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

DISSERTAÇÃO DE MESTRADO

Exposição subcrônica de ratos ao benzoato de emamectina: toxicidade neurocomportamental e hepática

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Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia e Biotecnologia do Instituto de Biociências da Universidade Estadual Paulista "Julio de Mesquita Filho", Campus Botucatu para a obtenção do título de Mestre em Farmacologia e Biotecnologia.

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1. Benzoatos. 2. Stress oxidativo. 3. Pesticidas - Toxicologia. 4. Toxicidade - Testes. 5. Rato como animal de laboratorio.

Palavras-chave: Benzoato de emamectina; Estresse oxidativo; Hepatotoxicidade; Neurotoxicidade; Praguicida.

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LISTA DE ABREVIATURAS E SIGLAS

Ab1a – Desmetil emamectina
ACA - Arena de campo aberto
ANVISA – Agência Nacional de Vigilância Sanitária
BE – Benzoato de emamectina
BHE – Barreira hematoencefálica
CAT - Catalase
CYP450 – Complexo enzimático citocromo P450
DL 50 – Dose letal em 50 % dos animais
EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária
ERO – Espécies reativas de oxigênio
GABA – Ácido gama-aminobutírico
GSHPX – Glutationa peroxidase
HB - Hole-board
INCA – Instituto Nacional do Câncer
IV – Via intra venosa
LCE - Labirinto em cruz elevada
MAB1a - Fração AB1a do benzoato de emamectina
SOD – Superóxido dismutase
VO – Via oral

RESUMO

HORTA, D. F. **Exposição sub-crônica de ratos ao benzoato de emamectina: toxicidade neurocomportamental e hepática**. 2017. 53f. Dissertação (Mestrado) – Instituto de Biociências, Universidade Estadual Paulista “Julio de Mesquita Filho”, Botucatu, 2017.

O Benzoato de emamectina é um inseticida da classe das avermectinas, os quais apresentam seu mecanismo de ação através de interações com o receptor ácido gama aminobutírico (GABA), e também por alterações do estresse oxidativo em tecidos o qual tem sido associado a diversas doenças crônicas. O objetivo deste projeto foi o de avaliar as alterações geradas pela exposição subcrônica (28 dias) a baixas doses do benzoato de emamectina, sobre os parâmetros neurocomportamentais, do estresse oxidativo em cérebro e fígado e biomarcadores séricos da função hepática e renal. Foram utilizados 64 ratos Wistar adultos machos, os quais receberam oralmente (gavage), diariamente, por 28 dias um dos seguintes tratamentos: Água destilada (controle); benzoato de emamectina 0.5 mg/Kg (BE0.5); benzoato de emamectina 1 mg/Kg (BE1); benzoato de emamectina 2 mg/Kg (BE2). No dia após a última exposição aos tratamentos os animais foram avaliados para atividade neurocomportamental, com o auxilio dos seguintes equipamentos: labirinto em cruz elevada (LCE) teste da arena de campo aberto (ACA) e o hole-board (HB). No dia seguinte os animais foram eutanasiados e coletadas amostras de fígado e cérebro para a avaliação do estresse oxidativo, via mensuração do hidroperóxido de lipídeo e da atividade das enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GSHPx) e sangue para a obtenção do plasma para análise dos biomarcadores da função hepática e renal. Os animais expostos ao BE nos testes neurocomportamentais apresentaram alterações comportamentais de caráter ansiolítico nos teste da ACA e LCE e prejuízo da coordenação motora no teste do hole board. Na avaliação do estresse oxidativo a exposição ao BE provocou estresse oxidativo nos tecidos cerebrais e hepáticos e redução na atividade das enzimas antioxidantes dos ratos expostos ao BE. Os biomarcadores da função hepática e renal não foram elevados pela exposição ao BE. A exposição subcrônica a baixas doses de benzoato de emamectina gerou alterações comportamentais do tipo ansiolítico, prejuízo da coordenação motora e estresse oxidativo nos tecidos cerebral e hepático em ratos.

Palavras-chave: benzoato de emamectina, neurotoxicidade, hepatotoxicidade, estresse oxidativo, praguicida.

ABSTRACT

HORTA, D. F. **Sub-chronic emamectin benzoate exposure in rats: neurobehavioral and hepatic toxicity**. 2017. 53f. Thesis (Master) – Instituto de Biociências, Universidade Estadual Paulista “Julio de Mesquita Filho”, Botucatu, 2017.

The emamectin benzoate is an insecticide of avermetins class which exerts your action mechanism by interactions with GABA receptors, and also by tissue oxidative balance alterations which can be associated with chronic diseases development. The purpose of this project was to evaluate possible alterations caused by sub-chronic exposure (28 days) to emamectin benzoate (EB) low doses, on the neurobehavioral parameters, brain and liver oxidative stress and liver and kidney function serum biomarkers. Was utilized 64 adult (70 days) Wistar male rats which received, daily, by gavage, during 28 days, one of the following treatments: distilled water (control); 0.5 mg/Kg of emamectin benzoate (EB0.5); 1 mg/Kg of emamectin benzoate (EB1); 2 mg/Kg of emamectin benzoate (EB2). The day after the last exposure, animals were assayed for neurobehavioral activity utilizing the following apparatus: elevated plus-maze, open-field and hole-board test. After behavioral evaluation, animals were euthanized, liver and brain samples were collected to evaluate oxidative stress, through the measurement of lipid hidroperoxide (LH) and the activity of antioxidants enzymes, superoxidodismutase (SOD), catalase (CAT) and gluthationa peroxidase (GSHPx). Blood also was collected for plasm biomarkers assay of kidney and liver function. Animals exposed to EB presented behavioral alterations with anxiolytic profile during the ACA and LCE tasks, and impairment of motor coordination, during the hole-board task. The EB exposure provoked oxidative stress demonstrated by the increase of LH in brain and hepatic tissues, and decreases of the activity of antioxidant enzymes. The kidney and liver function biomarkers were not elevated in plasm by the EB exposure. The sub-chronic EB low dose exposure provoked behavioral alterations with anxiolytic like behavior profile, impairment of motor coordination, and increase of liver and brain tissues oxidative stress of rats.

Keywords: emamectin benzoate, neurotoxicity, hepatotoxicity, oxidative stress, pesticide.

SUMÁRIO

1. Introdução

1.1. Panorama do uso de agrotóxicos no Brasil

O Brasil é o maior consumidor mundial de agrotóxicos desde 2008, 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. Este crescente aumento no uso de agrotóxicos é de se alarmar, pois o efeito deletério causado por estes produtos já é conhecido, podendo gerar tanto intoxicações agudas como ter relações com doenças crônicas como a diabetes, câncer e neurodegenerativas (ANVISA, 2009; LONDRES, 2011; IBGE, 2012).

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

Os agrotóxicos mais aplicados atualmente no Brasil são os herbicidas, seguido pelos inseticidas, fungicidas e acaricidas. Diversas formulações utilizadas aqui são proibidas em países da Europa, devido a sua toxicidade ou às alterações que promovem no ambiente, como a morte de insetos como exemplo, as abelhas, que são benéficos ao cultivo, e outros organismos não alvo (IBGE, 2012; CARNEIRO et al., 2015).

Alguns dados do censo agropecuário de 2006 tornam o assunto ainda mais preocupante, pois 77,6% dos responsáveis pelos estabelecimentos agropecuários tinham apenas o ensino fundamental incompleto, baixo percentual de adoção de práticas alternativas no controle de pragas e doenças, elevado numero de estabelecimentos que não utilizam equipamento de proteção individual (21,3%) e o altíssimo número de estabelecimentos que utilizam o pulverizador costal (70,7%), equipamento que gera o maior potencial de exposição ocupacional aos agrotóxicos (IBGE, 2009; IBGE, 2012).

Devido ao uso incorreto dos agrotóxicos não se utilizando de boas práticas agrícolas, cada vez mais as espécies alvo tem tido uma maior resistência

aos princípios ativos presentes no mercado, induzindo os produtores a exigir novos princípios ativos para sanar seus prejuízos (EMBRAPA, 2013).

1.2. Emergência fitossanitária e a "necessidade" do benzoato de emamectina no Brasil

No ano de 2013 foi permitida a importação para alguns estados do Brasil do principio ativo emamectina, o qual não obteve registro e sua comercialização não foi autorizada no país em 2007 pela ANVISA. Foi alegado que a emamectina apresenta um perfil toxicológico bastante desfavorável, tanto pelo ponto de vista agudo quanto crônico, devido à sua neurotoxicidade (ANVISA, 2007; BRASIL, 2013a; CARNEIRO et al., 2015).

Porém, em razão da emergência fitossanitária gerada em alguns estados brasileiros pela infestação da praga *Helicoverpa armigera* em diversas culturas, foi solicitada a importação e utilização deste produto mesmo sem registro no Brasil, em caráter emergencial, e o Ministério da Agricultura Pecuária e Abastecimento permitiu a importação desse princípio ativo através da Lei nº 12873/2013. No ano de 2013 foi declarada emergência fitossanitária no oeste da Bahia, seguida de Mato Grosso, Goiás, Minas Gerais, Piauí, Mato Grosso do Sul, Alagoas e Maranhão (BRASIL, 2013a; BRASIL, 2013b; CARNEIRO et al., 2015).

No ano de 2014 foi relatada a presença da *Helicoverpa* por todo o Brasil e alguns estados como Alagoas, Bahia, Goiás, Minas Gerais, Maranhão, Mato Grosso, Mato Grosso do Sul e Piauí tiveram o uso emergencial prorrogado até 18 de março de 2016. No estado do Rio Grande do Sul essa solicitação do uso da emamectina foi negada, relatando-se que outros produtos agrícolas são menos tóxicos e mesmo assim eficazes contra a praga, e que a utilização de boas práticas agrícolas têm sido eficiente no controle das infestações (BRASIL, 2015; PIAUÍ, 2015; RIO GRANDE DO SUL, 2014).

Porém, em dezembro do ano de 2016 o prazo para o uso do benzoato de emamectina em caso de emergência fitossanitária foi novamente prorrogado através da Portaria Nº 273 de 21 de dezembro de 2016, sendo prorrogado por mais um ano a partir de 15 de janeiro de 2017, podendo ser utilizado na safra 2016/2017 (BRASIL, 2016).

1.3. As moléculas da classe das avermectinas e o benzoato de emamectina

As moléculas da classe das avermectinas são um grupo de agentes anti-helmínticos produzidos por um actinomiceto do solo o *Streptomyces Avemetilis*. O qual foi primeiramente isolado pelo instituto Kitasato (Japão) de uma amostra de solo de um campo de golfe na cidade de Ito no Japão (BURG et al, 1979; CRUMP; OMURA, 2011).

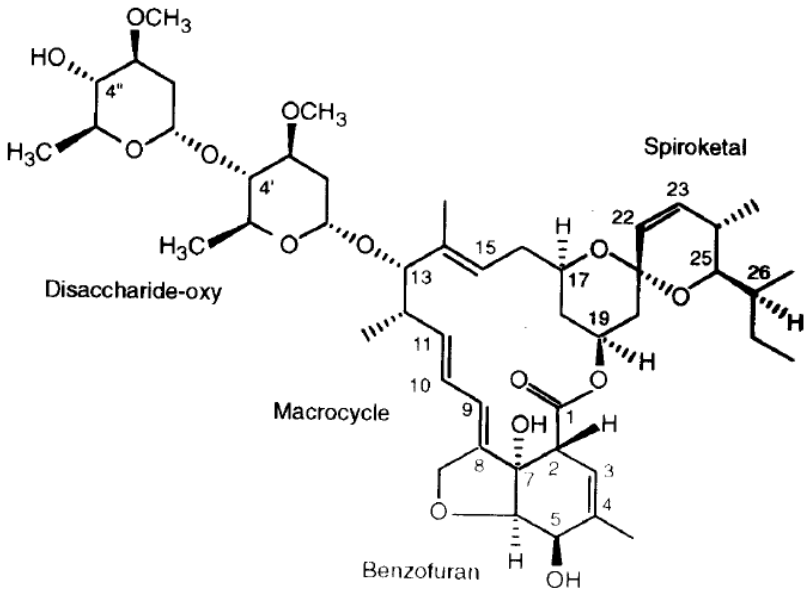
Os princípios ativos do grupo das Avermectinas podem ser formados pela associação de oito diferentes produtos da fermentação do *Streptomyces Avermitilis.* Esses compostos são $A1_a$, $A1_b$, $A2_a$, $A2_b$, $B1_a$, $B1_b$, $B2_a$ e $B2_b$, os quais têm diferentes espectros de ação e toxicidade nos seus organismos alvos. (BURG et al, 1979).

Dos oito homólogos da abamectina que são produzidos pelo S*treptomyces Avermitilis* apenas a $A2_a$, $B1_a$ e $B2_a$ são produzidos em quantidade significativa pela fermentação do fungo. O homologo B1 apresenta maior potência e maior espectro de ação, sendo seguido pelo B2, porém o B2 apresenta maior segurança. A estimativa da dose letal 50 (DL50) em ratos é de aproximadamente 15mg/Kg para B1 e 50 mg/KG para B2 (SHOOP; MROZIK; FISHER, 1995). As moléculas da classe das avermectinas apresentam atividade acaricida, inseticida e nematicida e contém diversos princípios ativos como a abamectina, ivermectina, doramectina o benzoato de emamectina dentre outros (LASOTA; DYBAS, 1991; DANAHER et al., 1996).

O benzoato de emamectina (4″*R*)-4″-deoxy-4″-(methylamino) avermectin B1 benzoate deve conter não mais de 90% 4”epimethylamino-4”-deoxyavermectin $B1_a$ ($AB1_a$) benzoato (1008,3 Kda) e não mais que 10% de 4”epimethylamino-4”deoxyavermectin B1b ($AB1_b$) benzoato (994.2 Kda) (FAO/WHO, 2011).

O Benzoato de emamectina é uma lactona macrocíclica semissintética a qual é sintetizada quimicamente a partir da abamectina pela substituição de um grupamento aminomethyl (-NCH3) por um hidroxyl (-OH) na 4” posição do dissacarídeo (MUSHTAQ et al., 1996). As principais estruturas das moléculas da classe das avermectinas são representadas na Figura 1.

Figura 1 - Avermectina B_{1a}, o componente mais abundante na fermentação do S*treptomyces Avermitilis*, mostrando as principais estruturas da molécula.



Fonte: SHOOP et al., 1995.

As lactonas macrocíclicas foram inicialmente introduzidas como uma droga veterinária para o controle de parasitas em animais de produção e de companhia, e depois passaram a ser utilizadas como praguicidas em plantações e para o controle de parasitas em humanos, neste último caso principalmente a ivermectina no controle de filarioses (CRUMP; OMURA, 2011).

1.4. Toxicocinética e Toxicodinâmica do benzoato de emamectina

A absorção, distribuição, excreção e metabolismo das moléculas de xenobióticos podem variar por diversos fatores como a via de exposição, formulação, espécie animal, idade e outros fatores (CANGA et al., 2009). O BE é uma molécula com alto peso molecular, sendo esperado que seja excretada principalmente pela via fecal e apresente uma baixa absorção pela via dermal, como o observado no trabalho de Wrzesinski et al. (1997) em macacos Rhesus.

Para analisar se há diferenças no metabolismo do BE pelo sexo ou via de exposição de ratos, Mushtaq et al. (1996) avaliaram a absorção, distribuição e excreção do BE em ambos os sexos e pelas vias de exposição intra venosa (IV) e via oral (VO). O Benzoato foi eliminado quase inteiramente nas fezes e se observou uma meia vida plasmática de 27 horas pela via IV e 22 horas pela VO.

O pico plasmático da VO ocorreu após 12 horas da exposição e o da IV antes de 2 horas pós a administração.

A exposição dermal e IV em macacos Rhesus foi analisada por Wrzesinski et al. (1997). A penetração dermal do BE foi muito baixa (1,6%) e os níveis plasmáticos permaneceram abaixo do limite de detecção do teste. Porém a exposição IV obteve um pico plasmático da droga com 5 minutos da administração o qual passou a cair até 15 minutos, sugerindo que o composto é removido rapidamente da circulação. Depois permaneceu até 8 horas pós-exposição em um estado de platô passando a declinar, estando presente no plasma até 10 dias.

A principal via de excreção do BE em ratos encontrada nos trabalhos de Zeng et al. (1996a) e Mushtaq et al. (1996) foi a fecal contendo mais de 98% dos resíduos do BE radiomarcado, um resultado esperado visto que o BE é um composto de alto peso molecular sendo preferencialmente excretado pela via hepatobiliar. Wrzesinski et al. (1997) também observaram a via fecal como a principal via de excreção em macacos *Rhesus* tanto pela exposição pela VO e IV. O pico de excreção do BE nas fezes dos animais expostos por via IV foi com 2 dias após a exposição e na dérmica entre 3 e 4 dias.

A emamectina apresenta diferentes dinâmicas entre os animais e respostas a diferentes concentrações. Mushtaq et al. (1996) analisaram os resíduos do BE pela exposição aguda a baixa dose de BE (0.5 mg/Kg do peso animal) pela via IV e VO e observou resíduos em uma concentração mais expressiva no rim, fígado e gordura os quais declinaram a níveis muito baixos após 7 dias. No entanto a exposição à alta dose do BE (20 mg/Kg do peso animal) gerou uma quantidade de resíduos relativamente alta após 7 dias da exposição, estando principalmente localizados no pulmão e baço.

A exposição de caprinos lactantes ao BE gerou resíduos em órgãos na seguinte ordem: fígado, rim e gordura. A concentração plasmática e do leite foi muito menor que a encontrada nos tecidos, e a concentração de resíduos no leite foram maiores 1.2-1.6 vezes que a do plasma (MUSHTAQ et al., 1997).

1.5. Metabolização do benzoato de emamectina

Uma das propriedades físicas de muitos xenobióticos para serem absorvidos pela pele, pulmão ou intestino é a lipofilicidade, porém é também um

obstáculo para sua excreção, pois estes compostos podem ficar sendo reabsorvidos. A biotransformação é a conversão metabólica de compostos endógenos e xenobióticos a compostos mais polares e mais solúveis em água para favorecer sua excreção (PARKINSON, 2001).

Esta biotransformação apresenta duas fases: Reações da fase I que envolvem hidrólise, redução e oxidação, estas reações introduzem um grupamento funcional (—OH, —NH_2, —SH, ou —COOH) a molécula, e usualmente resultam em um baixo aumento da hidrofilicidade; Reações de fase II da biotransformação envolvem glicuronidação, sulfonação, acetilação, metilação e conjugação com a glutationa, as quais resultam em aumento na hidrofilicidade e excreção do composto (PARKINSON; OGILVIE, 2010).

As transformações mais comuns que ocorrem na fase I da biotransformação são as reações oxidativas envolvendo o complexo enzimático citocromo P450 (CYP450), o qual contém oxidases, redutases e hidroxilases. As enzimas do CYP450 estão presentes nos hepatócitos do fígado, epitélio intestinal e também nos túbulos proximais renais (MITTAL et al., 2015).

As enzimas do CYP450 são também as principais envolvidas na metabolização das avermectinas em ratos (ZENG et al., 1996b; SKÁLOVÁ et al., 2000), humanos (ZENG et al., 1998), e aves (ZHU et al., 2014). Wrzesinski et al. (1998) observaram que as aves apresentam maior metabolização do BE e associou isto a maior atividade do CYP450 nestas.

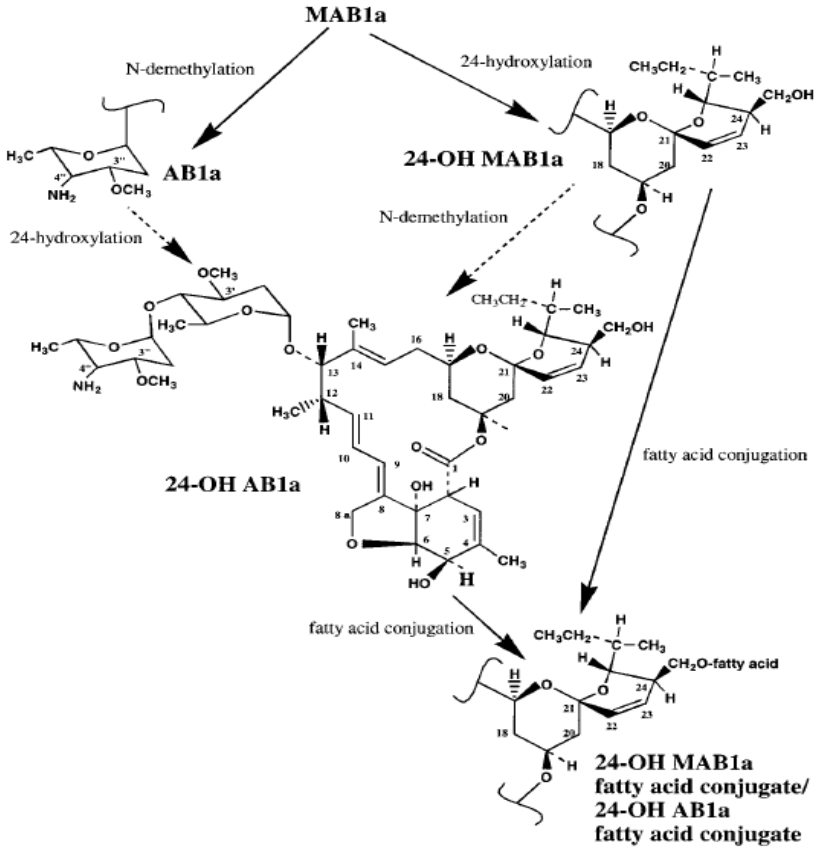
Os resíduos encontrados em fezes, leite e tecidos dos caprinos consistiam principalmente da fração AB1a (MAB1a) do benzoato de emamectina e o metabólico gerado pela N-desmetilação do BE (AB1a). Também foram encontrados outros dois metabólitos mais polares em concentrações muito pequenas os quais não foram caracterizados (MUSHTAQ et al., 1997).

A desmetil emamecticna (AB1a) também é considerada o principal metabólito em ratos (MUSHTAQ et al., 1996). Zeng et al. (1996a) identificou a maioria dos resíduos em fezes e tecidos como sendo o homólogo da emamectina B1a e o AB1a no entanto encontrou outros 5 diferentes metabólitos os quais não foram identificados. A metabolização do BE apresentou diferenças entre os sexos sendo encontrada uma maior quantidade do metabólito em tecidos nas fêmeas, mostrando que elas apresentam maior velocidade de metabolização.

Salmões expostos durante sete dias ao BE por VO, apresentaram resíduos do BE em rim, fígado, pele, músculo e ossos até 90 dias após a exposição, sendo os principais resíduos a emamectina B1a, desmetil emamectina B1a e traços do metabólito formil emamectina B1a em amostras de músculos (KIM-KANG et al., 2009).

Nestes trabalhos com mamíferos e salmões o principal resíduo encontrado foi a fração AB1a do BE mostrando que este não é completamente metabolizado e o único metabolito expressivo foi o AB1a. Em aves o principal resíduo também foi a fração AB1a do BE, porém a emamectina foi mais intensamente metabolizada tendo como principais metabolitos o 24-hydroxymethyl derivado do MAB1a (24-OH-MAB1a – 33%), uma quantidade menor comparada aos outros animais do AB1a (2-7%) e o derivado N-demetilado do 24-OH-MAB1a (24-OH-AB1a – 1%). Estes metabolitos estão exemplificados na Figura 2 (WRZESINSKI et al., 1998).

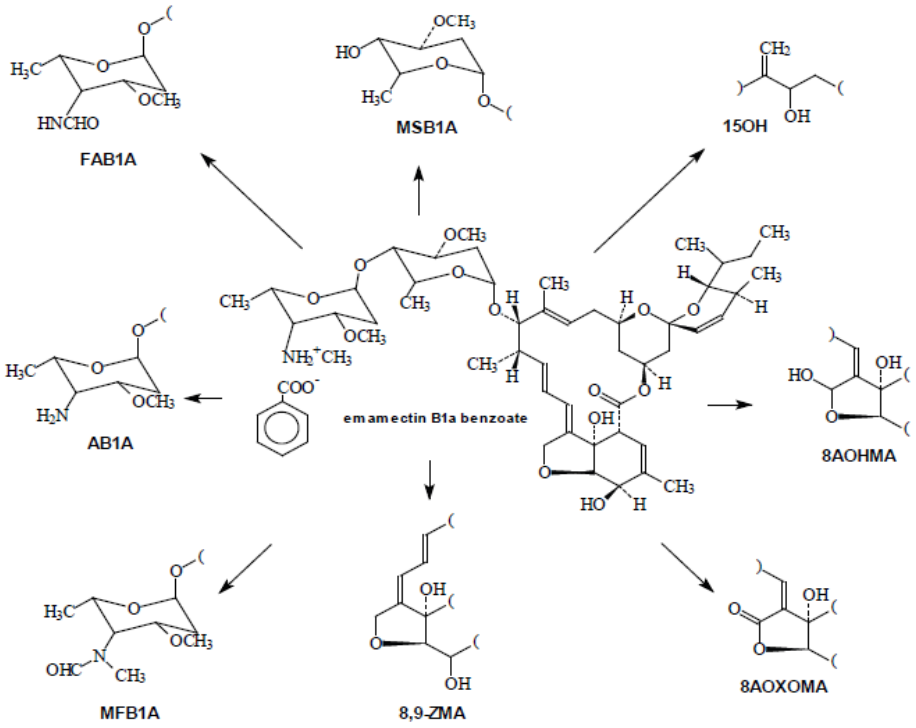
Figura 2 - Síntese do metabolismo in vivo do MAb1$_a$ benzoato em galinhas.



Fonte: Wrzensinski et al., 1998.

Drogas que são menos metabolizadas normalmente permanecem por um período maior no corpo e apresentam meia vida de eliminação maior que drogas que são rapidamente metabolizadas, se estas não são eliminadas por outro mecanismo (BENEDITE, 2009). Existem também diversos outros metabólitos do BE porem sua presença não foi relatada em animais, sendo encontrados em trabalhos da fotodegradação do BE e metabólitos presentes em plantas, água e solo e representados na Figura 3 (FAO/WHO, 2011).

Figura 3 - Esquema da degradação primária do benzoato $B1_a$ em partes da folha de culturas folhosas (alface, repolho) e cereais (forragem de milho). A abreviação " ─(" na formula estrutural indica que o resto da molécula é idêntica ao benzoato de emamectina B1a.



Fonte: FAO/WHO, 2011.

1.6. Mecanismo de ação e Toxicidade do benzoato de emamectina

A classe das avermectinas apresenta seu mecanismo de ação em mamíferos através da interação com receptores $GABA_A$ dos canais de cloreto agindo como um agonista parcial. Esta ativação dos canais de cloro promovem hiperpolarização do neurônio e consequente inibição da propagação do potencial de ação inibindo a neurotransmissão (ABALIS, et al., 1986). No entanto o mecanismo de ação das avermectinas não é tão específico podendo afetar uma variedade de outros canais de cloro ligantes e voltagem dependentes (STEVENS;

BRECKENRIDGE, 2001). Xu et al. (2016) em seu trabalho demonstrou que a emamectina também afeta outros canais iônicos dependente de ligante, sendo dois receptores nicotínicos seletivos a cátions (α7 e α4β2 nAChR) e outros dois canais de cloreto GABA dependentes (α1β2γ2 $GABA_A$ e ρ1 $GABA_C$).

Segundo Turner e Schaeffer (1989) a classe das avermectinas apresentam diferenças em sua ação sistêmica em vertebrados e invertebrados. Os mamíferos são menos sensíveis as avermectinas, pois elas possuem menor afinidade pelos receptores e uma relativa inabilidade de cruzar a barreira hematoencefálica (BHE) devido seu alto peso molecular.

Nos animais invertebrados as avermectinas agem nos canais de cloreto controlados por glutamato localizados no tecido periférico, podendo ser na junção interneuronal ou na junção neuromuscular, enquanto nos mamíferos estes sítios de ação estão compreendidos no sistema nervoso central (ALMEIDA; AYRES, 2006.).

Porém, algumas espécies podem apresentar alteração na glicoproteína P por uma mutação no gene MDR1 (ABCB1). Esta glicoproteína pode ser encontrada em tecidos que promovam absorção ou tenham função de barreira, em variados epitélios e tecidos excretores como pulmão, BHE, fígado, rim e ductos biliares. Na BHE de mamíferos sua função mais importante é a presença desta no lúmen da membrana plasmática do endotélio capilar, facilitando a prevenção de numerosos xenobióticos e compostos endógenos de cruzar a barreira hematoencefálica. Esta proteína também esta correlacionada com a capacidade de alguns parasitas em desenvolverem resistência a alguns princípios ativos (ALMEIDA; AYRES, 2006; CÁRCAMO, 2011; KENNEDY; TIERNEY; MITTELSTADT, 2014).

Mesmo o BE apresentando dificuldade em ultrapassar a BHE pelo seu alto peso molecular, resíduos da emamectina foram encontrados na medula espinhal e cérebro de ratos por Mushtaq et al. (1996). Wise et al. (1997) avaliaram o potencial da exposição gestacional ao BE em produzir neurotoxidade em prole de ratos. Foi observado que a exposição gestacional de ratos produziu evidências de neurotoxicidade na prole F1 e causou alterações comportamentais observadas na atividade locomotora e na resposta ao estímulo sonoro.

Existem poucos estudos com a avaliação neurocomportamental de animais expostos ao BE. Porém, a exposição a outras moléculas da classe das

avermectinas como a ivermectina e doramectina provocaram alterações ansiolíticas, prejuízo da coordenação motora e alterações na atividade locomotora. Spinosa et al. (2000) encontrou alterações nas neurotransmissões dopaminérgica, noradrenérgica e serotoninérgica no sistema nervoso central de ratos expostos a doramectina, e consideraram como consequência do fortalecimento na neurotransmissão Gabaérgica induzida pela doramectina.

Alterações histopatológicas em cérebro foram observadas em pombos expostos a avermectina no trabalho de Li et al. (2013), os quais sugeriram que o mecanismo que causou estas alterações foi que a exposição ao praguicida induziu o estresse oxidativo o qual alterou biomacromoléculas, função e estrutura dos tecidos, que por último irá influenciar as atividades fisiológicas normais da célula.

A estimulação da produção de radicais livres, prejuízo da capacidade antioxidante e a peroxidação lipídica são mecanismos de toxicidade dos pesticidas, e tem sido associado ao desenvolvimento de doenças pela exposição a praguicidas. E é reconhecido que o estresse oxidativo tem papel importante na etiologia e progressão de doenças crônicas (ABDOLLAHI et al., 2004; SOLTANINEJAD; ABDOLLAHI, 2009; RAJENDRAN, 2014).

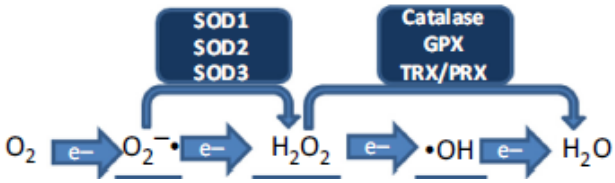
O estresse oxidativo ocorre quando a quantidade de espécies reativas de oxigênio (ERO) que são geradas excede a capacidade antioxidante da célula, o qual pode resultar em danos a componentes celulares como lipídeos de membrana, ácidos nucleicos, proteínas, enzimas e outras pequenas moléculas (MOSTAFALOU; 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 SOD, CAT e GSHPx pertencem ao sistema antioxidante enzimático, e estão envolvidas na manutenção do balanço REDOX e por neutralizar as ERO, como vemos na Figura 4. Porém, quando ocorre aumento no estresse oxidativo a capacidade de defesa contra as ERO pode colapsar (BIRBEN et al., 2012; NWANI et al., 2016).

A exposição de ratos ao BE no trabalho de EL-Sheik e Galal (2015) provocou um aumento da peroxidação lipidica em tecido hepático associado a redução da atividade da enzima antioxidante SOD. Li et al. (2013) também observaram aumento do estresse oxidativo em tecido cerebral de pombos

expostos ao BE, indução da peroxidação lipídica e redução da atividade das enzimas antioxidantes SOD e GSHPx.

Figura 4. Redução sequencial do O_2 pelas enzimas antioxidantes.



Fonte: Patel, 2016.

1.7. Estrutura da dissertação

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

O primeiro artigo foi submetido à revista Toxicology, intitulado “Sub-chronic emamectin benzoate exposure affects anxiety, motor coordination, and disturbs biomarkers of oxidative stress in rat brain tissue”. Ele consistiu em uma avaliação das alterações comportamentais provocadas pelo BE e alterações do estresse oxidativo em cérebro de ratos.

O segundo artigo será submetido à revista Free Radical Research, intitulado “Sub-chronic emamectin benzoate exposure disturbs liver oxidative stress but not hepatic and renal function biomarkers”. Ele consistiu em uma avaliação da influência do BE sobre o estresse oxidativo em tecido hepático de ratos e sobre as enzimas da função hepática e renal nesses animais.

No final é apresentada uma conclusão geral dos resultados obtidos.

2. Objetivo

O objetivo deste projeto de pesquisa experimental foi o de avaliar as possíveis alterações induzidas pela exposição subcrônica a baixas doses do BE, sobre parâmetros da atividade neurocomportamental, estresse oxidativo no cérebro e fígado e biomarcadores plasmáticos de alterações hepáticas e renais em ratos Wistar.

3. Artigos referentes à dissertação

3.1. Artigo submetido à revista Toxicology (Elsevier, ISSN 0300-483x)

Sub-chronic emamectin benzoate exposure affects anxiety, motor coordination, and disturbs biomarkers of oxidative stress in rat brain tissue

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Abstract

Emamectin benzoate (EB), a pesticide of the avermectins class, was initially introduced as a veterinary drug for control of internal and external parasites, and has being utilized in crops production and medical use for human parasite control. Avermectin class compounds act on mammals GABA-gated chloride channels. The aim of the present work was to evaluate effects of sub-chronic EB exposure on behavioral parameters and brain oxidative stress. Male Wistar rats received control (distilled water) or EB (0.5, 1, and 2 mg/kg) treatments, via gavage, during 28 days. Behavior was assessed using open field (OF), elevated plus maze (EPM), and hole-board (HB) apparatus. Oxidative stress assay was performed assessing brain hydroperoxide lipid (LH) levels and the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Overall, animals receiving EB presented anxyolitic-like behaviour and reduced motor coordination, accompanied by high hydroperoxide lipid levels and decreased antioxidant enzymes activity. In conclusion, sub-chronic EB exposure alters anxiety, motor coordination and disturbs biomarkers of oxidative stress in rats.

Key words: emamectin benzoate, behavior, oxidative stress, neurotoxicity, pesticide

1. Introduction

Pesticides are used in agriculture to protect crops and improve productivity, and are considered a quick, easy and inexpensive solution for controlling weeds and insect pests, however they posses potential to cause adverse human health and environmental effects, being able to contaminates soil, air and ground water. (Handford et al., 2015). Environmental contamination could affect the wild life affecting small invertebrates, crops beneficial mites like bees and also more evolved animals like birds and other animals (Aljedani, 2017; Grodnik et al., 1998; Lumaret et al., 2012).

The emamectin benzoate is a pesticide that belongs to the avermectins chemical agents class, which contains a macrocyclic lactone linked to an oleandrosyl disaccharide.

This class compounds are produced by the soil actinomycete *Streptomyces avermitilis* (Burg et al., 1979).

The avermectins class have many active principles like abamectin, ivermectin, doramectin, emamectin benzoate, eprinomectin, moxidectin, milbemycinoxime and selamectin (Danaher et al., 2006). These products demonstrated acaricidal, insecticidal and nematicidal activity (Lasota and Dybas, 1991).The macrocyclic lactones were initially introduced as veterinary drugs for use in commercial livestock and companion animals for control of internal and external parasites. Came to be utilized in crop production and medical use for parasites control in humans (Crump and Ōmura, 2011).

The EB is chemically synthesized from abamectin by a substitution of aminomethyl group for a hydroxyl group at the 4”-position on the disaccharide. It is composed of a mixture of two homologous compounds, 90 % or more of the constituent avermectin B1a (MAB1a) benzoate and not more than 10% constituent avermectin B1b (MAB1b) benzoate (Mushtaq et al., 1996). The EB is a molecule with higher molecular weight, low dermal absorption and water solubility of 24 ppm (Mushtaq, 1998; Wrzensinski et al, 1997).

The excretion main route of EB is fecal, and has plasma half life in male rats of 22 hours after oral administration and 27 hours after intravenous administration (Mushtaq, 1996). EB has in the soil a half life of 1.9 - 4.89 days (Wang, 2012); in tomatos 2.5 days, with residues decreasing below the maximal residue limit 10 days after spraying (Malhat, 2013). The EB is also utilized as an insecticide in fish farms, commonly used to control sea lice in salmon (Burridge, 2010). According Telfer et al. (2006), residues of EB are observed in marine sediment, 12 months after salmon treatment. In this form contamination of soils, water, and plants can reach the entire food chain and consequently reach the man.

Avermectins open the $GABA_A$ receptor chloride channel by binding to the GABA receptor acting as a partial agonist (Abalis, et al. 1986). This chloride channels activation develops a maintained negative charge at the motor neuron blocking signals for inhibitory or excitatory action. However, avermectins are less specific in their action and can affect a variety of other ligand and voltage gated chloride channels (Stevens and Breckenridge, 2001). Recent work of Xu et al. (2016) demonstrated the direct activation of multiple pentameric ligand-gated ion channels by emamectin, including two cation-selective nicotinic receptor and two GABA-gated chloride channels.

According to our knowledge, the current literature shows few works about mammals EB exposure and neurobehavioral alterations. Wise et al. (1997) evaluated the

developmental neurotoxicity of rats offspring's exposed to EB during gestation and lactation, and observed decreased locomotor activity and auditory startle response amplitude. Spinosa et al. (2002) showed that ivermectin exposure produced pharmacological profile of an anxiolytic drug with GABAergic properties and in the conflict behavior assessment was observed a classic effect of anxyolitic-like drug, reversing the conditioned response to shock.

Previous reports related that a mechanism for pesticide toxicity is provoking free radical production and lipid peroxidation. These effects are associated with injuries on endogenous antioxidant system (enzymatic and non-enzymatic) leading to oxidative stress (Abdollahi et al., 2004; Hernández et al., 2013). El-Sheikh and Galal (2015) and Li et al. (2013) observed decreased antioxidant enzyme activities and increased lipid peroxidation caused by EB and avermectin exposure on liver and brain tissues respectively.

Considering the potential of avermectins class for produce central nervous system effects the aim of present work was evaluate effects of sub-chronic EB exposure on specific neurobehavioral parameters and brain oxidative stress in rats.

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 753/2015. All procedures were performed in accordance with institutional guidelines for animal use and care.

The product used for experimentation was the technique formulation Killer 250 WDG® (water dispersible granule - Hailir Pesticides and Chemicals Group), containing emamectin benzoate 25g/kg.

2.1. Animals

Experiments utilized sixty four adult (70 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=16), receiving daily during 28 days, by gavage, respectively: distilled water (control -

Ct), and emamectin benzoate diluted in distilled water in concentrations of 0.5 mg/kg (EB0.5), 1 mg/kg (EB1), and 2 mg/kg (EB2).

Doses used were chosen based on lowest observed adverse effect level (LOAEL - 2.5 mg/kg) and on no observed adverse effect level (NOAEL - 1 mg/kg) for neuropathology assessment in rat repeated studies (FAO/WHO, 2014). This doses also were used for rat exposure in others similar works (El-Sheikh and Galal, 2015; Wise et al., 1997).

In order to adapt the logistics of the behavioral evaluation, to permit the assessment be realized between 9 and 12 a.m. The exposure started initially in half animals of each group (N=8), after two days the exposure started in the other half animals of each group (N=8).

During experiments animals were weighed for dose adjustment and food/water consumption was measured two times week. Every day observed for possible symptoms of toxicity.

Twenty four hours after the last EB exposure was realized the neurobehavioral assessment. After the end of the neurobehavioral tasks, the animals were euthanized by decapitation and brain tissue was collected for 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 dosing (Ct, EB0.5, EB1, EB2). At the end of each session with each animal, apparatus were cleaned with cotton soaked in ethyl alcohol (5% v/v) to eliminate traces of the predecessor animal. All tasks were recorded for posterior evaluation by three trained examiners and blind to treatment type.

2.3.1. Open field

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), exploratory rearing, number of

crossing the center of apparatus, the time to first cross through the center of the arena, which reflects anxiety (Prut and Belzung, 2003).

2.3.2. Elevated plus-maze

Elevated plus-maze behavior was assessed as described previously (Pellow and File, 1986) using an apparatus consisting of two open and two enclosed arms of equal lengths and widths (50 x 10 cm). The open arms have a 1-cm-high Plexiglas edge. The enclosed arms are not entirely enclosed, 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 elevated plus-maze facing one of the closed arms, and the number of entries with four paws and the time spent (in seconds) in the open and closed arms were recorded during a 5 minute test period. The EPM test is based on the principle that exposure to an elevated and open arm leads to an approach conflict that is considerably stronger than that evoked by exposure to an enclosed maze arm. Thus, the percent of entries and time spent in the closed arms provide a measure of anxiety (Carobrez and Bertoglio, 2005).

2.3.3. Hole Board

Motor coordination and exploration were evaluated according Godinho et al. (2014), using the hole board apparatus described by Meyer and Caston (2005), which consists of a square box (28 × 28 × 20 cm), taking the floor painted white and containing 36 holes of 2 cm diameter by 1 cm deep (arrangement of 6 × 6). 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's 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 (0.1M, 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 glutathione peroxidase, superoxide dismutase and catalase activities, also concentration of lipid hydroperoxide and total protein.

The activity of glutathione peroxidase was determined in a media containing 0.15 M sodium phosphate buffer pH 7.0, EDTA, NADPH, glutathione reductase, sodium azide (NaN_3) and glutathione reduced form. The reaction was started by the presence of hydrogen peroxide. The method was based on oxidation of NADPH at 340 nm, during the reduction of glutathione, with absorptivity of 6.22µmol. cm^{-1} (Nakamura et al., 1974).

For superoxide dismutase activity was used the reactive mixture composed of sodium phosphate buffer (50mM; 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).

Catalase activity was assayed in sodium and potassium phosphate buffer (50mM, pH 7.0) in the presence of hydrogen peroxide (Aebi, 1974).

The assay medium for determination of concentration of lipid hydroperoxide contained methanol 90% (v/v), iron sulphate, sulfuric acid, butylated hudroxytoluene and xylenol orange. The method consists in oxidation of Fe^{2+} under acid conditions and formation of Fe^{+3}– xylenol orange complex, which is read at 560 nm (Jiang et al, 1991).

Total protein was determined using biuret reagent that contains cupric ion, which react with peptide bonds resulting a violet color complex, whose intensity of coloration is proportional to concentration of protein (MOURA, 1982).

The analysis was performed at 25°C using a microplate reader (μQuant-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 using Graph Pad Instat Software (San Diego, California, USA). The results of the neurobehavioral task, oxidative stress and body weight gain are analyzed using one-way analysis of variance (ANOVA). Results of water and food consumption used repeated measures of ANOVA with a longitudinal design. Tukey–Kramer post hoc test was used for comparisons between means when ANOVA was significant at the $P< 0.05$ level (Snedecor and Cochran, 1991). Results were expressed as mean ± standard error of the mean (S.E.M).

3. Results

Treatments did not produced modifications in body weight gain or water and food consumption (Table 1) [$F_{3,60}$= 1.021; $F_{3,24}$= 2.262; $F_{3,24}$=2.369], nor produced symptoms of toxicity.

3.1. Neurobehavioral assessment

3.1.1 Open Field tasks

The Figure 1 shows the results obtained in the OF test. Animals exposed to EB1 and EB2 treatments increased significantly (P<0.05) the number of passages through the center of arena (Fig. 1A) in relation to control [$F_{3,60}$ = 3.603]. Exposure to EB2 significantly reduced (P<0.05) the latency to first cross the center of apparatus (Fig. 1B) compared to control [$F_{3,60}$ = 5.645]. In the Fig. 1C we observed decrease in the rearing number in all groups treated with EB , but only exposure to EB2 had a significant difference (P<0.05) in relation to control [$F_{3,60}$ = 3.485]. The locomotor activity did not change in any group of the treatment (Fig. 1D) [$F_{3,60}$ = 1.341].

3.1.2. Elevated plus-maze tasks

Exposure to EB0.5 and EB2 significantly increased (P<0.05) the time spent in the open arm of EPM (Fig. 2A) [$F_{3,60}$ = 4.720] and decreased significantly (P<0.05) the time spent on the closed arms of apparatus (Fig. 2A) compared to control [$F_{3,60}$ = 6.469]. The closed arms entries (Fig. 2B) significantly decreased (P<0.05) in the groups EB0.5 and EB2 [$F_{3,60}$ = 5.847], however the total entries on arms (Fig. 2C) and number of entries in the open arms (Fig. 2B) had no difference between groups (P>0.05) [$F_{3,60}$ = 0.395; $F_{3,60}$ = 0.705].

3.1.3. Hole board tasks

In the Fig. 3 we observed the results obtained in the HB tasks. The head dipping number (Fig. 3A) was significantly increased (P<0.05) in the EB0.5 group compared to control [$F_{3,60}$ = 3.575]. Although the paw-dip number had increased in all groups exposed to EB (Fig. 3B), just the group EB2 had a significantly difference (P<0.05) compared to control [$F_{3,60}$ = 3.235].

3.2. Oxidative stress assay

Figure 4 shows the biochemical assay of oxidative stress in brain tissue. The lipid hydroperoxide (Fig. 4A) significantly increased (P<0.05) in the group EB1 and EB2

compared to control [$F_{3,24}$ = 10.750]. The exposure to EB1 and EB2 also provoked a significantly reduction (P<0.05) in the SOD activity (Fig. 4B) compared to control [$F_{3,24}$ = 76.001]. All EB groups exposure presented a significant reduction (P<0.05) in CAT (Fig. 4C) [$F_{3,24}$ = 17.985] and GSHPx activity (Fig. 4D) compared to control [$F_{3,24}$ = 76.790]. The total protein had no difference (P>0.05) between groups (data not shown) [$F_{3,24}$ 2,505].

4. Discussion

Present results demonstrated that sub-chronic low doses EB exposure affects anxiety, motor coordination, and disturb biomarkers of oxidative stress in rat brain tissue.

The behavioral task assessment of animals in present work demonstrated an anxiolytic effect caused by EB exposure. Spinosa et al. (2000, 2002) also observed an anxiolytic effect caused by ivermectin and doramectin exposure, suggesting a relation between this effect and GABAergic properties of these compounds. GABA is the most important inhibitory neurotransmitter in the central nervous system (Liu et al., 2007). The macrocyclic lactones act on GABA receptor promoting an influx of chlorine ions, which hyperpolarize the neural membrane, modulating the generation of action potential (Ménez et al., 2012).

The main neural circuits anxiety related are spread over the brain and consist of amygdala, bed nucleus of the stria terminalis, medial prefrontal cortex, hippocampus, thalamus, hypothalamus, brainstem, basal forebrain and anterior cingulate cortex. The anxiety involves a integrated activity of numerous brain pathways and involves different neurotransmitters. GABA is recognized as central to the anxiety regulation but is not the only neurotransmitter important in the anxiety modulation (Nuss, 2015).

Animals EB exposed and tested in EPM apparatus had increased permanency time in open arms, and reduction in the closed arms permanency time and entries. Previous findings on avermectin class compounds exposure also observed these behavioral alterations in EPM and assigned it to an anxiolytic profile caused by these agents. The mode of action of these drugs was correlated with mammalian GABA receptor (Ménez et al., 2012; Spinoza et al., 2000).

The EPM is a recognized test to detect anxiolityc/anxiogenic effects of drugs acting on GABA receptors according Rägo et al. (1988), which observed an increase in time and numbers of entries in open arms of animals exposed to diazepam, a recognized anxiolytic drug. Additionally, the opposite was observed with the benzodiazepine inverse agonist

DMCM. These behavioral alterations were correlated with the density of GABA and benzodiazepinic binding sites in cerebral cortex (Rägo et al., 1988).

The increase in the crossings number and the reduction in the latency time to cross the center of OF arena observed in this work express a tendency of the animals to spent more time in the center of the apparatus. Since rodents spontaneously prefer staying in the periphery than in the apparatus central parts, the fact of animals spending more time in central area of apparatus is associated with an anxiolytic effect (Prut and Belzung, 2003).

The exploratory behavior can be measured with many unconditioned tests, such as rearing in OF (Prut and Belzung, 2003) and head-dip in HB (Brown and Nemes, 2008). Rearing is an exploratory behavior induced by the novelty, and is usually associated with anxiety, vertical activity and emotionality of rats (Costall et al, 1989; Prut and Belzung, 2003). In this work the rearing behavior assessed in the OF was reduced in the animals exposed to EB. Rats with smaller quantities of $GABA_A$ receptor present a higher rearing behavior (Alves et al., 2012). Since EB has an agonist effect on GABA, a reduction in the rearing behavior is expected. In a similar way, Spinoza et al. (2002) also observed a reduction in the rearing numbers caused by ivermectin exposure. In addition, the hole board test also permit a valid measure of exploration once the head-dip behavior express an animal attraction towards novelty (Brown and Nemes, 2008). Spinoza et al. (2000) observed anxiolytic effect of the doramectin associated with increased head-dip in the HB test and none alterations in locomotor activity. In the present work EB exposure provoked similar alteration.

In the present work we didn't observe locomotor activity alteration in EB exposed animals in any behavioral tests. A comparison of our results with general data of literature is difficult because lacking scientific information about effects of avermectin class on locomotor activity. Moreover, the rare existing data are contradictories. While Wise et al. (1997) evaluated the developmental neurotoxicity caused by perinatal EB exposure and observed that pups presented decreased locomotor activity and sensorimotor functions, Davis et al. (1999) observed increase in the locomotor activity induced by ivermectin exposure. Alterations in GABA system are recognized to disturb the locomotor and motor coordination (Milic et al., 2012).

The HB test can also be utilized to evaluate the locomotor activity, exploration, emotionality and motor coordination (Brown and Nemes, 2008; Takeda et al., 1998). In present work, animals receiving EB exposure and assessed in HB, had increased number of paw-dip, in a dose dependent manner, indicating a motor incoordination effect. Our results

corroborates with Trailovic et al. (2011), which observed a dose-dependent decrease of motor coordination using rats exposed to ivermectin and tested in a rota-rod apparatus.

The inhibitory motor neurons have an important function in the motor coordination, and alterations on 5-HT (serotonin), glycine and GABA systems are correlated with motor alterations (Jordan and Slawinska, 2011). Spinosa (2000) observed that alterations on central dopaminergic, noradrenergic and serotonergic neurotransmission, may be consequence of reinforcement in central GABAergic neurotransmission induced by doramectin.

Here, overall observations with behavior evaluations suggests that toxicological EB effects observed were not in a dose-dependent mode, but it is possible to affirm that all doses tested seems capable of provoking some biological alteration. This affirmation is possible once neurotoxicants can produce a non-linear dose-response relationship (U.S. EPA, 1998).

Data on NOAEL and LOAEL used to choose the dose utilized in the preset experiments were based in neuropathological studies in rats (FAO/WHO, 2014), but we observed that according our experiments a specific NOAL/LOAEL value from neurobehavioral effects could be necessary once behavior is consider a endpoint for neurotoxicity by environmental compounds (U.S. EPA, 1998).

Pesticides exposure is recognized to generate impact in human health, and is now being associated with chronic diseases like cancers, diabetes and neurodegenerative disorders, such as Alzheimer and Parkinson's disease. The present results obtained with animals exposed to EB could strength this presumed relationship. On the other hand, there is a huge body of literature findings on induction of oxidative stress by pesticides, linked to these health problems (Abdollahi et al., 2004; Braconi et al., 2010; Mostafalou and Abdollahi, 2013).

Mechanisms of pesticides toxicity include oxidative stress, which consists in increase in the reactive oxygen species (ROS) production and/or injury in the antioxidant defense. This disruption of the oxidative balance could damage all cell components because enhanced ROS production can react with membrane lipids, nucleic acids, proteins, enzymes and others small molecules (Mostafalou and Abdollahi, 2013; Rajendran et al., 2014).

The present work demonstrated increased lipid peroxidation and reduced antioxidant enzymatic activity in animals exposed to EB, indicating increase of the oxidative stress. The brain presents a high vulnerability to oxidative injury based on anatomic,

physiological and functional factors. The brain is an organ with high rate of metabolism accompanied with a higher availability of oxygen (O_2), utilized in the energy-consuming processes as action potentials, enzymatic reactions, synaptic machinery and neurotransmission. The O_2 is the major precursor of ROS generation and endogenous factors such as modest antioxidant defense, polyunsaturated fatty acids prone to peroxidation, limited regenerative capacity and others, becoming the brain highly vulnerable to oxidative damage (Patel, 2016).

The ROS production causes oxidation of polyunsaturated fatty acids and cholesterol, promoting the lipid peroxidation and by this, lipid hydroperoxide is produced and can be utilized as a biomarker of lipid peroxidation (Niki, 2013). Our results showed increase of this biomarker in the group EB1 and EB2, demonstrating enhanced lipid peroxidation. In these groups was also observed decreased activity of all antioxidant enzymes. The activity of the antioxidant enzymes SOD, CAT and GPx plays an important role in maintaining of the redox homeostasis (Birben et al., 2012).

The group EB0.5 did not presented increase on the lipid peroxidation, indicating minor oxidative stress compared to the higher doses group, however the GPx and CAT activity is reduced showing reduction on redox capability.

Our data are in accordance with others researchers which also observed deleterious effects on antioxidant enzymes activity and lipid peroxidation due sub-chronic exposure to pesticides (Badgujar et al., 2015; Sankwar et al., 2016).

5. Conclusions

The present work demonstrated clearly that sub-chronic emamectin benzoate exposure in rats alters anxiety and motor coordination, together with increased oxidative stress in brain tissue. These findings could strength the suggested relationship between pesticides exposure and neurodegenerative diseases. Future directions for a mechanistic action causing the effects here observed are need, including pharmacological strategies, and research on neurotransmitters and oxidative stress in specific brain regions responsible to behavior modulation.

Conflict of interest

Authors declare that there are no conflicts of interest.

Funding

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Figures and table

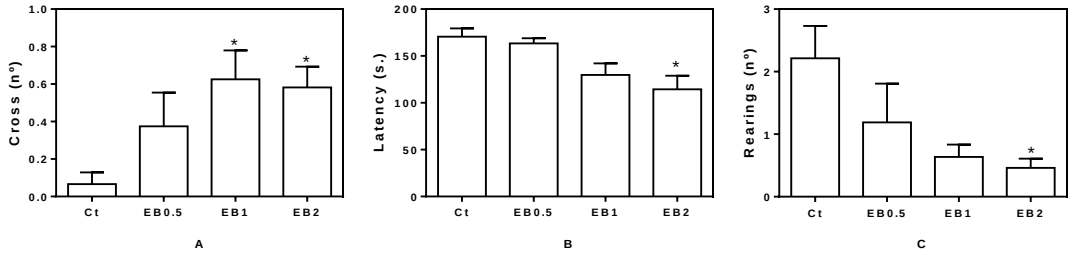

Figure 1. Assessment of the open field tasks in animals exposed to emamectin benzoate. Number of crossings through OF center (A), latency to first cross through OF center (B), rearing number (C). Values represent the mean ± S.E.M of 16 animals per group. Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB1= Emamectin benzoate 1 mg/Kg; EB2= Emamectin benzoate 2 mg/Kg. *P<0.05 vs. Ct; **P<0.05 vs. EB0.5 (ANOVA).

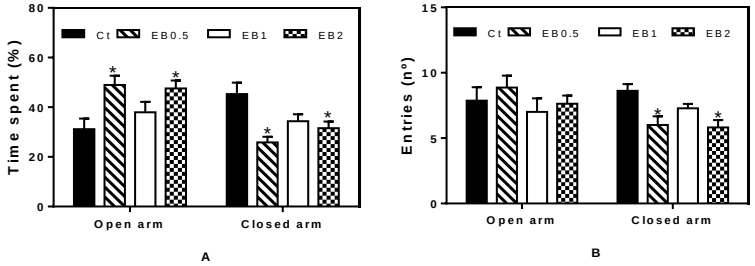

Figure 2. Assessment of the elevated plus maze tasks in animals exposed to emamectin benzoate. Time spent (A) and number of entries (B) in the open and closed arms. Values represent the mean ± S.E.M of 16 animals per group. Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB1= Emamectin benzoate 1 mg/Kg; EB2= Emamectin benzoate 2 mg/Kg. *P<0.05 vs. Ct (ANOVA).

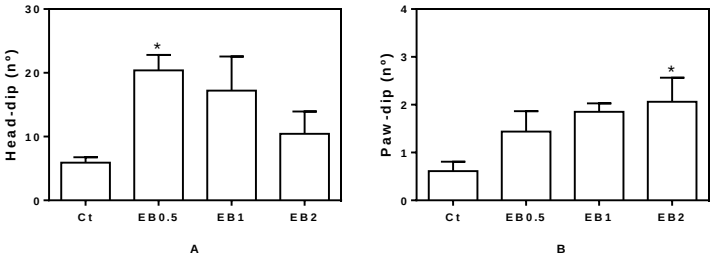

Figure 3. Assessment of the hole-board tasks in animals exposed to emamectin benzoate. Head-dip number (A) and Paw-dip number (B) in the HB test. Values represent the mean ± S.E.M of 16 animals per group. Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB 1= Emamectin benzoate 1 mg/Kg; EB 2= Emamectin benzoate 2 mg/Kg. *P<0.05 vs. Ct (ANOVA).

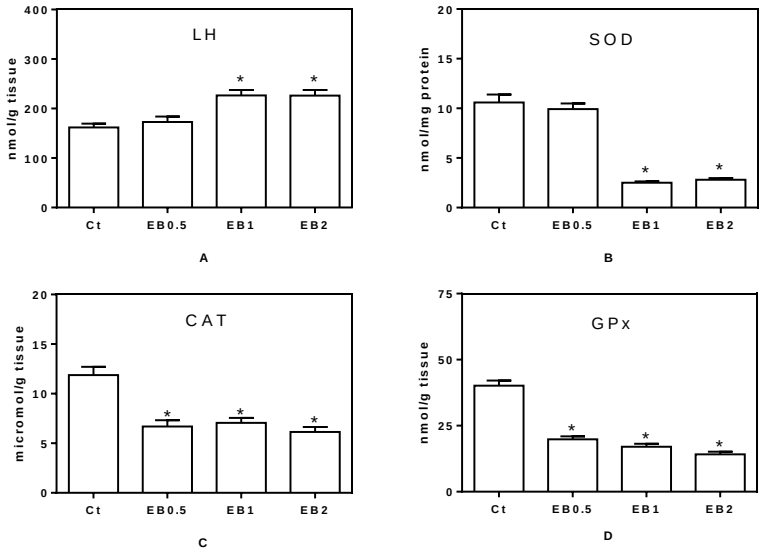

Figure 4. Assessment of biomarkers of oxidative stress in brain tissue of animals exposed to emamectin benzoate. Lipid hydroperoxide (A), Superoxide dismutase (B), Catalase (C), Glutathione peroxidase (D). Values represent mean ± S.E.M of 7 samples per group of treatment. Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB1= Emamectin benzoate 1 mg/Kg; EB2= Emamectin benzoate 2 mg/Kg. *P<0.05 vs. Ct (ANOVA).

Table 1 – Body weight gain and water/food consumption during experiment.

	Ct	EB0.5	EB1	EB2
Body weight gain	69.75±3.02	72.27±3.33	76.26±5.95	66.43±3.40
Water consumption	36.91±0.64	41.62±1.04	39.54±1.87	38.45±1.13
Food consumption	25±0.33	25.48±0.26	25.83±0.25	25.05±0.28

Values represent mean ± S.E.M. (N = 16). The data relative to body weight gain were analyzed using ANOVA. The data relative to water and food consumption were analyzed using repeated measures of ANOVA; Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB1= Emamectin benzoate 1 mg/Kg; EB2= Emamectin benzoate 2 mg/Kg.

3.2. Artigo que será submetido à revista Free Radical Research (Taylor e Francis, ISSN 1071-5762)

Sub-chronic emamectin benzoate exposure disturbs liver oxidative stress but not hepatic and renal function biomarkers

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Abstract

Emamectin benzoate (EB) is a pesticide of the avermectins class which posses acaricidal, insecticidal and nematicidal activities being initially utilized to control animal parasites. Actually, they are used in the crops production and to control human parasites. Pesticide exposure is associated with many deleterious effects and could damage organs like liver and kidney which are very exposed due to their metabolizing and excretion functions. One mechanism of pesticides toxicity is the oxidative stress (OS) which affects many biological structures and cause alterations on membrane permeability. The present work aim was to evaluate in rats, effects of sub-chronic EB low doses exposure (28 days) on liver oxidative stress and serum biomarkers of hepatic and kidney function. The oxidative stress was evaluated by the liver lipid hydroperoxide levels (LH) and antioxidant enzymes activity. The liver and kidney function was evaluated using serum biomarkers evaluation. The EB exposure provoked increase of lipid hydroperoxide, and reduction of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) enzymes activity in liver. Serum biochemical parameters of kidney and liver function were unchanged by EB exposure. In conclusion sub-chronic emamectin benzoate exposure in rats provoked

oxidative stress in the liver tissue without increase of the serum biochemical parameters of liver and kidney. The oxidative stress could occur by an increase of the ROS production and/or alteration on the antioxidant defense. It is necessary more research to establish the specific manner by which EB provoked these OS alterations.

Key words: emamectin benzoate, hepatotoxicity, liver, kidney, oxidative stress, pesticide.

1.Introduction

Pesticides are compounds used in the crops production for controlling weeds and insect pests, and in the cities for control building infestations being able to contaminate soil, air and ground water [1,2]. The pesticides exposure is associated with many deleterious effects, like non-specific symptoms as dizziness, nausea and/or vomiting, fatigue, weakness and others symptoms [3], being also correlated with chronic diseases like cancers, diabetes, neurodegenerative and reproductive disorders [4].

The avermectins class represent compounds produced by the soil actinomycete *Streptomyces avermitilis*, and contain molecules with a macrocyclic lactone linked to an oleandrosyl disaccharide [5]. Some active principles of this class are abamectin, ivermectin, doramectin and emamectin benzoate (EB) [6]. These chemicals possess acaricidal, insecticidal and nematicidal activity [7]. Initially introduced as veterinary drugs for use in animal parasites control and came to be utilized in crop production and medical use for parasites control in humans [8].

The EB is structurally similar to abamectin and ivermectin, which is composed of a mixture of two homologous compounds, 90 % or more of the constituent avermectin B1a (MAB1a) benzoate and not more than 10% constituent avermectin B1b (MAB1b) benzoate [9].

The pesticide exposure is recognized to enhance free radical (FR) generation, promoting oxidative stress (OS). Free radicals are atoms or molecules that contain one or more unpaired electrons, by this they are generally more reactive than non radicals and their reactivity vary widely in different FR types. The oxygen molecule (O_2) is itself a radical, with two unpaired electrons, but it is not particularly reactive due to a special electron arrangement. When O_2 is partially reduced, many different reactive oxygen species (ROS) may be produced, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO^-)[10,11].

The increased production of reactive oxygen species (ROS) and/or decreased capacity antioxidant defense (enzymatic and non-enzymatic) can disrupt the oxidative balance and result in damages to cellular components like membrane lipids, nucleic acids, proteins, enzymes and others small molecules leading to the OS. [4,12]

El-Sheikh and Galal [13] and Li [14] observed increase of the oxidative stress and decrease of the antioxidant enzyme activities caused by EB and avermectin exposure on liver and brain tissues respectively. Works with others pesticides like cypermethrin [15] and carbosulfan [16] also observed an increase in the oxidative stress in rats exposed to these compounds and liver damage.

The liver plays the dominant role in the first pass clearance of ingested xenobiotic compounds and controls the systemical level of drugs and chemicals [17]. The drug metabolism is typically realized by enzymes like the cytochrome P450, and results in the formation of more hydrophilic compounds to be excreted by the liver, kidney and/or gut [18].

Recent works of El-sheik and Galal [13] and Khaldoum-Oularbi [19] observed hepatotoxicity caused by EB exposure showed by the increase of hepatic biomarkers and alterations on hepatic architecture. Another work of Khaldoun-oularbi [20] with EB observed alteration on hepatic and renal function, evidenced by an increase in liver and kidney toxicity biomarkers and histopathological changes in the kidney parenchyma. The kidney has many physiological functions, including their principal action as an excretory organ for xenobiotics and their polar metabolites but it is also involved in the biotransformation of these compounds [21].

Considering the potential of pesticides exposure to produce various levels of organs toxicity and oxidative stress, the aim of present work was to evaluate effects of sub-chronic EB low doses exposure on liver oxidative stress and biomarkers of hepatic and kidney function in rats.

2.Material and Methods

2.1. Animals and treatments

The Ethics Committee on Use of Animals (CEUA) of the Bioscience Institute, São Paulo State University, has approved the experimental protocols under the number 753/2015. All procedures were performed in accordance with institutional guidelines for animal use and care.

Experiments utilized thirty two adult (70 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*.

The product used for the experiment was the technique formulation Killer 250 WDG® (water dispersible granule - Hailir Pesticides and Chemicals Group), containing emamectin benzoate 25g/kg.

For experiments, animals were randomly divided into four experimental groups (N=8), receiving daily during 28 days, by gavage, respectively: distilled water (control - Ct), emamectin benzoate 0.5 mg/kg (EB0.5), emamectin benzoate 1 mg/kg (EB1), and emamectin benzoate 2 mg/kg (EB2). Doses used were chosen based on lowest observed adverse effect level (LOAEL - 2.5 mg/kg) and on no observed adverse effect level (NOAEL - 1 mg/kg) for EB exposure in rat repeated studies [22]. These doses also were used in another works with EB [13,23].

During experiments animals were weighed, food and water consumption were measured two times week, and observed for possible symptoms of toxicity. One day after last exposure the animals were anesthetized with Ketamin:Xilazyn and the blood were collected by cardiac puncture and euthanized by decapitation and liver tissue was weighted for oxidative stress assay. Tissues and plasma were stored in a freezer -80°C until the analyses.

2.4. Oxidative stress assay (liver)

The tissue samples (~100 mg) were homogenized in 2 mL of cold 0.1M phosphate buffer, pH 7.4, with a motor-driven Teflon *Potter-Elvehjem*. The homogenate was centrifuged at 10.000 g, for 15 min at 4°C, and the supernatant was used for analysis of GSHPx [24], SOD [25], and CAT [26] activities, and quantification of LH [27] and total protein (TP) [28]. The analysis was performed at 25°C using a microplate reader (μQuant-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).

2.3. Blood biochemistry

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), creatinine, urea and total protein

were assayed using a Dimension EXL 200 Integrated Chemistry System (Siemens, Erlangen, Germany), according to the manufacturer's specifications.

2.5. Statistical analysis

Results were statistically analyzed using Graph Pad Instat Software (San Diego, California, USA) using the one-way analysis of variance (ANOVA). Tukey–Kramer post hoc test was used for comparisons between means when ANOVA was significant at the $P< 0.05$ level [29]. Values were expressed as mean ± standard error mean (S.E.M.).

3. Results

Treatments did not produce symptoms of toxicity, nor alterations in water and food consumption, or in body weight gain (data not shown).

3.1. Liver oxidative stress

Figure 1 shows the result of oxidative stress assay in liver. The lipid hydroperoxide (Fig. 1A) was significantly increased ($P<0.05$) in all EB exposure groups compared to control. The SOD activity (Fig. 1B) was significantly decreased ($P<0.05$) in EB1 and EB2 exposure groups compared to control. The CAT (Fig. 1C) and GPX (Fig. 1D) activities were significantly reduced ($P<0.05$) in all EB exposure groups compared to control. The total protein of liver tissue had no difference ($P>0.05$) between groups (data not shown).

3.2. Blood biochemistry

Figure 2 shows the biochemical serum assay of kidney biomarkers. We observe that the creatinine level (Fig. 2A) in the groups EB1 and EB2 was significantly decreased ($P<0.05$) compared to control. The urea level (Fig. 2B) don´t showed differences ($P>0,05$) between treatment groups.

Table 1 shows the biochemical serum assay of liver biomarkers. We observe that the activity of ALT, AST and ALP enzymes were unchanged ($P>0.05$) in relation to control. The activity of GGT enzyme was significantly reduced ($P<0.05$) in the groups EB1 and EB2 compared to control. The total liver protein in serum had no significant alterations ($P>0.05$) between groups.

4. Discussion

Present results demonstrated that sub-chronic emamectin benzoate exposure provoked liver oxidative stress without increase of liver and renal biochemical biomarkers.

Stimulation of free radical production, disturbance of the antioxidant capability of the body and induction of lipid peroxidation are pesticides mechanisms of toxicity [30], and it has been implicated in the development of health problems mediated by pesticide exposure [31]. It is recognized that oxidative stress has an important role in the etiology and progression of chronic diseases [12].

In the present work we observed increase of the lipid hydroperoxide concentration in all EB exposure groups. El sheik and Galal [13] studying the EB exposure and also observed an increase of the lipid peroxidation. Others works also observed this same alteration caused by the pesticide exposure [14,15].

The high rate of lipid peroxidation observed in the present work indicate that occurs oxidative stress in the liver of animals EB exposed, suggesting that free radicals produced due to EB presence can induce oxidative damage in this tissue.

The oxidative stress occurs when the rate at which ROS are generated exceeds the capacity of the cell to remove them by anti-oxidants systems. In biological membranes the OS results in injury of membrane structure and loss of activity. Free radical mediated oxidation of polyunsaturated fatty acids and cholesterol, promoting the lipid peroxidation and the formation of hydroperoxides [32,33]. The lipid peroxidation involves complex reactions and a great variety of substrates. The lipids hydroperoxides are formed as the major primary product of lipid peroxidation and can be used as a biomarker to assess the oxidative stress status [33].

The ROS production comes from many intracellular sources. The larger contributor to intracellular oxidant production in most cell types is the mitochondria. The ATP production in an oxygen-dependent manner privilege the generation of superoxide anion, which is generated by the addition of one electron to O_2. The superoxide production is believed to be more influenced by the complex I and complex III of the electron transport chain [34].

The pesticides can disturb oxidative homeostasis through direct or indirect pathways, including mitochondrial or extra-mitochondrial generation of free radicals, thiol oxidation and depletion of cellular antioxidant reservoirs [4,35]. Zanoli et al. [36] in your work with abamectin observed impairment of mitochondrial bioenergetics, caused by inhibition on the adenine nucleotide translocator and F_0F_1-ATPsyntase which is a mitochondrial component involved in the ATP synthesis.

For neutralizing these ROS the tissues posses an antioxidant capability including enzymatic and non-enzymatic antioxidants with the function of blocking harmful effects of ROS. The SOD, CAT and GSHPx are enzymes involved in the maintenance of the redox balance and responsible to neutralize the ROS, since the primary ROS produced the superoxide [37], when there is a high increase in oxidative stress the defense capabilities against ROS could collapse [16].

The exposure to EB in our work provoked decrease on the liver enzyme antioxidant activity. The SOD activity is reduced by the exposure to 1mg/Kg and 2 mg/Kg of EB, the GSHPx and CAT activity is reduced in all EB dose of exposure. The SOD is an enzyme capable of enhancing the dismutation of the superoxide radicals with formation of H_2O_2 and O_2^- [38]. The hydrogen peroxide produced by SODs activity is reduced to water by CAT and GSHPx [37]. CAT is another antioxidant enzyme with a heme active site responsible for its catalytic action, which transforms two H_2O_2 into two H_2O and O_2 [39]. GSHPx catalyzes the reduction of lipid or nonlipidic hydroperoxides as well as H_2O_2, with reduced glutathione to form oxidized glutathione disulfide, a product of hydroperoxide and water. Both GSHPx and CAT have an important cooperativity in the protection to H_2O_2, and GSHPx also to organic hydroperoxides [39,40].

The present work shows an impairment of the enzymatic antioxidant system caused by EB exposure in agreement with other works which also observed decrease of these antioxidant enzymes caused by compounds of the avermectins class [13,41]. These enzymatic antioxidant systems are susceptible to the reactive molecules. The SOD activity can be reduced by H_2O_2; GSHPx activity by hydrogen peroxide and hydroxyl radicals and the CAT by hydroxyl radicals and O_2^- [42]. This enzymatic system can also be affected by changes in your structure caused by ROS [43]. The paraquat pesticide exposure in the work of Takiwaza et al. (2007) [44] also provoked a decrease in the GSHPx activity, and these are associated to an injury of activity enzyme by ROS.

The pesticide exposure is able to provoke liver and kidney toxicity [45]. The liver is a crucial organ in the first line of defense and metabolism, and appears to be the most common target organ damaged by chemicals [46]. The kidney, another organ involved in lower degree with the metabolic activity, plays an important role in the elimination of numerous xenobiotics and endogenous compounds [20].

The liver toxicity can be assayed by evaluation of serum biochemical parameters, which can provide information of the extent and severity of liver damage and the type of damage (membrane injury versus cholestasis and hepatic function) [47].

The evaluation of serum aminotransferases activities like ALT and AST are utilized as biomarkers of liver damage [48]. These enzymes are present in the hepatocytes, and hepatocellular injury and/or alterations on liver membrane permeability permit these enzymes “leak-out” of the membrane into peripheral blood. For this reason the measurement of their activities in serum can be used to detect liver injury [47].

The alkaline phosphatase and gamma-glutamyltransferase are considered cholestatic induction enzymes of hepatobiliary origin. ALP and GGT could be elevated by impaired bile flow and due to some drugs exposure. The increased synthesis of these enzymes occurs and they are released into circulation [49,50]. The GGT have the function of metabolize extracellular reduced glutathione, allowing for precursor amino acids to be assimilated and reutilized for intracellular glutathione synthesis and could be involved in the generation of ROS [50].

In the present work the biochemical liver evaluation not detected none increase in the level of biomarker enzymes in all EB exposure groups. However, was observed OS in the hepatic tissue which may not have so intense to cause alterations on the membrane permeability and overflow of the enzymes. However, these findings not agree with other works using EB exposure, which observed hepatotoxicity correlated to an increase of the hepatic enzymes and alterations on hepatic architecture [13,19].

The pesticide exposure also is correlated to alterations of renal function and the elevations of urea and creatinine concentrations in serum may be associated with impaired kidney function, since this organ excrete this substances by filtration process [51]. In the present work we did not observed increase of the urea or creatinine concentrations indicating that EB did not caused alterations of kidney excretory function. However, Khaldoun-Oularbi (2015) [20] in your work with EB observed nephrotoxicity caused by EB. Increase of the urea concentration are not observed in the treatments however exposure to 10 mg/Kg of EB caused increase of the uric acid and creatinine associated to histopathological kidney alterations. The low dose (5 mg/Kg) provoked just increase of the uric acid but also associated to disruption of the renal architecture.

In the present work we did not found increase of the serum biochemical parameters of liver and kidney. The EB exposure could be not so drastic to cause elevations of this parameters which occur when these organs are damaged. However the presence of the lipid peroxidation and prejudice of the antioxidant enzyme defense indicates a possible liver damage.

5. Conclusions

The present work demonstrated that sub-chronic emamectin benzoate exposure in rats provoked oxidative stress in the liver tissue without increase of the serum biochemical parameters of liver and kidney. The oxidative stress could occur by an increase of the ROS production and/or alteration on the antioxidant defense. It is necessary more research to establish the specific manner by which EB provoked these OS alterations.

Disclosure statement

Authors declare that there are no conflicts of interest.

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Figures and table:

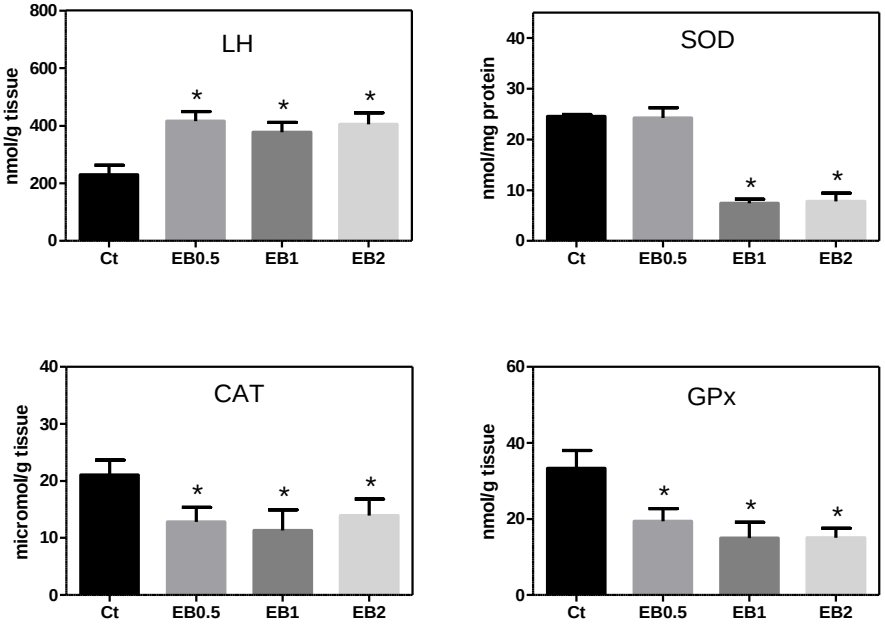

Figure 1 - Assay of oxidative stress biomarkers in liver tissue of animals exposed to emamectin benzoate. LH= Lipid hydroperoxide, SOD= Superoxide dismutase, CAT= Catalase, GPx= Glutathione peroxidase. Values represent mean ± S.E.M. of 7 samples per group of treatment. Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB1= Emamectin benzoate 1 mg/Kg; EB2= Emamectin benzoate 2 mg/Kg. *P<0.05 vs. Ct (ANOVA).

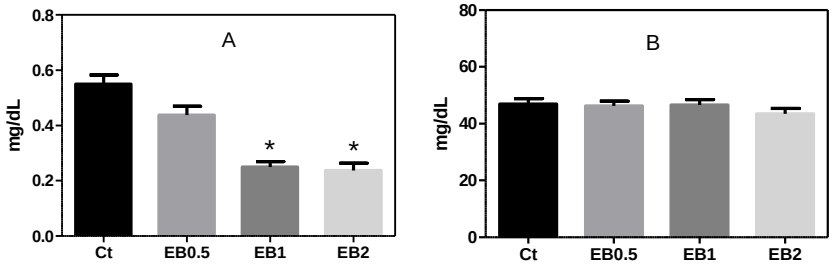

Figure 2 - Assay of kidney biomarkers in serum of animals exposed to emamectin benzoate. (A) creatinine, (B) urea. Values represent mean ± S.E.M (N= 8 samples per group of treatment). Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB1= Emamectin benzoate 1 mg/Kg; EB2= Emamectin benzoate 2 mg/Kg. *P<0.05 vs. Ct (ANOVA).

Table 1 - Quantification of hepatic biomarkers in serum of rats exposed to emamectin benzoate.

Biomarkers	Ct	BE0.5	BE1	BE2
ALT (U/L)	58.00±3.62 [a]	53.13±2.72 [a]	69.75±6.15 [a]	70.13±2.14 [a]
AST (U/L)	116.00±10.59 [a]	82.25±8.19 [a]	102.63±16.51 [a]	106.13±19.50 [a]
ALP (U/L)	205.50±18.91 [a]	190.38±14.35 [a]	185.13±18.48 [a]	195.00±25.11 [a]
GGT (U/L)	5.75±0.45 [a]	5.75±0.37 [a]	2.42±0.18 [b]	2.75±0.31 [b]
TP (g/dL)	6.50±0.64 [a]	6.79±0.18 [a]	6.24±0.23 [a]	6.21±0.07 [a]

Values represent mean ± S.E.M. (N = 8). Values with different superscript (a, b) in each row are significantly different at $P<0.05$; Ct= control; EB0.5= Emamectin benzoate 0.5 mg/Kg; EB1= Emamectin benzoate 1 mg/Kg; EB2= Emamectin benzoate 2 mg/Kg. ALT= alanine aminotransferase, AST= aspartate aminotransferase, ALP= alkaline phosphatase, GGT= gamma-glutamyltransferase, TP= total protein.

4. Conclusão geral

O presente trabalho de pesquisa permitiu avaliar os efeitos da exposição subcrônica de ratos ao BE, com a elaboração de dois artigos científicos. O primeiro artigo descreveu a avaliação da neurotoxicidade causada pela exposição ao BE e o segundo, a hepatotoxicidade e a quantificação dos biomarcadores da função hepática e renal. Juntos, os artigos demonstraram que a exposição subcrônica de ratos ao BE provocou alterações neurocomportamentais com um perfil ansiolítico, prejuízo da coordenação motora e estresse oxidativo nos tecidos cerebral e renal, sem aumentar a concentração sérica de biomarcadores das funções hepática e renal.

Estes resultados reforçam a relação entre a exposição à praguicidas e o desenvolvimento de doenças crônicas e neurodegenerativas, visto que alterações do estresse oxidativo e tais doenças têm sido correlacionadas.

São necessários trabalhos adicionais para melhor compreensão do mecanismo de ação determinante para as alterações comportamentais aqui observadas, incluindo pesquisas sobre neurotransmissores e estresse oxidativo em regiões cerebrais específicas responsáveis pela modulação comportamental e elucidação das vias moleculares específicas responsáveis pelas alterações oxidativas observadas.

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