UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E BIOTECNOLOGIA

TESE DE DOUTORADO

ESTUDO EXPERIMENTAL DOS EFEITOS DA EXPOSIÇÃO REPETIDA AO HERBICIDA ATRAZINA SOBRE A ATIVIDADE NEUROCOMPORTAMENTAL, BIOMARCADORES DO ESTRESSE OXIDATIVO E SISTEMA DOPAMINÉRGICO

FÁBIO ANSELMO

Botucatu - SP

2020

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia e Biotecnologia do Instituto de Biociências de Botucatu da Universidade Estadual Paulista "Júlio de Mesquita Filho" para obtenção do título de Doutor em Farmacologia e Biotecnologia.

Orientador: Prof. Dr. Antonio Francisco Godinho

Co-orientadora: Profª. Drª. Ana Angélica Henrique Fernandes

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"A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê."

(Arthur Schopenhauer)

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LISTA DE ABREVIATURAS E SIGLAS

AAPH - 2',2-azobis-(2- amidinopropane) dihydrochloride
ACA - Arena de campo aberto
AGROFIT - Sistema de Agrotóxicos Fitossanitários
ANOVA - Analysis of variance
ANVISA - Agência Nacional de Vigilância Sanitária
ATR - Atrazina/atrazine
ATR+LEV - Atrazine plus levodopa
ATR+VE - Atrazine plus Vitamin E
BODIPY - Boron-dipyrromethene
CAH - Capacidade antioxidante hidrofílica
CAT - Catalase
CEUA - Ethics Committee on Use of Animals
CNS - Central nervous system
CPF - Córtex pré-frontal
Ct - Control
DA - Dopamina/Dopamine
DDT - Dicloro-difenil-tricloroetano
EDTA - Ethylenediamine tetraacetic acid
EO - Estresse oxidativo
EPA - Environmental Protection Agency
EPM - Elevated plus-maze
ERO - Espécies Reativas de Oxigênio
EU - União Europeia/European Union
FAO - Food and Agricultural Organization of the United Nations
FIOCRUZ - Fundação Oswaldo Cruz
FSI - Fotossistema I
FSII - Fotossistema II
GPx - Glutationa peroxidase
HAC - Hydrophilic antioxidant capacity
HB - Hole-Board
HIP - Hipocampo/hippocampus
HPLC - High performance liquid chromatography
IBGE - Instituto Brasileiro De Geografia e Estatística
INCA - Instituto Nacional de Câncer
LCE - Labirinto em cruz elevado
LD 50 - Lethal Dose 50
LEV - Levodopa
LOAEL - Lowest observed adverse effect level
MAPA - Ministério da Agricultura, Pecuária e Abastecimento
MDA - Malondialdeído/Malondialdehyde

NADH - Nicotinamide adenine dinucleotide (NAD) carrying two electrons and bonded with a hydrogen (H) ion
NBT - Nitro blue tetrazolium
Nrf2 - Nuclear Factor Erythroid 2-Related Factor 2
OE - Oxidative stress
OECD - Organisation For Economic Co-Operation and Development
OF - Open field arena
OPAS - Organização Pan-Americana da Saúde
PC - Phosphatidylcholine
PFC - Prefrontal cortex
ROS - Reactive oxygen species
S.E.M. - Standard error of the mean
SINITOX - Sistema Nacional de Informações Tóxico-Farmacológicas
SNC - Sistema nervoso central
SOD - Superóxido dismutase/Superoxide dismutase
TBA - Thiobarbituric acid
USEPA - Agência de Proteção Ambiental Americana
VE - Vitamina E/Vitamin E
VTA - Ventral tegmental área

JUSTIFICATIVA PARA A REALIZAÇÃO DO TRABALHO

Juntamente com a ampla utilização de praguicidas no mundo, as preocupações sobre seus impactos na saúde cresceram rapidamente. Nas últimas décadas estudos epidemiológicos têm ligado a exposição a praguicidas com o aumento de vários distúrbios neurológicos, como por exemplo a ansiedade, incluindo também uma maior incidência de doenças neurodegenerativas como a doença de Parkinson e Alzheimer. Além disso, na literatura são encontrados poucos e inconclusivos trabalhos que abordam, em caráter neurocomportamental, a exposição repetida a herbicidas, incluindo a atrazina. A atrazina é usada em muitas culturas que são produzidas em grandes quantidades (milho, soja, sorgo, cana-de-açúcar e outras), a fim de aumentar a produtividade. Devido seu uso comum na agricultura, a atrazina chega à água (de superfície e subterrânea) e afeta organismos não-alvo, inclusive seres humanos. Logo, pesquisar o potencial de neurotoxicidade da atrazina trará informações que podem ser de interesse clínico no que diz respeito ao seu modo de ação e a avaliação de danos causados aos seres humanos. Além disso, espera-se que este trabalho suscite questões pertinentes à relação entre saúde ambiental, saúde ocupacional e saúde pública e possa vir a influenciar as tomadas de decisão quanto aos impactos potenciais da exposição à praguicidas.

RESUMO

ANSELMO, F. **Estudo experimental dos efeitos da exposição repetida ao herbicida atrazina sobre a atividade neurocomportamental, biomarcadores do estresse oxidativo e sistema dopaminérgico.** 2020. 71f. Tese (Doutorado) - Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu, 2020.

O herbicida atrazina (ATR) é um praguicida usado intensivamente na agricultura, que persiste no meio ambiente e causa toxicidade em espécies não-alvo, inclusive seres humanos. Considerando o potencial da ATR para produzir efeitos no sistema nervoso central (SNC) e a falta de informações na literatura corrente sobre esse tipo de toxicidade, o objetivo deste projeto de pesquisa foi avaliar os efeitos da exposição subcrônica ao herbicida ATR sobre parâmetros neurocomportamentais específicos e estabelecer sua correlação com o estresse oxidativo (EO) em tecido cerebral de ratos e com o sistema dopaminérgico em regiões específicas do cérebro, nominalmente córtex pré-frontal (CPF), hipocampo (HIP) e corpo estriado. Ratos Wistar machos receberam solução salina (0,3 ml; controle - Ct), atrazina (ATR; 100 mg/kg), atrazina mais vitamin E (ATR; 100 mg/Kg + VE; 200 mg/kg), apenas vitamina E (VE; 200 mg/kg), atrazina mais levodopa (ATR; 100 mg/Kg + LEV; 20 mg/kg) e apenas levodopa (LEV; 20 mg/kg), por gavage, durante 28 dias. O comportamento foi avaliado utilizando arena de campo aberto (ACA), labirinto em cruz elevado (LCE) e hole-board (HB). O teste de estresse oxidativo no tecido cerebral avaliou os níveis de malondialdeído (MDA), capacidade antioxidante hidrofílica (CAH) e atividade das enzimas superóxido dismutase (SOD) e catalase (CAT). A dopamina (DA) foi quantificada por HPLC, em áreas cerebrais específicas (CPF, HIP e estriado). Exposição à ATR provocou ansiedade, diminuiu a coordenação motora e não alterou a atividade locomotora dos animais. A ATR não modificou os níveis de MDA, a CAH nem as atividades da SOD e CAT. No entanto, ATR+VE aumentou os níveis de MDA e a CAH enquanto a VE aumentou a atividade da SOD. Além disso, o tratamento com ATR não alterou os níveis de DA no estriado cerebral, mas aumentou sua concentração no CPF e no HIP. Como conclusão, observou-se que a exposição subcrônica ao herbicida ATR foi capaz de prejudicar parâmetros comportamentais específicos, principalmente aqueles relacionados a ansiedade e a coordenação motora. Esses prejuízos não estão relacionados com a indução de EO no tecido cerebral de ratos. Além disso, sugere-se atenção em situações de suplementação oral por VE, pois, quando combinada com substâncias do tipo praguicidas, pode exercer efeitos pró-oxidantes a nível de SNC. Adicionalmente a ATR foi capaz de causar perturbações nos sistemas dopaminérgicos cerebrais de ratos, principalmente nas regiões do CPF e HIP, não alterando os níveis de DA na região do estriado. Esses resultados reforçam a relação entre exposição à praguicidas e efeitos de neurotoxicidade.

Palavras-chave: atrazina; neurotoxicidade; comportamento; estresse oxidativo; sistema dopaminérgico

ABSTRACT

ANSELMO, F. **Experimental study of the effects of repeated exposure to atrazine herbicide on the neurobehavioral activity, oxidative stress biomarkers and dopaminergic system.** 2020. 71f. Thesis (PhD) - Institute of Biosciences, São Paulo State University "Júlio de Mesquita Filho", Botucatu, 2020.

Atrazine (ATR) herbicide is a pesticide used intensively in agriculture that persists in environment and causes toxicity on non-target species, include humans. Considering the potential of ATR to produce effects on the central nervous system (CNS) and the lack of information in the current literature about this type of toxicity, the aim of this research project was to evaluate the effects of subchronic exposure to ATR herbicide on specific neurobehavioral parameters and to establish its correlation with oxidative stress (OE) in rat brain tissue and with the dopaminergic system in specific brain regions, nominally prefrontal cortex (PFC), hippocampus (HIP) and striatum. Male Wistar rats received saline (0.3 ml; control - Ct), atrazine (ATR; 100 mg/kg), atrazine plus vitamin E (ATR; 100 mg/Kg + VE; 200 mg/kg) only vitamin E (VE; 200 mg/kg), atrazine plus levodopa (ATR; 100 mg/Kg + LEV; 20 mg/kg) and only levodopa (LEV; 20 mg/kg), via gavage, during 28 days. Behavior was assessed using open field arena (OF), elevated plus-maze (EPM) and hole-board (HB) apparatus. Oxidative stress assay in brain tissue evaluated malondialdehyde (MDA) levels, hydrophilic antioxidant capacity (HAC), and superoxide dismutase (SOD) and catalase (CAT) enzymes activity. Dopamine (DA) was quantified using HPLC, in specific brain areas (PFC, HIP and striatum). ATR exposure caused anxiety-like behavior, decreased motor coordination and did not alter the locomotor activity of the animals. ATR did not change the MDA levels, the HAC, or SOD and CAT activities. However, ATR+VE increased MDA levels and the HAC while VE increased SOD activity. Additionally, ATR treatment did not change DA levels in the striatum cerebral, but increased its concentration in PFC and HIP. As a conclusion, it was observed that subchronic exposure to ATR herbicide was able to impair specific behavioral parameters, especially those related to anxiety and motor coordination. These impairs are not related to OE induction in brain tissue of the rats. Moreover, attention is suggested in situations of oral VE supplementation, because, when combined with substances like pesticides, it can exert pro-oxidant effects in the CNS. Additionally, ATR was able to cause disturbances in the brain dopaminergic systems of rats, mainly in the regions of PFC and HIP, without altering the DA levels in the striatum region. These results reinforce to relation between pesticides exposure and neurotoxic effects.

Keywords: atrazine; neurotoxicity; behavior; oxidative stress; dopaminergic system

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INTRODUÇÃO

1. Praguicidas

Praguicida, denominação utilizada para designar um grupo de agentes químicos tóxicos, são substâncias ou misturas de substâncias que tem como objetivo prevenir, destruir ou combater qualquer tipo de praga presente em cultivo agrícola. São frequentemente utilizados no meio agrícola para controlar uma variedade de insetos e pragas, e nos animais, por via tópica ou oral, para o controle de parasitas (Poppenga e Oehme, 2010; Amaral, 2012).

Praga (do latim *plaga*) refere-se a tudo aquilo que ataca, lesa ou transmite enfermidade às plantas, aos animais e ao homem, como ervas daninhas, fungos, insetos, carrapatos, aracnídeos ou qualquer outra forma de vida vegetal ou animal considerada danosa à saúde e ao bem-estar do homem, à lavoura, à pecuária e aos seus produtos e matérias-primas alimentares (Spinosa e Xavier, 2008).

O uso de praguicidas iniciou-se na década de 1920, quando havia pouco conhecimento sobre seu potencial tóxico. Na Segunda Guerra Mundial (1939-1945) essas substâncias foram empregadas como armas químicas (OPAS, 1996). No período do pós-guerra houve um crescimento populacional e econômico que exigiu um aumento pela demanda de alimentos e matérias-primas. Desta forma, o emprego dos praguicidas tornou-se necessário para aumentar a produtividade agrícola (Moragas e Schneider, 2003). Esse aumento no uso de praguicidas, juntamente com o desenvolvimento de novas sementes (resistentes a doenças, com maior rendimento) e aplicação de novas práticas (mudança de irrigação, por exemplo) foram denominados Revolução Verde, a qual ocorreu por volta de 1960 (Raley-Susman, 2014).

As primeiras substâncias utilizadas como praguicidas foram agentes tóxicos de origem natural, tais como nicotina, piretro, além de substâncias inorgânicas como o mercúrio e o enxofre. O uso de praguicidas mais modernos teve seu início por volta de 1940, quando Paul Müller descobriu o inseticida dicloro-difenil-tricloroetano (DDT). O DDT era muito eficaz, transformando-se rapidamente no praguicida mais utilizado no mundo. Entretanto, na década de 1960, descobriu-se que o DDT provocava danos à saúde de diversas espécies de aves, prejudicando sua reprodução e oferecendo grandes riscos à biodiversidade (Amaral, 2012).

No Brasil, os praguicidas tiveram primeiramente seu uso em programas de saúde pública, no combate a vetores de parasitas, sendo mais utilizados de forma intensiva na agricultura somente a partir de 1960 (OPAS, 1996). Atualmente, grandes empresas

produtoras de praguicidas, fertilizantes e sementes, dominam o agronegócio (Raley-Susman, 2014).

De acordo com a Agência Nacional de Vigilância Sanitária - ANVISA, em 2008 o Brasil alcançou o primeiro lugar no *ranking* de maior consumidor de praguicidas do mundo, ultrapassando os Estados Unidos. No ano de 2009 o consumo continuou aumentando, observando-se uma média de uso de 5,2 Kg de agrotóxicos por habitante. O consumo não parou de crescer e no ano de 2011 foram consumidos 852,8 milhões de litros. (ANVISA, 2012; IBGE, 2012). Segundo o Ministério da Agricultura, Pecuária e Abastecimento - MAPA, em 2017, as exportações brasileiras do agronegócio somaram US$ 96,01 bilhões, registrando 13% de crescimento em relação ao ano de 2016. O setor foi responsável por 44,1% do total das vendas externas do Brasil. O superávit foi de US$ 81,86 bilhões sendo o segundo maior da história, inferior apenas ao registrado em 2013 (R$ 82,91 bilhões). Este crescente consumo é alarmante, pois já são conhecidos os efeitos deletérios causados por estes produtos (MAPA, 2018).

Os praguicidas de uso agrícola somados aos de uso doméstico foram causadores de 5,67% dos casos de intoxicações registrados no ano de 2016 no Brasil. Com relação aos óbitos provocados por intoxicação humana, os praguicidas foram responsáveis por 45,07% dos casos (SINITOX, 2016). Em 2016, o Sistema Nacional de Informações Tóxico-Farmacológicas (SINITOX) registrou mais de trinta e nove mil casos de intoxicação humana e mais de cem casos de óbitos decorrentes de intoxicação, sendo os praguicidas os agentes de maior taxa de letalidade. Esses dados, entretanto, mostram parcialmente a realidade do Brasil, uma vez que segundo o Ministério da Saúde, para cada evento de intoxicação por praguicidas, existem outros cinquenta casos não notificados (FIOCRUZ, 2015).

Em consequência da sua elevada utilização no Brasil e aos seus comprovados efeitos deletérios, o Instituto Nacional do Câncer (INCA) se posicionou contra as atuais práticas de uso de praguicidas e ressaltou seus riscos à saúde e principalmente à exposição crônica, a qual pode gerar infertilidade, impotência, abortos, malformações, desregulação hormonal, neurotoxicidade, efeitos sobre o sistema imunológico e câncer (INCA, 2015).

Os praguicidas mais aplicados atualmente no Brasil são os herbicidas, seguido pelos inseticidas, fungicidas e acaricidas. Diversas formulações utilizadas aqui são proibidas em países da Europa, em decorrência da sua toxicidade ou às alterações que promovem no ambiente (IBGE, 2012; Carneiro, 2015). Os herbicidas estão entre as

substâncias mais tóxicas devido à sua elevada estabilidade química, resistência ao metabolismo, e a sua eficiência em penetrar em ambientes celulares em razão das suas propriedades lipofílicas (El-Amrani et al., 2012). Neste contexto, um problema que surge da utilização generalizada de herbicidas é a bioacumulação, o que afeta a estabilidade dos ecossistemas, a cadeia alimentar, e também a saúde animal e humana (Bolle et al., 2004). Segundo a União Europeia (EU) e a Agência de Proteção Ambiental Americana (USEPA), o herbicida atrazina é um dos compostos que estão na lista de poluentes prioritários por causa da sua persistência ambiental e sua toxicidade (Oliveira, 2008).

2. Atrazina

A atrazina (2-cloro-4-(etilamino)-6-isopropilamino-s-triazina) (figura 1) é um herbicida sistêmico pertencente ao grupo das cloro-s-triazinas amplamente utilizado como pré e pós-emergente no controle de ervas daninhas. (LeBaron et al., 2008). A ATR age nas plantas alvo como inibidor da fotossíntese, mais precisamente inibindo o fotossistema II (FSII) que faz parte da fase fotoquímica da fotossíntese, quando a energia luminosa é absorvida e convertida em energia química. O FSII é o primeiro sistema de proteínas envolvido na fotossíntese e está associado a um complexo de proteínas receptoras e doadoras de elétrons (Roman, 2005).

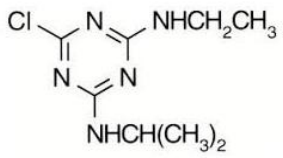


Figura 1. Fórmula estrutural da atrazina (Prade et al., 1998)

Simplificadamente, o mecanismo de ação da ATR consiste no bloqueio do processo fotossintético da planta a ser controlada, através da interferência no fluxo fisiológico normal de elétrons do FSII para o fotossistema I (FSI) nos cloroplastos. Além da inibição da fotossíntese, como a captação de energia solar é contínua e não há transferência de elétrons, as clorofilas atingem uma carga energética elevada, resultando na produção de radicais livres que atuam desencadeando a peroxidação de lipídios de membrana. Como consequência destes processos, ocorre clorose e subsequente necrose de tecidos levando à planta a morte. Nas espécies tolerantes a ATR, como é o caso do

milho por exemplo, o herbicida é eficientemente metabolizado em formas não tóxicas (Marchi et al., 2008).

A ATR possui massa molecular de 215,7 g/mol, sendo considerada uma base fraca (pKa ~1,7), com grau de volatilização, expresso em pressão de vapor, igual a $4x10^{-5}$ Pa (a 20 °C). Apresenta solubilidade em água de 33 mg/L (a 20 °C), que está vinculada à sua baixa polaridade (log Kow de 2,2-2,7). As propriedades da ATR resultam em moderada mobilidade e elevada persistência, contribuindo para a acumulação a níveis contaminantes tanto na superfície como em profundidade nos solos e potencialmente atingindo águas superficiais e subterrâneas (Prosen, 2012).

A Tabela 1 apresenta alguns produtos comercializados no Brasil, juntamente com a dosagem recomendada, e suas classificações toxicológicas e de risco ambiental. Os acréscimos de rendimento, oriundos do eficiente controle de plantas daninhas por meio do uso da ATR, variam de acordo com o sistema de cultivo sendo que, segundo Carlson (1998), o impacto parece ser maior em sistema de plantio direto do que no plantio convencional. Além do sistema de cultivo, a dosagem e a época de aplicação da ATR também devem considerar o tipo de solo, conteúdos de matéria orgânica e argila e também o clima (Pike et al., 2008).

Tabela 1. Alguns produtos comercializados no Brasil que possuem a atrazina como ingrediente ativo

Nome comercial	**Dosagem recomendada***	**Classificação toxicológica****	**Classificação de risco ambiental*****
Atrazina Nortox 500 SC®	1,5 a 4 Kg/ha	III - Medianamente tóxico	II - Produto muito perigoso ao meio ambiente
Proof®	2 a 2,5 Kg/ha	IV - Pouco tóxico	II - Produto muito perigoso ao meio ambiente
Atrazina Atanor 50 SC®	2 a 3 Kg/ha	III - Medianamente tóxico	III - Produto perigoso ao meio ambiente

* As doses podem variar de acordo com o tipo de solo a cultivar.
** Os produtos são classificados em ordem decrescente de toxicidade, de I (extremamente tóxico) a IV (pouco tóxico).
*** Os produtos são classificados em ordem decrescente de risco, de I (produto altamente perigoso) a IV (produto pouco perigoso).
Fonte: AGROFIT (2020).

2.1. Atrazina e meio ambiente

As abordagens ambientais no estudo da ATR visam avaliar o risco ambiental do herbicida, seus resíduos e sua acumulação na água e em solos (Hang et al., 2007). Usualmente, quanto maior o tempo de meia-vida no solo, maior tende a ser o potencial de contaminação das águas (Rebelo e Caldas, 2014). Tradicionalmente, o tempo de

meia-vida da ATR no solo, ou seja, o tempo necessário para que 50% do herbicida aplicado sejam dissipados, é considerado como sendo 60 dias. No entanto, os valores de meia-vida podem variar de alguns dias até anos, dependendo das condições e fatores do meio (Graymore et al., 2001; Krutz et al., 2010). Problemáticas surgem pelo fato de muitos estudos de persistência serem realizados em laboratório e/ou em condições de campo, com o tempo de meia-vida sendo estimado a partir de experimentos de curta duração. Neste sentido, a persistência da ATR no solo pode ser subestimada.

Em estudos na Alemanha, a ATR e seus resíduos foram extraídos de solos mesmo depois de mais de duas décadas após a sua última aplicação (Jablonowski et al., 2009; Vonberg et al., 2014). Assim, quando aplicada por muitos anos, solos agrícolas potencialmente acumulam ATR e seus resíduos, que podem ser mobilizados, por exemplo, através de ciclos entre períodos secos e úmidos, e então ser lixiviados e contaminar águas subterrâneas (Jablonowski et al., 2009). Portanto, considerando as elevadas quantidades aplicadas anualmente, juntamente com pesquisas que demonstram sua elevada persistência nos solos, a ATR representa potencial ameaça ao ambiente (Jablonowski et al., 2011).

Nesse mesmo sentido, estudos mostram que a ATR e/ou seus metabólitos são frequentemente detectados no solo, águas subterrâneas, superficiais e na água de beber, (Mosquin et al., 2012), em famílias de agricultores (Lozier et al., 2012), em amostras de urina de aplicadores de praguicidas, além da população em geral (Curwin et al., 2007). Logo, sua ampla utilização e detecção frequente origina preocupações sobre seus potenciais efeitos adversos a saúde.

2.2. Efeitos toxicológicos da atrazina

Embora haja muito a aprender sobre os efeitos da atrazina, muitos trabalhos demonstram uma série de efeitos adversos consistentes da ATR que devem ser pesados contra qualquer um de seus benefícios, ou seja, ela causa efeitos negativos para diversas espécies (Rohr e McCoy, 2010).

A ATR é absorvida por via oral, inalatória e dérmica, somente penetrando na pele numa área muito limitada. Em ratos, a meia vida é de 1,3 dias, e 95% da dose é eliminada dentro de sete dias. Mesmo doses baixas, administradas diariamente em ratos, possibilitou a detecção da substância nos tecidos desses animais. É biotransformada principalmente no fígado e sua excreção ocorre pela urina (75%) e pelas fezes (20%) (Coutinho, 2005).

Estudos mostraram que a ATR é capaz de causar diversas alterações graves em anfíbios, principalmente alterações hormonais, sendo comum ocorrer o fenômeno da feminilização ou até a "castração química" desses animais. Nestes casos a ATR interferiu no desenvolvimento sexual favorecendo a conversão da testosterona em estradiol causando efeitos estrogênicos e levando os anfíbios feminilizados a serem capazes de produzir ovos viáveis. (Carr et al., 2003; Kloas et al., 2009; Hayes et al., 2010).

Estudos com ratos também mostraram que a exposição à ATR é prejudicial para diversos sistemas do organismo, incluindo o sistema imune (Filipov et al., 2005), sistema endócrino (Ghiselli e Jardim, 2007) e principalmente sistema reprodutor com adição para alterações de caráter genotóxico (Feyzi-Dehkharagni et al, 2012). Existem muitas evidências de que em roedores, a ATR produz efeitos adversos tanto no sistema reprodutor masculino quanto no feminino (Kale et al., 2018).

Além disso, vários estudos têm demonstrado certa relação entre a contaminação por ATR ou seus produtos de degradação e anomalias pré-natais na população humana em geral como nascimentos prematuros e/ou abortos espontâneos, interferência no ciclo menstrual e redução da fertilidade (Hase et al., 2008; Chevrier et al., 2011; Cragin et al., 2011). Munger et al. (1997) constataram altas taxas de nascimento de crianças prematuras em Iowa (EUA) em comunidades que consumiam água com altas concentrações de ATR. Cragin et al. (2011) mostraram que a ATR pode ter provocado desregulação no ciclo menstrual das mulheres que consumiam água contaminada por este herbicida sendo evidenciada sua ação antiestrogênica e Hase et al. (2008) relataram que a exposição a ATR pode levar a redução da qualidade do sêmen. Adicionalmente, inúmeros relatórios sugerem exposição prolongada à ATR como um potencial fator de risco para distúrbios neurológicos particularmente entre trabalhadores agrícolas (Dardiotis et al., 2013; Abarikwu e Farombi, 2015; Song et al., 2015; Liu et al., 2016).

Por outro lado, se tratando de efeitos neurotóxicos, a quantidade de estudos relacionando esses efeitos com a exposição à ATR são ainda pouco encontrados. Bardullas et al. (2011) mostraram que exposição oral à ATR em roedores produz alterações neurocomportamentais que são acompanhadas por uma diminuição dos níveis de dopamina no corpo estriado do cérebro. Lin et al. (2013) também chegaram a conclusões parecidas, mostrando que a exposição durante um curto período à ATR causou diminuição na concentração de DA na região estriada do cérebro de roedores. Adicionalmente, Li et at. (2014) demonstraram que no caso de alta exposição, como por

exemplo a ocupacional, a ATR pode inibir a captação vesicular e o armazenamento de DA. Logo, as reduções de DA sugerem que a ATR é um tóxico dopaminérgico e pode ser um fator de risco para doença de Parkinson, uma vez que esta doença caracteriza-se pelo déficit de dopamina nas vias nigroestriatais do cérebro.

A levodopa ou L-dopa é um medicamento destinado ao tratamento da doença de Parkinson. A L-dopa, sendo um precursor da dopamina entra no cérebro através da barreira hemato-encefálica e após ser catalisada pela enzima dopa-descarboxilase forma a DA, aumentando assim, a concentração intracraniana deste neurotransmissor e restaurando o sistema de transmissão dopaminérgico (Wang et al., 2017). Como a dopa-descarboxilase existe tanto perifericamente quanto centralmente, a L-dopa é combinada com um inibidor da dopa-descarboxilase (a carbidopa, por exemplo) para evitar os efeitos periféricos.

Embora os efeitos da ATR sobre a dopamina no estriado e células produtoras de catecolaminas serem relatados em alguns estudos (Filipov et al., 2007; Bardullas et al., 2011; Lin et al., 2013; Li et al., 2014), pouco se sabe sobre os seus efeitos nas outras monoaminas, como a serotonina. Também é desconhecido se ATR afeta outras estruturas cerebrais, tais como o córtex pré-frontal e hipocampo, que recebem projeções dopaminérgicas da área tegmental ventral (Alcaro et al., 2007).

Se tratando de comportamento, a exposição crônica de ratos a ATR prejudica a coordenação motora, diminui a atividade locomotora espontânea e altera a memória espacial desses animais (Bardullas et al., 2011). Kale et al. (2018) também observaram prejuízo nas funções motoras dos animais expostos à ATR. Já os resultados de Lin et al. (2013) indicaram que exposição a curto prazo à ATR induz múltiplas anomalias comportamentais envolvendo alterações motoras, cognitivas e também emocionais.

O fato do desempenho alterado no teste de reconhecimento de um novo objeto, utilizado para avaliar memória cognitiva, ocorrer em doses que não alteraram a atividade motora e o sistema de monoaminas cerebrais, indica que determinados efeitos comportamentais podem ser particularmente sensíveis à ATR e poderia haver o envolvimento de outras regiões do cérebro, além da parte estriada. Contudo, a dose-dependência dos efeitos da ATR em diferentes comportamentos em roedores adultos não foi ainda muito bem investigada (Lin et al., 2013).

Adicionalmente, alguns estudos demonstraram que a ATR pode promover estresse oxidativo, aumentando a concentração de espécies reativas de oxigênio (ERO) e produtos de danos oxidativos, tais como peróxidos de lipídios e malondialdeído e

consequentemente influenciando a atividade de enzimas antioxidantes, como a superóxido dismutase, glutationa peroxidase (GPx) e catalase (Abarikwu et al., 2010; Adesiyan et al., 2011). Singh et al. (2011) mostraram que a ATR pode induzir respostas ao estresse oxidativo no fígado de ratos após administração por um período de vinte e oito dias. Além disso, Griboff et al. (2014) observaram em um estudo com camarões que a suplementação na dieta com vitamina E pode aumentar a resistência a exposição ATR, como indicado pela reversão nas respostas dos biomarcadores do EO avaliados, uma vez que a VE é um dos antioxidantes mais eficazes encontrado no sistema biológico.

Apesar desses estudos mostrarem que a ATR causa estresse oxidativo, este efeito de toxicidade foi observado principalmente em tecidos como fígado e rins, havendo ainda carência de estudos que avaliem o EO no tecido cerebral e sua relação com efeitos neurocomportamentais, uma vez que o cérebro pode ser vulnerável ao EO pois consome grandes quantidades de oxigênio, resultando em aumento da produção de EROs (Patel, 2016). Além disso, a maioria dos estudos em animais utiliza altas concentrações de ATR, concentrações essas que normalmente não são encontradas no meio ambiente (Semren, 2018).

Portanto, de maneira geral, existem ainda poucos trabalhos de pesquisa relacionando exposição à ATR com possíveis efeitos de neurotoxicidade e os que existem são ainda pouco conclusivos. Por isto tornam-se necessários mais dados experimentais no sentido de comprovar estes achados ou encontrar outros efeitos ligados à neurotoxicidade provocada pela atrazina.

3. Bioindicadores e biomarcadores

Estudos de alterações fisiológicas, comportamentais ou de sobrevivência em um organismo, ou em uma população, constituem-se como bioindicadores (Jonsson e Castro, 2005). Esta determinação deve estabelecer uma relação entre à exposição a agentes químicos e os seus efeitos tóxicos refletidos em alterações pré-clínicas e ainda permitir entender os mecanismos relacionados à ação tóxica nos indivíduos (Castro, 2006). Estas investigações neurofisiológicas são importantes, nos casos em que os indivíduos (animais ou humanos) são expostos a produtos químicos tanto a baixas doses durante um longo período, quanto a uma única dose elevada (Szegedi et al., 2005).

Já biomarcador compreende toda substância ou seu produto de biotransformação ou ainda qualquer alteração bioquímica precoce cuja determinação possa ser realizada

nos fluidos biológicos, tecidos ou ar exalado. Há várias evidências experimentais mostrando que, na progressão de doenças neurotóxicas, os eventos bioquímicos precedem as alterações estruturais e danos permanentes ao sistema nervoso central (Castro, 2006).

3.1. Avaliações neurocomportamentais

Comportamento tem sido utilizado como um bioindicador de função para estudar efeitos tóxicos de agentes químicos. Alterações do comportamento em animais expostos a um determinado agente químico podem servir como indicativo de que a substância provoca neurotoxicidade. A importância de estudos neurocomportamentais reside no fato de que comportamento é considerado como um produto final funcional de vários processos sensoriais, motores e integrativos, que ocorrem no sistema nervoso (Terçariol, 2007). Embora vários estudos procurem compreender os mecanismos de neurotoxicidade dos praguicidas em mamíferos, há poucos relacionados aos efeitos neurocomportamentais e estes podem ser úteis para avaliar os efeitos das exposições às substâncias químicas (Martins, 2009).

Um dos aspectos da neurotoxicidade que pode ser estudado através dos testes comportamentais é o da ansiedade/emocionalidade. Algumas tarefas experimentais têm sido utilizadas para estudar este componente comportamental. A arena de campo aberto (Figura 2) é o mais antigo dos testes de ansiedade que são baseados na exploração espontânea de um novo ambiente (Behr et al., 2009). O campo aberto foi criado em 1934 e é utilizado para avaliar principalmente a atividade locomotora, exploração e emocionalidade de roedores, porém também pode ser utilizado para avaliar ansiedade (Martins, 2009).



Figura 2. Arena de Campo Aberto. **Fonte:** Arquivo pessoal

Um dos fatores relevantes na observação do comportamento do animal é a sua tendência a movimentar-se, conhecida como atividade geral. A quantificação dessa locomoção pode refletir o comportamento, uma vez que o animal tenderá a permanecer imóvel *(freezing)* quando apresentar alto grau de ansiedade. Nesse sentido, a exploração do roedor é inversamente proporcional ao medo ou emocionalidade (Udo, 2012). Os organismos, quando expostos a situações de ameaça e perigo, respondem com uma série de respostas adaptativas, físicas e mentais que se opõe aos estímulos estressores na tentativa de manter a homeostasia (Benetti, 2005).

Além da locomoção, o comportamento de levantar (animal apoiado nas patas traseiras, eleva o tronco para farejar o ambiente verticalmente) é a manifestação de um processo exploratório de aproximação das regiões modificadas do ambiente (Silva, 2008; Martins, 2009; Udo, 2012). Além disso, o teste em ACA é comumente utilizado para triagem farmacológica de drogas que atuam sobre o sistema nervoso central (Terçariol, 2007).

O teste do labirinto em cruz elevado (Figura 3) foi criado por Montgomery em 1955 e é um dos principais modelos usados no estudo da ansiedade. Esse teste é baseado no comportamento exploratório de locais novos e na observação de respostas comportamentais em situações que poderiam ou não provocar perigo. Ele consiste em analisar o grau de aversão do animal aos braços abertos, em conflito com o comportamento exploratório (Udo, 2012), já que ambientes abertos, sem proteção lateral, são naturalmente aversivos para o roedor (Martins, 2009).



Figura 3. Labirinto em Cruz Elevado. **Fonte:** Arquivo pessoal

A exposição aos braços abertos do LCE produz significativamente mais ansiedade quando comparada à exposição aos braços fechados. Drogas ansiolíticas

elevam a procura dos roedores pelos braços abertos quando comparadas com a procura pelos braços fechados e, em contraste, drogas ansiogênicas diminuem a procura pelos braços abertos (Silva, 2008; Martins, 2009).

O aparelho Hole-Board (Figura 4) oferece um método simples de medir as respostas de um rato a um ambiente novo e tem sido amplamente utilizado para avaliar os efeitos de drogas (File e Wardill, 1975). Trata-se de um aparelho que tem sido utilizado para avaliar comportamentos de coordenação motora (deslizamento de patas) e comportamento exploratório (introduções de cabeça) frente à exposição a agentes químicos neurotóxicos (Kuc et al., 2006; Godinho et al. 2014).

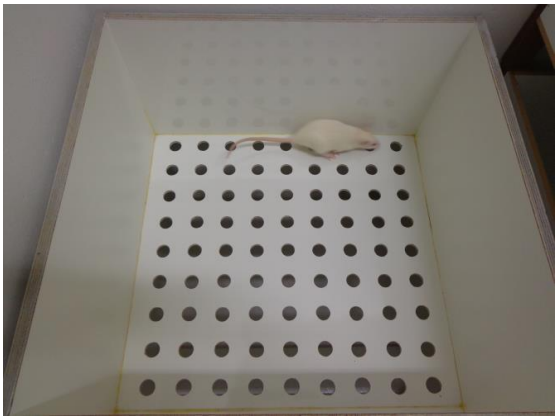


Figura 4. Hole-Board. **Fonte:** Arquivo pessoal

3.2. Avaliação dos biomarcadores de estresse oxidativo

Na experimentação animal, esta avaliação bioquímica pode ajudar a entender e às vezes, caracterizar a relação de processos danosos que compõem o quadro geral de intoxicação. A estimulação da produção de radicais livres, prejuízo da capacidade antioxidante e a peroxidação lipídica são mecanismos de toxicidade dos praguicidas, e tem sido associado ao desenvolvimento de doenças causadas pela exposição a estes agentes tóxicos. Além disso, é reconhecido que o estresse oxidativo tem papel importante na etiologia e progressão de doenças crônicas (Abdollahi et al., 2004; Soltaninejad e Abdollahi, 2009; Rajendran, 2014; Gallegos et al., 2018).

O estresse oxidativo ocorre quando há um desequilíbrio entre as espécies reativas de oxigênio e a capacidade antioxidante da célula. Esse desequilíbrio resulta na produção de radicais livres e danos a componentes celulares, como lipídios de membrana, proteínas, ácidos nucléicos e outras pequenas moléculas (Mostafalou e Abdollahi, 2013; Rajendran, 2014).

O sistema antioxidante é divido em enzimático e não enzimático sendo responsável por neutralizar as espécies reativas de oxigênio. As enzimas superóxido dismutase, catalase, e glutationa peroxidase pertencem ao sistema antioxidante enzimático, e estão envolvidas na manutenção do balanço REDOX e por neutralizar as ERO, como vemos na Figura 5. Porém, quando ocorre aumento do estresse oxidativo a capacidade de defesa contra as ERO pode colapsar (Birben et al., 2012; Nwani et al., 2016).

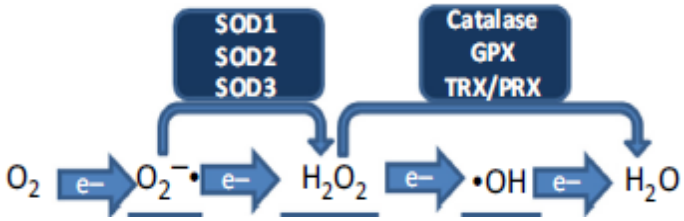


Figura 5. Redução sequencial do O_2 pelas enzimas antioxidantes (Patel, 2016).

3.3. Quantificação de dopamina

Da mesma maneira que o estresse oxidativo é reconhecido como um dos mecanismos de toxicidadade dos praguicidas, diversos estudos mostram que a alteração dos níveis de dopamina em partes específicas cerebrais pode indicar efeito de neurotoxicidade e estar associado a diversos tipos de doenças (Filipov et al., 2007; Bardullas et al., 2011; Lin et al., 2013; Li et al., 2014).

Os sistemas dopaminérgicos são conhecidos por participarem do controle motor do indivíduo (Schultz, 2007), desse modo, não é surpreendente que a exposição à agentes tóxicos do tipo praguicidas induzam a déficits motores, provavelmente devido a perturbações nos circuitos de DA, principalmente pela via nigroestriatal (Lin et al., 2013). Dentre as funções da dopamina produzida na via nigroestriatal estão, além do controle motor, a regulação do humor, do estresse, da ansiedade entre outras (Delucia, 2006).

Além disso, a DA é um neurotransmissor produzido em várias regiões cerebrais, além da via nigroestriatal, como por exemplo a via mesolímbica e mesocortical, logo várias funções podem ser comprometidas em decorrência de perturbações nos circuitos dopaminérgicos (Standaert, 2009). Desta maneira, fica evidente a importância de investigar possíveis alterações deste neurotransmissor em estudos de exposição à agentes tóxicos.

4. Estrutura da tese

Esta tese de doutorado foi elaborada seguindo estrutura de artigo científico, a qual possibilitou a elaboração de dois artigos científicos.

O primeiro artigo foi submetido à revista Pharmacology Biochemistry and Behavior, intitulado "Subchronic exposure to atrazine herbicide in rats induces neurobehavioral effects not associated to oxidative stress in brain tissue". Ele consistiu em uma avaliação dos efeitos da exposição subcrônica à ATR sobre parâmetros neurocomportamentais específicos e sua relação com o estresse oxidativo em tecido cerebral de ratos.

O Segundo artigo será submetido a revista Toxicology and Applied Pharmacology, intitulado "Neurobehavioral and neurochemical effects caused by subchronic exposition to atrazine herbicide in rats". Ele consistiu em uma avaliação dos efeitos da exposição subcrônica à ATR sobre parâmetros neurocomportamentais específicos e sua relação com o sistema dopaminérgico em áreas específicas do cérebro de ratos.

OBJETIVO

O objetivo deste projeto de pesquisa foi avaliar os efeitos da exposição subcrônica ao herbicida atrazina sobre parâmetros neurocomportamentais específicos e estabelecer sua correlação com o estresse oxidativo em tecido cerebral de ratos e com o sistema dopaminérgico em regiões específicas do cérebro, nominalmente córtex pré-frontal, hipocampo e corpo estriado.

CAPÍTULO 1

Trabalho submetido à revista Pharmacology Biochemistry and Behavior (Elsevier, ISSN: 0091-3057; IF: 2.773)

Subchronic exposure to atrazine herbicide in rats induces neurobehavioral effects not associated to oxidative stress in brain tissue

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Abstract

Atrazine (ATR) herbicide is a pesticide used intensively in agriculture that persists in environment and causes toxicity on non-target species, include humans. Considering the potential of ATR to produce effects on the central nervous system (CNS) and the lack of information in the current literature about this type of toxicity, the aim of the present study was to evaluate the effects of subchronic exposure to ATR on specific neurobehavioral parameters and its relation with oxidative stress in rat brain tissue. Male Wistar rats received saline (0.3 ml; control - Ct), atrazine (ATR; 100 mg/kg), atrazine plus vitamin E (ATR; 100 mg/Kg + VE; 200 mg/kg) and only vitamin E (VE; 200 mg/kg), via gavage, during 28 days. Behavior was assessed using open field arena (OF), elevated plus-maze (EPM) and hole-board (HB) apparatus. Oxidative stress assay in brain tissue evaluated malondialdehyde (MDA) levels, hydrophilic antioxidant capacity (HAC), and superoxide dismutase (SOD) and catalase (CAT) enzymes activity. ATR exposure caused anxiogenic-like behavior, decreased motor coordination and rearing behavior with no change MDA levels, the HAC, or SOD and CAT activity in brain. However, ATR+VE increased MDA levels and HAC and VE increased SOD activity. The results suggest that, although ATR produces neurotoxicity, evidenced by impaired behavioral parameters, it is not related to induction of brain tissue oxidative stress. In addition, attention is required in situations of oral VE supplementation, once VE combined with substances like pesticides a pro-oxidant effect may be observed in the CNS.

Keywords: atrazine; vitamin E; behavior; oxidative stress; neurotoxicity

1. Introduction

Herbicides are among the most toxic substances due to their high chemical stability, metabolism resistance and their ability to penetrate cellular environments because of their lipophilic properties (El-Amrani et al., 2012). According to the European Union (EU) and the U.S. Environmental Protection Agency (EPA), atrazine herbicide is one of the compounds on the priority pollutant list due to its environmental persistence and toxicity (Grube et al., 2011).

Atrazine herbicide (ATR; 2-chloro-4-(ethylamino)-6-isopropylamino-s-triazine) belongs to the group of chloro-s-triazines (LeBaron et al., 2008) and is widely used worldwide, mainly for control annual grass and broadleaf weeds in crops such as corn, sorghum and sugar cane. Due to its intensive use in agriculture, ATR reaches surface waters and groundwater, affecting non-target organisms, including humans (Barbash et al., 2001; Dooley et al., 2013).

Previous reports indicate that the risk of exposure to ATR is not only occupational, since it is also found in food and farming households or near rural areas (Lioy et al., 2000; Curwin et al., 2005; Gammon et al., 2005). Therefore, its widespread use and frequent detection result in concerns about its potential adverse health effects.

Studies have shown that ATR causes several serious changes in amphibians, especially hormonal changes, being common occur the phenomenon of feminization or even "chemical castration" of several amphibian species (Hayes et al., 2010). Studies with rats also show that exposure to ATR is detrimental to a variety of body systems, including the immune (Filipov et al., 2005), endocrine (Ghiselli and Jardim, 2007) and reproductive system, with addition for alterations of genotoxic character (Feyzi-Dehkharagni et al., 2012). In addition, other studies have shown some relation between ATR contamination or its degradation products and prenatal abnormalities in general human population such as premature births and/or spontaneous abortions, interference with the menstrual cycle and reduced fertility (Hase et al., 2008; Chevrier et al., 2011; Cragin et al., 2011).

On the other hand, in the case of neurotoxic effects, studies relating these effects with exposure to ATR are still rarely found. Bardullas et al. (2011) showed that oral exposure to ATR impaired motor coordination and modulated spontaneous locomotor activity in rats. The results of Lin et al. (2013) indicated that short-term exposure to ATR induces multiple behavioral anomalies in rodents involving motor, cognitive and emotional changes.

Additionally, some studies have shown that ATR can promote oxidative stress by increasing the concentration of reactive oxygen species (ROS) and products of oxidative damage such as lipid hydroperoxide and malondialdehyde, and consequently influencing the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Abarikwu et al., 2010; Adesiyan et al., 2011). According to Singh et al. (2011) ATR may induce oxidative stress in rat liver in terms of enhanced lipid peroxidation.

Moreover, Griboff et al. (2014) suggested in a study with shrimps that vitamin E diet supplementation may increase the resistance to ATR exposure, as indicated by the reversal in response of the oxidative stress biomarkers evaluated.

These toxicity effects of ATR involving oxidative stress were mainly observed in tissues such as liver and kidney. However, there is a lack of studies evaluating oxidative stress in brain tissue and especially its relation with neurobehavioral effects, considered as biomarkers of CNS effect. In addition, most animal studies use high concentrations of ATR, which are not normally found in the environment (Semren, 2018).

Therefore, considering the potential of ATR to produce effects on the CNS and the lack of information in the current literature about this type of toxicity, the aim of the present study was to evaluate the effects of subchronic exposure to ATR on specific neurobehavioral parameters and its relation with oxidative stress in rat brain tissue.

2. Material and methods

The experimental protocol was approved by the Ethics Committee on Use of Animals (CEUA) of the Bioscience Institute, São Paulo State University, under the number 897/2016. All procedures were performed in accordance with institutional guidelines for animal use and care. The product used for the experiments was the technique formulation Atrazina 500 SC®, produced by Nortox S/A, containing atrazine 500 g/L (2-chloro-4-(ethylamino)-6-isopropylamino-s-triazine). Vitamin E was acquired of the Purifarma-Brazil.

2.1. Animals

Experiments utilized sixty adult (80 days old) male Wistar rats obtained from the colony housed at the Sao Paulo State University, and kept under a constant 12 h

light/dark cycle and controlled temperature (22 ± 2 °C). Standard pellet chow (Presence Animal Nutrition, Paulínia/SP, Brazil) and tap water were available *ad libitum*.

2.2. Experimental Procedure

For experiments, animals were randomly divided into four experimental groups (N=15), receiving daily during 28 days (OECD, 1997), by gavage, respectively: saline (0.3 mL; control - Ct), atrazine (ATR; 100 mg/kg), atrazine plus vitamin E (ATR; 100 mg/Kg + VE; 200 mg/kg) and only vitamin E (VE; 200 mg/kg). The choice of oral route (gavage) was based in the principle of that general population receives low doses of atrazine through contaminated food and water.

According to FAO (2005), for oral rat studies, atrazine LOAEL ranged from 10-70 mg/kg but there was no study to evaluate LOAEL specifically for neurotoxicity. On the other hand, atrazine LD50 in orally exposed rats was 1870 to 3090 mg/kg. Based on these data and on the behavioral studies by Lin et al. (2013) and Rodríguez et al. (2013) the dose chosen for this study was 100 mg/kg. The vitamin E dose was chosen based on the experiments of An et al. (2015).

During the period of ATR exposure, animals were observed for possible symptoms of toxicity following exposure (mood swings, aggression, seizure, death, etc.), and were weighed weekly for dose adjustment.

Twenty-four hours after the last exposure, neurobehavioral evaluations were performed. After these evaluations were completed the animals were anesthetized using a 1:1 solution of 10% ketamine hydrochloride (Dopalen® Ceva, Brazil) and 2% xylazine hydrochloride (Anasedan® Ceva, Brazil). After the loss of pupillary and nociception reflexes the animals were euthanized by decapitation and brain tissue was collected for the oxidative stress assay. Tissues were stored at -80 °C until the analyses.

2.3. Neurobehavioral Assessments

The neurobehavioral tasks were realized between 9 and 12 a.m., and animals were tested in a counter balanced order in the same alternating sequence used for exposure (Ct, ATR, ATR+VE and VE). At the end of each session with each animal, apparatus was cleaned with cotton soaked in ethyl alcohol (5% v/v) to eliminate traces of the predecessor animal. All tasks were recorded through filming for posterior evaluation by three trained examiners and blind to treatment type.

2.3.1. Open field (OF)

Open field behavior was assessed using a wooden box measuring 97 x 32.5 cm (diameter x height), as described previously (Broadhurst, 1960). The box was divided into three concentric circles, which were subdivided by painted black lines into 18 similar spaces. For OF observations, each rat was placed in the center of the arena and scored on the following parameters for 3 minutes: ambulation frequency (number of floor units entered with four paws, which reflects locomotors activity) and exploratory rearing (which reflects anxiety).

2.3.2. Elevated plus-maze (EPM)

Elevated plus-maze behavior was assessed as described previously (Pellow and File, 1986) using an apparatus consisting of two open and two closed arms of equal lengths and widths (50 x 10 cm). The open arms have a 1-cm-high Plexiglas edge. The closed arms are not entirely closed, but rather have walls that extend 40 cm high. The EPM was elevated 50 cm above the floor. Each rat was placed in the center of the EPM facing one of the closed arms and the following parameters were analyzed for 5 minutes: total number of entries in apparatus arms (number of open arms entries + number of closed arms entries) and the percentage of entries in the closed arms in relation to the total number of entries (which provides a measure of anxiety).

2.3.3. Hole-Board (HB)

Motor coordination and exploration were evaluated according Godinho et al. (2014), using the hole board HB apparatus described by Meyer and Caston (2005), which consists of a square box (28 × 28 × 20 cm), taking the floor painted white and containing 81 holes of 2 cm diameter by 1 cm deep (arrangement of 9 × 9). An acrylic lid covers the box to prevent animal escape and allow viewing inside the box. The tasks were filmed by the underside of the box floor, during 5 minutes. The motor coordination was assessed according to the number of times the animal paw dives into a hole (paw-dip number) and exploratory activity, according to the number of times the animal stuck his head in a hole (head-dip number).

2.4. Oxidative stress assay

The brain samples (~100 mg) were homogenized in 2 ml of cold sodium phosphate buffer (0.1 M, pH 7.4), with a motor-driven Teflon *Potter-Elvehjem*. The

homogenate was centrifuged at 10,000 x g, for 15 min at 4 °C. The supernatant was used for determination of malondialdehyde (MDA) levels, Hydrophilic Antioxidant Capacity (HAC), and superoxide dismutase (SOD) and catalase (CAT) activities.

2.4.1. Malondialdehyde levels

For MDA quantification, 250 μL of supernatant was used for 750 μL of 10% trichloroacetic acid for precipitation of proteins. Samples were centrifuged (3000 rpm; for 5 minutes; Eppendorf® Centrifuge 5804-R, Hamburg, Germany) and the supernatant removed. Thiobarbituric acid (TBA) was added 0.67% in ratio (1:1) and the samples heated for 45 minutes at 100 °C. MDA reacts with TBA in the ratio 1:2 MDA-TBA, absorbed at 535 nm. After cooling, the reading at 535 nm was performed on Spectra Max 190 microplate reader (Molecular Devices®, Sunnyvale, CA, USA). The MDA concentration was obtained by the molar extinction coefficient ($1.56 \times 10^5 M^{-1} cm^{-1}$) and the absorbance of the samples and the final result expressed in nmol/g protein (Samarghandian et al., 2016).

2.4.2. Hydrophilic Antioxidant Capacity measurement

The HAC was determined fluorometrically, as described by Beretta et al. (2006) using a VICTOR X2 reader (Perkin Elmer - Boston, MA). The antioxidant activity was quantitated by comparing the area under the curve relating to the oxidation kinetics of the suspension phosphatidylcholine (PC), which was used as reference biological matrix. The peroxyl radical 2',2-azobis-(2-amidinopropane) dihydrochloride (AAPH) was used as an initiator of the reaction. The results represent the percent inhibition (4,4-difluoro-5-(4-phenyl 1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) (BODIPY) 581/591 plasma with respect to the control sample of BODIPY 581/591 PC liposome. All analyses were performed in triplicate. The results are reported as percentage of protection.

2.4.3. Superoxide dismutase and catalase activities

For SOD activity was used the reactive mixture composed of sodium phosphate buffer (50 mM; pH 7.4), EDTA, NADH and NBT (nitro blue tetrazolium). After addition of phenazine methosulfate the inhibition of reduction of NBT by the enzyme was measured at 560nm according with method described by Ewing and Janero (1995). CAT activity was assayed in sodium and potassium phosphate buffer (50 mM, pH 7.0)

in the presence of hydrogen peroxide (Aebi, 1974). The analysis was performed at 25°C using a microplate reader (Quant-MQX) with Gen5 2.0 software for computer system control (Bio-Tec Instruments, Winooski, Vermont, USA). All reagents were purchased from Sigma - Aldrich (St. Louis, MO, USA).

2.5. Statistical analysis

Results were statistically analyzed by one-way analysis of variance (ANOVA) using Graph Pad Instat Software (San Diego, California, USA). The post hoc Tukey-Kramer multiple comparisons test was used to identify differences between groups if means were considered significantly different at the $p<0.05$ (Snedecor and Cochran, 1991). Results were expressed as mean ± standard error of the mean (S.E.M.).

3. Results

Treatments did not produce modifications in the animals body weight gain, nor did they produce symptoms of toxicity (data not shown).

3.1. Neurobehavioral Assessments

3.1.1. Open Field and Elevated Plus-Maze tests

Table 1 shows the effects of treatments on tasks evaluated in animals in OF and EPM. In OF, treatments did not produce modifications in the locomotor activity (ambulations). We observed that ATR, ATR+VE and VE treatments significantly decreased ($p<0.05$) rearing frequency compared to control animals. In EPM, treatments did not produce changes in total entries in the arms of apparatus (closed arms entries plus open arms entries), however ATR and ATR+VE treatments significantly increased ($p<0.05$) the percentage of entries in closed arms.

Table 1. Neurobehavioral assessment in open field and elevated plus-maze tests in rats exposed during 28 days to ATR, ATR+VE and VE (N=10-15/group).

Tasks	Ct	ATR	ATR+VE	VE
Open field				
Ambulations (counts)	60.13±3.48	64.07±5.60	57.9±4.49	59.13±3.39
Rearing (counts)	19.26±1.98	12.35±1.61*	8.72±0.78*	11.33±1.41*
Elevated plus-maze				
Total entries in the arms (counts)	11.38±0.77	13.55±1.12	11.2±1.04	11.6±1.14
Entries in closed arms (%)	42.0±3.85	59.6±2.63*	57.4±3.58*	44.93±5.09

Ct=control; ATR=atrazine; ATR+VE=atrazine+vitamin E; VE=vitamin E. Values represents the mean ± S.E.M. (ANOVA). *p<0.05 vs. Ct.

3.1.2. Hole-Board test

The exploration behavior, assessed by the number of times the animal stuck his head in a hole (head-dip number) of the HB, was not changed by any of the treatments (data not shown). Figure1 shows the effects of treatments on paw-dip task assessed in HB. We observed that there was a significant increase ($p<0.05$) in paw-dip number of the animals of the ATR group in relation to the Ct group. The animals ATR+VE treated showed a significant decrease ($p<0.05$) in paw-dip number when compared to the ATR group.

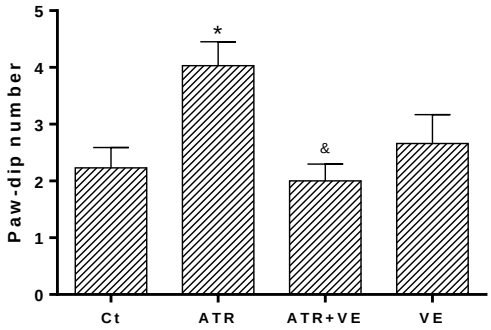


Figure 1. Paw-dip number assessed in Hole-Board test in rats exposed during 28 days to ATR, ATR+VE and VE (N=15/group). Ct=control; ATR=atrazine; ATR+VE=atrazine+vitamin E; VE=vitamin E. Values represents the mean ± S.E.M. (ANOVA), *$p<0.05$ vs. Ct; $^{\&}p<0.05$ vs. ATR.

3.2. Oxidative stress biomarkers assay

3.2.1. Malondialdehyde levels

Figure 2 shows MDA levels in brain tissue of treated animals. We observed a significant increase ($p<0.05$) in MDA levels in the ATR+VE treated group in relation to the Ct group.

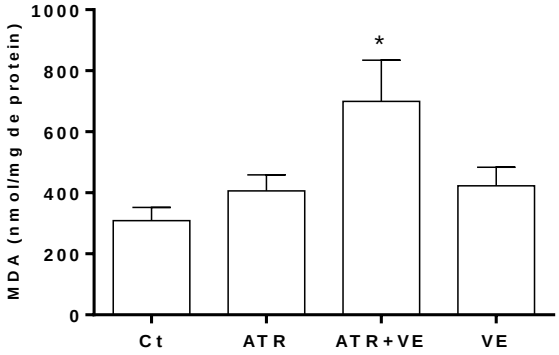


Figure 2. Malondialdehyde levels in brain tissue of rats exposed during 28 days to ATR, ATR+VE and VE (N=7/group). Ct=control; ATR=atrazine; ATR+VE=atrazine+vitamin E; VE=vitamin E. Values represents the mean ± S.E.M. (ANOVA), *$p<0.05$ vs. Ct.

3.2.2. Hydrophilic Antioxidant Capacity measurement

Figure 3 shows HAC measurement in brain tissue of treated animals. We observed a significant increase ($p<0.05$) in HAC in the ATR+VE treated group when compared to all other groups.

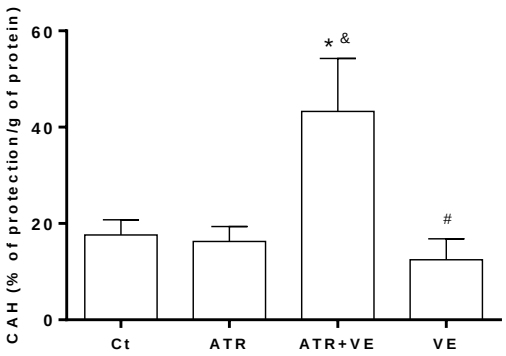


Figure 3. Hydrophilic antioxidant capacity (%) in brain tissue of rats exposed during 28 days to ATR, ATR+VE and VE (N=7/group). Ct=control; ATR=atrazine; ATR+VE=atrazine+vitamin E; VE=vitamin E. Values represents the mean ± S.E.M. (ANOVA), *$p<0.05$ vs. Ct; $^{\&}p<0.05$ vs. ATR; $^{\#}p<0.05$ vs. ATR+VE.

3.2.3. Superoxide dismutase and catalase Activities

The CAT activity assay in the animals brain was not altered in any of the treatments (data not shown). Figure 4 shows SOD activity assay in brain tissue of treated animals. We observed a significant increase ($p<0.05$) in SOD activity in the VE treated group in relation to other groups.

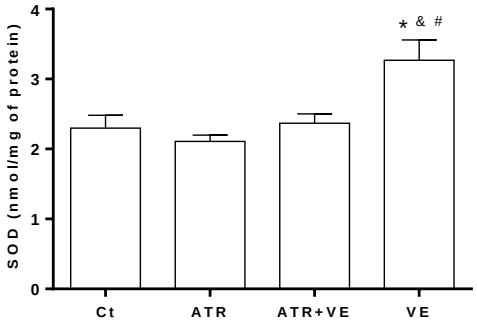


Figure 4. Superoxide dismutase activity assay in brain tissue of rats exposed during 28 days to ATR, ATR+VE and VE (N=7/group). Ct=control; ATR=atrazine; ATR+VE=atrazine+vitamin E; VE=vitamin E. Values represents the mean ± S.E.M. (ANOVA), *$p<0.05$ vs. Ct; $^{\&}p<0.05$ vs. ATR; $^{\#}p<0.05$ vs. ATR+VE.

4. Discussion

The main results obtained in the experiments showed that: ATR promoted behavioral changes in animals; ATR did not induced oxidative stress in the brain tissue; When VE was combined with ATR an increased oxidative stress was observed in the brain tissue, probably associated to increased lipid peroxidation and impaired antioxidant capacity; VE was not able to reverse the behavioral effects caused by ATR, except for motor coordination. In addition, all treatments had no effect in animals weight during the exposure period studied and no signals of toxicity were observed. These results suggest low toxicity by the dose used in the experiment and are in agreement with results from Bardullas et al. (2011) and Márquez-Ramos et al. (2017).

Previous studies have shown that ATR is able to decrease the locomotor activity of animals (Rodríguez, 2013; Lin et al., 2013; Márquez-Ramos et al., 2017). However, the present study does not confirm these previous findings. These discrepancies may be associated to different doses and administration routes employed in the present study. Nevertheless, in the tasks assessed in the OF, evaluated by the number of rearing, only exploratory activity was decreased by ATR, and corroborates with previous findings reported by Lin et al. (2013). When animal behavior was assessed by EPM, the total number of entries in apparatus arms (open + closed) was not modified by the different treatments, thus showing that the animals of the different groups circulated through the apparatus in a similar manner. On the other hand, the percentage of entries in closed arms in relation to the total number of entries increased significantly in the animals that received ATR, indicating an anxiogenic potential caused by the herbicide. This result is similar to previous findings by Lin et al. (2013) and Walters (2015) who also observed anxiety-like behavior caused by ATR exposure.

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidant capacity of the cell. This imbalance results in free radicals production and damage of cellular components such as membrane lipids, proteins, nucleic acids, and other small molecules (Mostafalou and Abdollahi, 2013; Rajendran, 2014).

Pesticides are known to produce oxidative stress and lipid peroxidation of body tissues, processes that are responsible for a variety of adverse biological effects (Singh et al., 2011; Cattani et al., 2014; Gallegos et al., 2018). Increased lipid peroxidation has been proposed as one of the mechanisms associated to ATR toxicity (Abarikwu, 2010; Adesiyan, 2011; Liu et al., 2014). However, in previous studies, has been observed that

the treatment of experimental animals with VE was able to attenuate the toxic effects of ATR by reducing lipid peroxidation (Singh et al., 2011; Griboff et al., 2014).

The malondialdehyde levels, a biological marker of oxidative stress, were not modified by ATR administration. Other markers of oxidative stress as hydrophilic antioxidant capacity and the levels of endogenous antioxidant enzymes SOD and CAT were similar to control group. These results indicate that behavioral changes in the animals, induced by ATR were apparently not linked to oxidative stress in brain tissue. In previous studies, the toxic action of ATR was attributed to oxidative stress (Abarikwu, 2010 Singh et al., 2011; Liu et al., 2014); however, the organs that showed this effect were different from the brain tissue in our study.

Semren, et al. (2018) suggests that changes in oxidative stress biomarkers in rats are observed only with exposure to high doses of ATR, which are not normally found in the environment. As here a relatively low dose of ATR was used, this could explain the lack of oxidative stress in brain tissue in this experiment.

The hydrophilic antioxidant capacity assay can be considered a biomarker of oxidative stress, once it measures the antioxidant capacity in biological fluids. This assay provides a deeper insight into the involvement of oxidative stress in several pathophysiological conditions, but also monitors the effectiveness of antioxidant interventions (Fraga, 2014). Another approach to assess antioxidant capacity is to measure antioxidants activity individually. However, there are many antioxidants and this would require longer dosing time and a variety of analytical techniques, instruments and procedures (Beretta et al., 2006).

The administration of ATR or VE isolated did not alter MDA and HAC, however, their combined administration significantly increased the concentration of these biomarkers in the brain. One possible explanation for this finding would be that some chemical or toxicokinetic interaction between ATR and VE may have occurred by modifying their actions on brain tissue, with increased oxidative stress effect. The literature reports that a diet rich in vitamins prevents several diseases (Mangge, 2014; Fiedor, 2014). This is because exogenous and endogenous antioxidants act in synergism to combat free radicals (Montezano et al., 2014). However, it is important to emphasize that this intake needs to be constant and regulated, as the intake of vitamins in excess, mainly through supplements, may result in pro-oxidant effects (Niki, 2014).

In the same sense, the results of Nascimento et al. (2010) indicated that the administration of VE, in the dose of 250 mg/kg, induced cardiotoxicity in healthy

Wistar rats. These adverse effects on the myocardium may be related to the high non-physiological dose of VE. The antioxidant function of VE depends on several factors, such as the dose and the presence of co-antioxidants. It is plausible that increased VE concentrations resulted in increased α-tocopheroxyl radicals, which in turn may initiate a lipid peroxidation cascade (Nascimento et al., 2010). The VE mediated peroxidation process has been demonstrated in several experimental studies where its oxidation was initiated and promoted by α-tocopheryl radicals (Witting, 1996; Neuzil, 1997; Upston, 2002).

Vitamin E administration did not alter CAT activity, but was able to increase SOD activity. This may be associated to the ability of VE to activate the Nrf2 (Nuclear Factor Erythroid 2-Related Factor 2) by increasing its expression (Zhu et al., 2016; Duan et al., 2017). Nrf2 is a critical transcription factor considered one of the major regulators of oxidative stress as it modulates the expression of hundreds of genes, including antioxidant enzymes such as SOD (Hybertson et al., 2011).

The preference of animals by the closed arms of the EPM and the decrease of rearing activity in OF are related to an increase in anxiety naturally caused by these tests, since exploration in the rodent is inversely proportional to fear or emotionality (Archer, 1973). This anxiogenic effect could be due to the fact that ATR increased fear and modulated the animal emotionality pattern. The fact that VE, used in this experiment for its antioxidant potential, did not modify these behavioral changes induced by ATR would indicate this anxiogenic effect have not direct relation with the production of oxidative stress.

Interestingly, VE also decreased the rearing number when administered alone to animals and still enhanced the effect of ATR when administered in association. This may suggest a certain toxic effect of oral VE supplementation.

Neurotoxic chemicals like pesticides are capable of altering animal motor coordination (Kuc et al., 2006; Godinho et al., 2014). In the present study, the task analysis in HB showed that ATR decreased the motor coordination of the animals, with no changes in the exploration behavior. This behavior, when increased, can influence the motor coordination, as previously demonstrated by Bardullas et al. (2011). In addition, the VE administration was unable to attenuate the toxic effects induced by ATR, whit exception to the motor coordination that was reestablished in the group treated by ATR in association to VE. This effect may not be attributed to the antioxidant

capacity of VE, and probably is associated to other cellular mechanisms activated in specific areas of the brain.

In conclusion, although ATR produces neurotoxicity, evidenced by impaired behavioral parameters, it is not related to induction of brain tissue oxidative stress. In addition, attention is required in situations of oral VE supplementation, once VE combined with substances like pesticides a pro-oxidant effect may be observed in the CNS. Further studies, using different doses of VE, are needed to better understand their interaction with different pesticides.

Conflict of interest statement

The authors declare no conflicts of interest.

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CAPÍTULO 2

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Neurobehavioral and neurochemical effects caused by subchronic exposition to atrazine herbicide in rats.

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Abstract

Atrazine (ATR) herbicide is a pesticide used intensively in agriculture that persists in environment and causes toxicity on non-target species, include humans. Considering the potential of ATR to produce effects on the central nervous system (CNS) and the lack of information in the current literature about this type of toxicity, the aim of the present study was to evaluate the effects of subchronic exposure to ATR on specific neurobehavioral parameters and its relation with the dopaminergic system in specific brain areas. Male Wistar rats received saline (0.3 ml; control - Ct), atrazine (ATR; 100 mg/kg), atrazine plus levodopa (ATR; 100 mg/Kg + LEV; 20 mg/kg) and only levodopa (LEV; 20 mg/kg), via gavage, during 28 days. Behavior was assessed using open field arena (OF), elevated plus-maze (EPM) and hole-board (HB) apparatus. Dopamine (DA) was quantified using HPLC, in specific brain areas (prefrontal cortex, hippocampus and striatum). ATR exposure caused anxiety-like behavior, decreased motor coordination and did not alter the animals locomotor activity. Additionally, ATR treatment did not change dopamine levels in the striatum cerebral. However, ATR increased the DA concentration in the prefrontal cortex and hippocampus. In conclusion, although ATR produces neurotoxicity, evidenced by impaired behavioral parameters and perturbations in brain dopaminergic systems, the damages ATR induced dopaminergic systems is more related to anxiety than motor activity and prefrontal cortex and hippocampus regions were more affected than striatum region. Further studies with longer ATR exposure and/or higher doses are needed to better understand its interaction with the dopaminergic system.

Keywords: atrazine; behavior; neurotoxicity; dopaminergic system; dopamine

1. Introduction

Herbicides are among the most toxic substances due to their high chemical stability, metabolism resistance and their ability to penetrate cellular environments because of their lipophilic properties (El-Amrani et al., 2012). According to the European Union (EU) and the U.S. Environmental Protection Agency (EPA), atrazine herbicide is one of the compounds on the priority pollutant list due to its environmental persistence and toxicity (Grube et al., 2011).

Atrazine herbicide (ATR; 2-chloro-4-(ethylamino)-6-isopropylamino-s-triazine) belongs to the group of chloro-s-triazines (LeBaron et al., 2008) and is widely used worldwide, mainly for control annual grass and broadleaf weeds in crops such as corn, sorghum and sugar cane. Due to its intensive use in agriculture, ATR reaches surface waters and groundwater, affecting non-target organisms, including humans (Barbash et al., 2001; Dooley et al., 2013).

Previous reports indicate that the risk of exposure to ATR is not only occupational, since it is also found in food and farming households or near rural areas (Lioy et al., 2000; Curwin et al., 2005; Gammon et al., 2005). Therefore, its widespread use and frequent detection result in concerns about its potential adverse health effects.

Studies have shown that ATR causes several serious changes in amphibians, especially hormonal changes, being common occur the phenomenon of feminization or even "chemical castration" of several amphibian species (Hayes et al., 2010). Studies with rats also show that exposure to ATR is detrimental to a variety of body systems, including the immune (Filipov et al., 2005), endocrine (Ghiselli and Jardim, 2007) and reproductive system, with addition for alterations of genotoxic character (Feyzi-Dehkharagni et al., 2012). In addition, other studies have shown some relation between ATR contamination or its degradation products and prenatal abnormalities in general human population such as premature births and/or spontaneous abortions, interference with the menstrual cycle and reduced fertility (Hase et al., 2008; Chevrier et al., 2011; Cragin et al., 2011).

On the other hand, in the case of neurotoxic effects, studies relating these effects with exposure to ATR are still rarely found. Bardullas et al. (2011) showed that oral exposure to ATR impaired motor coordination and modulated spontaneous locomotor activity in rats and these effects were accompanied by a decrease in dopamine levels in the brain striatum. The results of Lin et al. (2013) indicated that short-term exposure to ATR induced multiple behavioral abnormalities involving motor, cognitive and

emotional functions also accompanied by decreased DA concentration in the striated brain region of rodents.

While effects of ATR on DA in the striatum are reported in some studies (Filipov et al., 2007; Bardullas et al., 2011; Lin et al., 2013), little is known about its effects on other brain structures, such as prefrontal cortex (PFC) and hippocampus (HIP), which receive dopaminergic projections from the ventral tegmental area (VTA) (Alcaro et al., 2007).

Additionally, levodopa is a known drug for the treatment of Parkinson's disease, a disorder characterized by DA deficiency in the nigrostriatal pathways of the brain. Levodopa reaches brain tissue through the blood-brain barrier and forms DA after being catalyzed by dopa decarboxylase enzyme increasing the intracranial concentration of this neurotransmitter and restoring the dopaminergic transmission system (Wang et al., 2017).

Therefore, considering the potential of ATR to produce effects on the CNS and the lack of information in the current literature about this type of toxicity, the aim of the present study was to evaluate the effects of subchronic exposure to ATR on specific neurobehavioral parameters and its relation with the dopaminergic system in specific brain areas.

2. Material and methods

The experimental protocol was approved by the Ethics Committee on Use of Animals (CEUA) of the Bioscience Institute, São Paulo State University, under the number 897/2016. All procedures were performed in accordance with institutional guidelines for animal use and care. The product used for the experiments was the technique formulation Atrazina 500 SC®, produced by Nortox S/A, containing atrazine 500 g/L (2-chloro-4-(ethylamino)-6-isopropylamino-s-triazine). Levodopa was acquired of the Purifarma-Brazil.

2.1. Animals

Experiments utilized sixty adult (80 days old) male Wistar rats obtained from the colony housed at the Sao Paulo State University, and kept under a constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C). Standard pellet chow (Presence Animal Nutrition, Paulínia/SP, Brazil) and tap water were available *ad libitum*.

2.2. Experimental Procedure

For experiments, animals were randomly divided into four experimental groups (N=15), receiving daily during 28 days (OECD, 1997), by gavage, respectively: saline (0.3 ml; control - Ct), atrazine (ATR; 100 mg/kg), atrazine plus levodopa (ATR; 100 mg/Kg + LEV; 20 mg/kg) and only levodopa (LEV; 20 mg/kg). The choice of oral route (gavage) was based in the principle of that general population receives low doses of atrazine through contaminated food and water.

According to FAO (2005), for oral rat studies, atrazine LOAEL ranged from 10-70 mg/kg but there was no study to evaluate LOAEL specifically for neurotoxicity. On the other hand, atrazine LD50 in orally exposed rats was 1870 to 3090 mg/kg. Based on these data and on the behavioral studies by Lin et al. (2013) and Rodríguez et al. (2013) the dose chosen for this study was 100 mg/kg. The levodopa dose (20 mg/kg) was chosen based on the experiments of Upadhya et al. (2016).

During the period of ATR exposure, animals were observed for possible symptoms of toxicity following exposure (mood swings, aggression, seizure, death, etc.), and were weighed weekly for dose adjustment.

Twenty-four hours after the last exposure, neurobehavioral evaluations were performed. After these evaluations were completed the animals were anesthetized using a 1:1 solution of 10% ketamine hydrochloride (Dopalen® Ceva, Brazil) and 2% xylazine hydrochloride (Anasedan® Ceva, Brazil). After the loss of pupillary and nociception reflexes the animals were euthanized by decapitation and the brain was quickly removed and the prefrontal cortex, hippocampus and striatum were dissected on an ice plate. The dissected parts were frozen in liquid nitrogen and kept at -80 °C until the analysis (Costa et al., 2011; Costa et al., 2013).

2.3. Neurobehavioral Assessments

The neurobehavioral tasks were realized between 9 and 12 a.m., and animals were tested in a counter balanced order in the same alternating sequence used for exposure (Ct, ATR, ATR+LEV and LEV). At the end of each session with each animal, apparatus was cleaned with cotton soaked in ethyl alcohol (5% v/v) to eliminate traces of the predecessor animal. All tasks were recorded through filming for posterior evaluation by three trained examiners and blind to treatment type.

2.3.1. Open field (OF)

Open field behavior was assessed using a wooden box measuring 97 x 32.5 cm (diameter x height), as described previously (Broadhurst, 1960). The box was divided into three concentric circles, which were subdivided by painted black lines into 18 similar spaces. For OF observations, each rat was placed in the center of the arena and scored on the following parameters for 3 minutes: ambulation frequency (number of floor units entered with four paws, which reflects locomotors activity) and exploratory rearing (which reflects anxiety).

2.3.2. Elevated plus-maze (EPM)

Elevated plus-maze behavior was assessed as described previously (Pellow and File, 1986) using an apparatus consisting of two open and two closed arms of equal lengths and widths (50 x 10 cm). The open arms have a 1-cm-high Plexiglas edge. The closed arms are not entirely closed, but rather have walls that extend 40 cm high. The EPM was elevated 50 cm above the floor. Each rat was placed in the center of the EPM facing one of the closed arms and the following parameters were analyzed for 5 minutes: total number of entries in apparatus arms (number of open arms entries + number of closed arms entries) and the percentage of entries in the closed arms in relation to the total number of entries (which provides a measure of anxiety).

2.3.3. Hole-Board (HB)

Motor coordination and exploration were evaluated according Godinho et al. (2014), using the hole board HB apparatus described by Meyer and Caston (2005), which consists of a square box (28 × 28 × 20 cm), taking the floor painted white and containing 81 holes of 2 cm diameter by 1 cm deep (arrangement of 9 × 9). An acrylic lid covers the box to prevent animal escape and allow viewing inside the box. The tasks were filmed by the underside of the box floor, during 5 minutes. The motor coordination was assessed according to the number of times the animal paw dives into a hole (paw-dip number) and exploratory activity, according to the number of times the animal stuck his head in a hole (head-dip number).

2.4. Extraction and Characterization of the Dopamine by HPLC

Dopamine was extracted and isolated according to the method described by Flores and Galston (1982) modified by Lima et al. (2008). Briefly, the samples were

homogenized in perchloric acid (5% v/v) centrifuged (5,000 × g, 10 min at 4 °C), and then 4.5 mol/L Na_2CO_3 and 9.3 mmol/L dansyl chloride in acetone were added to supernatant. The samples were incubated in the dark during 1 hour at room temperature. After the addition of 0.87 mol/L proline (99%), the samples were kept at room temperature for 60 min. The toluene was used to extract the dopamine. Finally, the toluene aliquots of the samples were dried under nitrogen line and resuspended in 150 µL of acetonitrile and injected (20 µL) onto HPLC (Ultimate 3000 BioRS, Dionex-Thermo Fisher Scientific Inc., USA), equipped with a diode array detector (set to 225 nm), column ACE 5 C18 (Advanced Chromatography Technologies, UK) (5 µm, 25 cm × 4.6 mm) and gradient flow of 0.7 mL/min, according to Dadáková et al. (2009). The chromatograph gradient was established with different proportions of (A) acetonitrile 50% and (B) acetonitrile 100% as follows: 0-2 min, 60% A; 2-4 min, 40% A; 4-8 min, 35% A; 8-12 min, 15% A; 12-15 min, 5% A; 15-21 min, 15% A; 21-22 min, 25% A; 22-25 min, 60% A. Dopamine identification was performed by comparing the retention time and the UV spectrum of the compound with commercial standard. Calibration curves were prepared by commercial standard.

2.5. Statistical analysis

Results were expressed as the mean ± standard error of the mean (S.E.M.) or as median with the minimum and maximum values given in parentheses. Results were statistically analyzed by one-way analysis of variance (ANOVA) with a post hoc Tukey-Kramer test or the Kruskal-Wallis with a post hoc Dunn test. Values were considered significant for $p<0.05$ (Snedecor and Cochran, 1991).

3. Results

Treatments did not produce modifications in the animals body weight gain, nor did they produce symptoms of toxicity (data not shown).

3.1. Neurobehavioral Assessments

3.1.1. Open Field test

Figure 1 shows the effects of treatments on tasks evaluated in animals in OF. The treatments did not produce modifications in the locomotor activity (ambulations) (fig. 1A). We observed that ATR and ATR+LEV treatments significantly decreased ($p<0.05$) rearing frequency compared to control animals (fig. 1B).

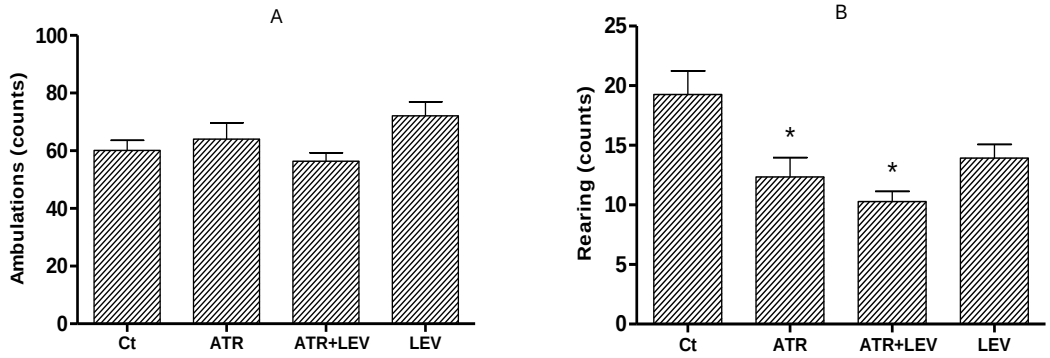


Figure 1. Ambulations (A) and rearing (B) behaviors assessed in Open field test in rats Ct and exposed to ATR, ATR+LEV and LEV (N=15/group). Ct=control; ATR=atrazine; ATR+LEV=atrazine+levodopa; LEV=levodopa. Values represents the mean ± S.E.M. (ANOVA), *p<0.05 vs. Ct.

3.1.2. Elevated Plus-Maze test

Figure 2 shows the effects of treatments on tasks evaluated in animals in EPM. The treatments did not produce changes in total entries in the arms of apparatus (closed arms entries plus open arms entries) (fig. 2A), however ATR treatment significantly increased ($p<0.05$) the percentage of entries in closed arms (fig. 2B).

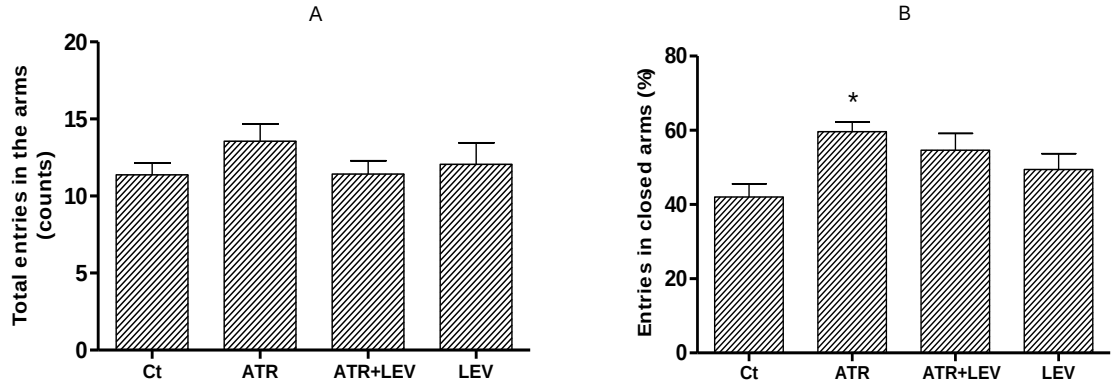


Figure 2. Total arms entries (A) and percentage of entries in closed arms (B) assessed in Elevated Plus-Maze test in rats Ct and exposed to ATR, ATR+LEV and LEV (N=15/group). Ct=control; ATR=atrazine; ATR+LEV=atrazine+levodopa; LEV=levodopa. Values represents the mean ± S.E.M. (ANOVA), *p<0.05 vs. Ct.

3.1.3. Hole-Board test

The exploration behavior, assessed by the number of times the animal stuck his head in a hole (head-dip number) of the HB, was not changed by any of the treatments (data not shown). Figure 3 shows the effects of treatments on paw-dip task assessed in HB. We observed that there was a significant increase ($p<0.05$) in paw-dip number of the animals of the ATR group in relation to the Ct group.

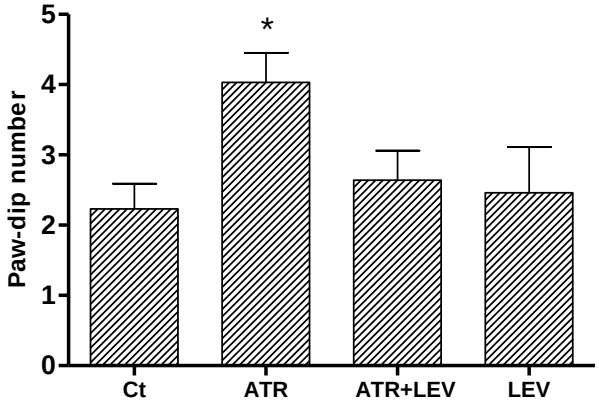


Figure 3. Paw-dip number assessed in Hole-board test in rats Ct and exposed to ATR, ATR+LEV and LEV (N=15/group). Ct=control; ATR=atrazine; ATR+LEV=atrazine+levodopa; LEV=levodopa. Values represents the mean ± S.E.M. (ANOVA), *p<0.05 vs. Ct.

3.2. Dopamine quantification

Table 1 shows the dopamine quantification in specific brain areas in the different treatment groups. In the prefrontal cortex, there was a significant increase ($p<0.05$) in the dopamine level in the ATR and LEV groups compared to Ct. The same occurred in the hippocampus, where the ATR and LEV treated groups had a significant increase ($p<0.05$) of dopamine level compared to Ct. Already in striatum there was no significant difference between the groups.

Table 1. Dopamine quantification (pg/mg) in specific brain areas in rats Ct and exposed to ATR, ATR+LEV and LEV (N=7/group).

Brain areas	**Experimental groups**			
	Ct	**ATR**	**ATR+LEV**	**LEV**
Prefrontal cortex	2.09 (<0.01; 7.02)	4.89 (4.29; 6.23)*	4.53 (0.93; 7.44)	4.07 (1.38; 106.99)*
Hippocampus	0.65 (<0.01; 10.03)	2.52 (1.37; 4.13)*	2.15 (<0.01; 4.52)	3.85 (2.45; 26.07)*
Striatum	35.11 (2.81; 194.90)	36.85 (8.90; 173.12)	58.96 (0.86; 357.38)	34.20 (19.20; 108.23)

Ct=control; ATR=atrazine; ATR+LEV=atrazine+levodopa; LEV=levodopa. Values represents the median with the minimum and maximum values given in parentheses, *p<0.05 vs. Ct.

4. Discussion

The main results obtained in the experiments showed: ATR promoted neurobehavioral and neurochemical effects after subchronic exposure; the damages to dopaminergic systems induced by ATR is more related to anxiety than motor activity; prefrontal cortex and hippocampus regions were more affected than striatum region.

The different treatments had no effect in animals weight during the exposure period studied and no signals of toxicity were observed. These results suggest low

toxicity by the dose used in the experiment and are in according to results from Bardullas et al. (2011) and Márquez-Ramos et al. (2017).

Previous studies have shown that ATR is able to decrease the locomotor activity of animals (Lin et al., 2013; Rodríguez, 2013; Márquez-Ramos et al., 2017). However, the locomotor activity data obtained in the present study, through the OF analysis, does not confirm these previous findings. These discrepancies may be associated to different doses and administration routes employed in the present study. Analysis of the tasks in HB showed that the ATR decreased the animals motor coordination without changing the exploration behavior, since the exploration behavior, when increased, can influence the motor coordination. This damage in motor coordination of animals treated with ATR corroborates with the previous findings by Bardullas et al. (2011). Kuc et al. (2006) and Godinho et al. (2014) also suggest that neurotoxic chemicals of the pesticides type are capable of altering animal motor coordination.

Some studies have demonstrated that ATR targets dopaminergic pathways (Filipov et al., 2007; Bardullas et al., 2011; Rodríguez et al., 2013). Dopaminergic systems are known for participating mainly in motor control (Schultz, 2007). Therefore, it is not surprising that exposure to ATR induces motor deficits, probably due to disturbances in dopamine levels, especially in the striated brain region. In this present study, ATR impaired motor coordination of animals without changing DA levels in the striatum. However, a disturbance of DA homeostasis in the prefrontal cortex and hippocampus was observed, both areas that are not directly related to motor control, but receive dopaminergic projections from the ventral tegmental area (Alcaro et al., 2007).

In this regard, treatment of animals with levodopa lost relevance in our study, since we expected a DA levels decrease in the striatum as reported in previous studies (Filipov et al., 2007; Bardullas et al., 2011; Lin et al., 2013; Rodriguez et al., 2013). In the present study, ATR not only did not modify DA levels in the striatum, but also increased the levels of this neurotransmitter in the prefrontal cortex and hippocampus.

In the Walters study (2015), the decreases in striatal DA concentrations occurred after seven months of exposure to ATR and were correlated with changes in locomotor activity and motor coordination of rats. Litteljohn (2009) studying another dopaminergic toxic, the herbicide paraquat, showed that short-term exposure increased striatum DA levels, while longer exposure decreased this neurotransmitter levels. Perhaps here, the period of exposure to ATR (28 days) was not sufficient to cause a decrease in striatum dopamine concentrations.

On the other hand, previous data of our laboratory showed that calcium ion and the channels in which it is involved can be important target for the toxic effects of pesticides on motor coordination in rats (Godinho et al., 2014). Is know that dopaminergic D2 receptor-mediated inhibition of voltage-gated Ca^{2+} channels occurs through both voltage-dependent and -independent mechanisms (Brown and Seabrook, 1995; Kisilevsky and Zamponi, 2008; Dong et al., 2018). Aditionally, activation of D2 receptors inhibited voltage-gated Ca^{2+}current in mammalian interneurons (Cabrera-Vera et al., 2004). As activity-induced Ca^{2+} influx is a key determinant of presynaptic vesicle release (Zucker and Regehr, 2002), this likely contributes to dopaminergic inhibition of interneuron synaptic output. Therefore, it would be possible that the increase in dopamine levels observed in present work, in brain areas, could be related to motor effects observed in animals exposed to ATR.

However, as in the present work the increase in dopamine was observed only in prefrontal cortex and hippocampus but not in striatum, and as in the mammalian brain, dopaminergic D2 receptor-dependent inhibition of neurotransmitter release from interneurons in the striatum are critically involved in motor control (DeBoer and Abercrombie, 1996; Ikarashi et al, 1997), it is difficult to establish a correlation between the findings found in the present experiments about motor activity and synergistic changes in dopaminergic system and calcium function caused by ATR.

When the exploratory activity was assessed in the OF, exposure to ATR impaired this behavior, since there was decrease in the number of rearing of animals in this group, corroborating with previous findings reported by Lin et al. (2013). Additionally, in behaviors assessed in EPM, the total number of entries in apparatus arms (open + closed) was not modified by the different treatments, thus showing that animals of the different groups circulated through the apparatus in a similar manner. On the other hand, the percentage of entries in closed arms in relation to the total number of entries increased significantly in the animals that received ATR, indicating an anxiogenic potential caused by the herbicide. This result is similar to previous findings by Lin et al. (2013) and Walters (2015) who also observed anxiety-like behavior caused by ATR exposure.

The decrease of rearing activity in OF and the preference of animals by the closed arms of the EPM are related to an increase in anxiety naturally caused by these tests, since exploration in the rodent is inversely proportional to fear or emotionality

(Archer, 1973). This anxiogenic effect may be due to the fact that ATR increased the fear and modulated the animal emotionality pattern.

Experimental studies in animal models revealed that many brain regions acting in concert mediate the symptoms of anxiety, both normal and abnormal. However, some areas such as the hippocampus (Rostami et al., 2006; Ashabi et al., 2011), amygdale (Palomares-Castillo et al., 2012), septum (Molina-Hernandez et al., 2006), prefrontal cortex (Peleg-Raibstein et al., 2005; Fogaça et al., 2012), and nucleus accumbens (Kochenborger et al., 2012) seem to be specially involved in anxiety-like behavior.

Some studies have indicated that the mesolimbic and mesocortical dopaminergic system are involved in the mediation of stress (Bananej et al., 2012) and anxiety (Nasehi et al., 2011; Zarrindast et al., 2012). There are data showing that stress, such as stress due to chemical exposures, activates the dopaminergic mesolimbic system (Nasehi et al., 2010; Trainor, 2011), increasing DA levels in the synaptic cleft (inhibiting the dopamine reuptake for example), which may induce anxiety-like behavioral effects (Simon et al., 1993; Nasehi et al., 2010). Cox et al. (2010) also suggest that alterations in dopaminergic systems may be one of the mechanisms of the anxiogenic effect.

In the present study, ATR increased the hippocampus DA concentration. The HIP is the main part of the mesolimbic system that is involved in modulation of fear and anxiety-related behaviors (Modol et al., 2011; Nasehi et al., 2015). It is well known that the dorsal hippocampus plays a vital role in the learning and spatial memory (Khakpai et al., 2013), while the ventral hippocampus is predominantly involved in the modulation of fear and anxiety, and projects to the prefrontal cortex (Calixto et al., 2010). Lesions of the ventral hippocampus induce anxiolytic-like effects similar to those observed after treatment with anxiolytic drugs in different animal models of anxiety (Nasehi et al., 2015).

When we quantified dopamine levels in the prefrontal cortex, there was increase in the concentration of this neurotransmitter in the ATR exposed group. The PFC has been associated with several emotional, cognitive, attentional processes, among others (Vertes, 2006). In rats, the PFC receives projections of sensory cortical areas, as well as motor and limbic areas (Uylings and Van Eden, 1990). Thus, the disturbances in the DA levels in the PFC may be associated with the increased anxiety observed in our study.

In conclusion, although ATR produces neurotoxicity, evidenced by impaired behavioral parameters and perturbations in brain dopaminergic systems, the damages to

dopaminergic systems induced by ATR seem to be more related to anxiety than motor activity. In addition, prefrontal cortex and hippocampus regions were more affected than striatum region. Further studies with longer ATR exposure and/or higher doses are needed to better understand its interaction with the dopaminergic system.

Conflict of interest statement

The authors declare no conflicts of interest.

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CONCLUSÃO GERAL

Como conclusão desta tese de doutorado, observou-se que a exposição subcrônica ao herbicida atrazina foi capaz de prejudicar parâmetros comportamentais específicos, principalmente aqueles relacionados a ansiedade e a coordenação motora.

O primeiro artigo demonstrou que os prejuízos dos parâmetros comportamentais não estão relacionados com a indução de estresse oxidativo no tecido cerebral de ratos. Além disso, sugere-se atenção em situações de suplementação oral por vitamina E, uma vez que a vitamina E, quando combinada com substâncias do tipo praguicidas, pode exercer efeitos pró-oxidantes a nível de sistema nervoso central.

O segundo artigo demonstrou que além dos prejuízos neurocomportamentais, a exposição à atrazina foi capaz de causar perturbações nos sistemas dopaminérgicos cerebrais de ratos, principalmente nas regiões do córtex pré-frontal e hipocampo, não alterando os níveis de dopamina na região do estriado.

Esses resultados reforçam a relação entre exposição à praguicidas e efeitos de neurotoxicidade.

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