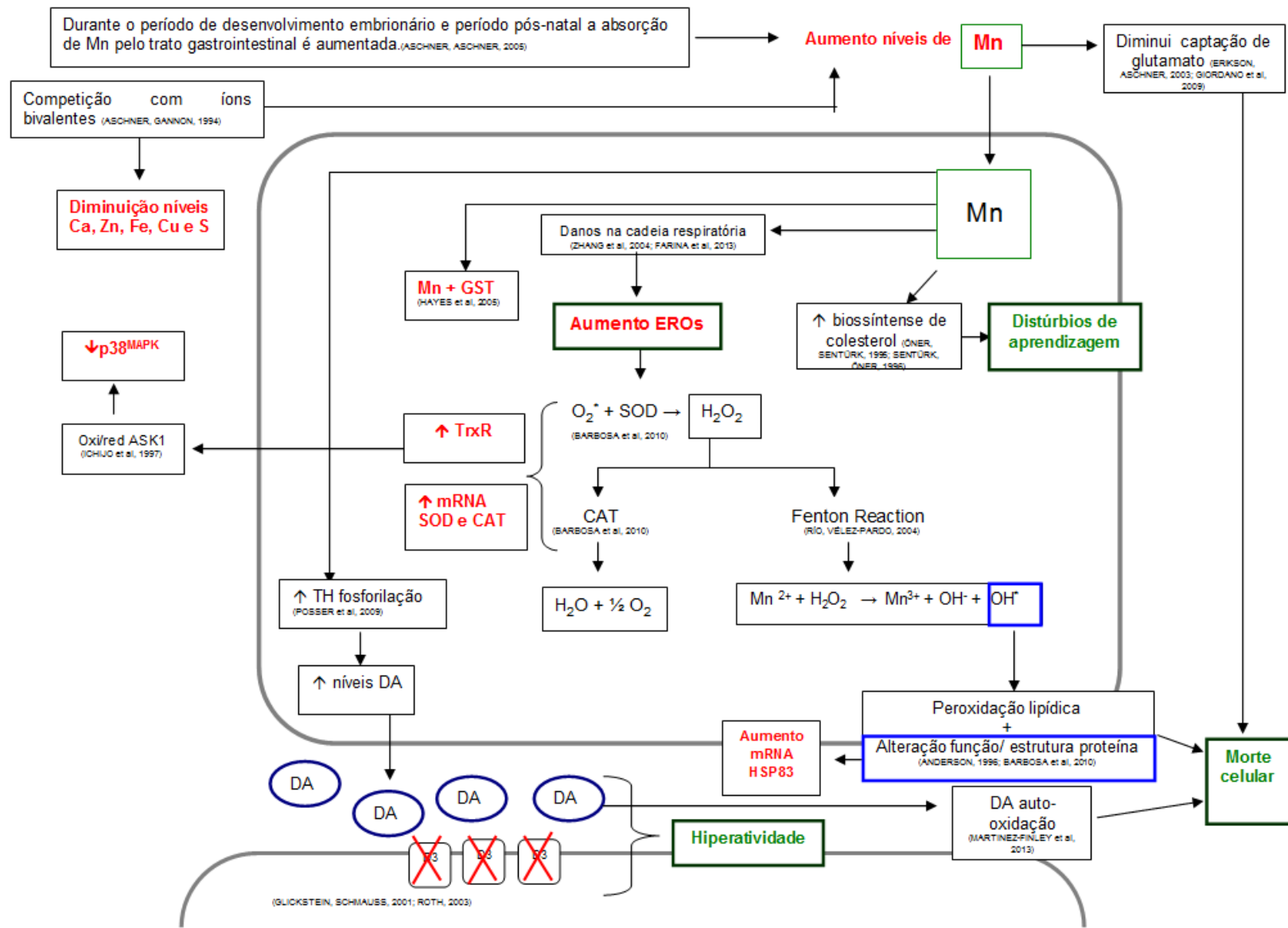
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Declaration of interest

The authors declare that there are no conflicts of interest.

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ANEXO B – Esquema sintetizando os resultados obtidos no trabalho e os conhecimentos disponíveis na literatura da área.

Durante o período de desenvolvimento embrionário e período pós-natal a absorção de Mn pelo trato gastrointestinal é aumentada.(ASCHNER, ASCHNER, 2005)

Aumento níveis de **Mn**

Diminui captação de glutamato (ERIKSON, ASCHNER, 2003; GIORDANO et al, 2009)

Competição com íons bivalentes (ASCHNER, GANNON, 1994)

Diminuição níveis Ca, Zn, Fe, Cu e S

Mn

Danos na cadeia respiratória (ZHANG et al, 2004; FARINA et al, 2013)

Mn + GST (HAYES et al, 2005)

↓p38^{MAPK}

Aumento EROs

↑ biossíntense de colesterol (ÖNER, SENTÜRK, 1995; SENTÜRK, ÖNER, 1996)

Distúrbios de aprendizagem

Oxi/red ASK1 (ICHIJO et al, 1997)

↑ TrxR

$O_2^* + SOD \rightarrow$ H_2O_2 (BARBOSA et al, 2010)

↑ mRNA SOD e CAT

CAT (BARBOSA et al, 2010)

Fenton Reaction (RÍO, VÉLEZ-PARDO, 2004)

↑ TH fosforilação (POSSER et al, 2009)

$H_2O + \frac{1}{2} O_2$

$Mn^{2+} + H_2O_2 \rightarrow Mn^{3+} + OH^- + OH^-$

↑ níveis DA

Peroxidação lipídica + Alteração função/ estrutura proteína (ANDERSON, 1996; BARBOSA et al, 2010)

Aumento mRNA HSP83

Morte celular

DA

DA

DA

DA

DA auto-oxidação (MARTINEZ-FINLEY et al, 2013)

Hiperatividade

(GLICKSTEIN, SCHMAUSS, 2001; ROTH, 2003)

Durante os períodos de desenvolvimento embrionário e pós-natal a absorção de Mn pelo trato gastrointestinal é aumentada, tendo em vista que o sistema de excreção biliar ainda não está completamente desenvolvido (ASCHNER; ASCHNER, 2005). Além disso, é conhecido que os íons Mn^{2+} e Mn^{3+} competem com outros íons nos sistemas de transporte através da membrana celular tais como DMT1 e canais de cálcio (ASCHNER; GANNON, 1994), o que pode explicar a diminuição nos níveis corporais de Ca, Zn, Fe, Cu e S observada no presente estudo.

Esse aumento na absorção e a competição do Mn com outros elementos acarreta em maiores concentrações desse metal no organismo. É relatado que níveis aumentados de Mn no cérebro estão associados à dimuição da recaptação do glutamato da fenda sináptica pelos astrócitos, contribuindo para morte celular por excitotoxicidade (ERIKSON; ASCHNER, 2003; GIORDANO et al., 2009). Além disso, prévios estudos associam os níveis de Mn com o aumento da biossíntese de colesterol, diretamente associada a distúrbios de aprendizagem em roedores (ÖNER; SENTÜRK, 1995; SENTÜRK; ÖNER, 1996).

O Mn é também responsável por causar danos através da inibição de alguns complexos da cadeia respiratória (ZHANG et al., 2004; FARINA., et al, 2013). Esse processo está relacionado com um aumento na produção de ERO, com isso o organismo tende a aumentar suas defesas antioxidantes a fim de minimizar os danos inerentes ao estresse oxidativo. A enzima antioxidante SOD é responsável por catalisar a reação que converte o radical superóxido em H_2O_2. Já a CAT catalisa a reação que converte o H_2O_2 em água e oxigênio (BARBOSA et al., 2010). Com isso, pode-se explicar os aumentos nos níveis de RNAm de SOD e CAT, embora deva existir um mecanismo pós-transcricional que controle a tradução dessas enzimas uma vez que neste estudo não foi constatado aumento na atividade das mesmas.

O H_2O_2, por sua vez, pode também reagir com o Mn^{2+} através da Reação de Fenton, produzindo radicais hidroxila (RÍO; VÉLEZ-PARDO, 2004). Esses radicais são altamenente reativos e ocasionam peroxidação lipídica e alterações na função e estrutura de proteínas, o que em última instância leva à morte celular (ANDERSON, 1996; BARBOSA et al., 2010). No presente estudo, constatou-se um aumento nos níveis de RNAm de Hsp83, uma proteína conhecida genericamente como chaperona que auxilia no enovelamento de outras proteínas, bem como encaminha para a destruição proteínas caso não seja possível atingir a configuração correta. Esse aumento nos níveis de Hsp83, pode significar uma resposta do organismo visando minimizar os danos causados pelos radicais hidroxila.

A enzima antioxidante GST participa do processo de detoxificação de substâncias eletrofílicas (HAYES et al., 2005). O aumento na atividade da GST, verificada nos experimentos, é um indício de que essa enzima está atuando na detoxificação do Mn do organismo. Outra enzima que teve sua atividade aumentada foi a TrxR. Ela é responsável pela redução da tioredoxina. É sabido que o estado redox da tioredoxina está relacionado com a ativação da ASK1 (ICHIJO et al., 1997), que por sua vez faz parte da cascata de sinalização celular que ativa a fosforilação da p38^{MAPK}. Assim, sugere-se que o aumento na atividade na TrxR é um possível mecanismo adaptativo do organismo, visando inibir a fosforilação da p38^{MAPK}, uma vez que, quando ativada, essa proteína ativa fatores relacionados à morte celular por apoptose.

Prévios estudos, demonstraram que o Mn contribui para o aumento da fosforilação da tirosina hidroxilase (TH), enzima passo-limitante da síntese de catecolaminas como a dopamina (POSSER et al., 2009). Assim, níveis aumentados de Mn acarretam em maior síntese de dopamina, que por sua vez sofre auto-oxidação, levando à morte celular (MARTINEZ-FINLEY et al., 2013).

Por fim, é relatado na literatura que em situações de toxicidade crônica de Mn ocorre uma diminuição na densidade de receptores de dopamina do tipo D2 (ROTH, 2003). Além disso, em roedores, quando os receptores de dopamina do tipo D3 foram inibidos, os animais apresentaram comportamentos hiperativos (GLICKSTEIN; SCHMAUSS, 2001). Assim, o comportamento hiperativo apresentado pelas moscas pode estar relacionado a uma diminuição na densidade de receptores de dopamina, assim como com os níveis reduzidos de Fe (KONOFAL et al., 2008).