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EFEITO DA OXIMA (3Z)-5-CLORO-3-(HIDROXIIMINO)-INDOLIN-2-ONA SOBRE A HEPATOTOXICIDADE E OS DISTÚRBIOS NA HOMEOSTASE DA GLICOSE INDUZIDOS PELA INTOXICAÇÃO POR ORGANOFOSFORADO

DISSERTAÇÃO DE MESTRADO

EDINA DA LUZ ABREU

Uruguaiana, RS, Brasil

2019

EFEITO DA OXIMA (3Z)-5-CLORO-3-(HIDROXIIMINO)-INDOLIN-2-ONA SOBRE A HEPATOTOXICIDADE E OS DISTÚRBIOS NA HOMEOSTASE DA GLICOSE INDUZIDOS PELA INTOXICAÇÃO POR ORGANOFOSFORADO

Por

Edina da Luz Abreu

Dissertação apresentada ao Programa de Pós-graduação em Bioquímica, da Universidade Federal do Pampa (UNIPAMPA), como requisito parcial para obtenção do grau de **Mestre em Bioquímica**.

Orientadora: Profª Drª Simone Pinton
Coorientador: Prof. Dr. Cristiano Ricardo Jesse

Uruguaiana, RS, Brasil
2019

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elaborada por

Edina da Luz Abreu

Como requisito parcial para obtenção do grau de

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COMISSÃO EXAMINADORA:

Profª Drª Simone Pinton
(presidente, orientador)

Prof Dr. Vanderlei Folmer

Drª Caroline Brandão Quines

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EPÍGRAFE

"O cientista não é o homem que fornece as verdadeiras respostas; é quem faz as verdadeiras perguntas". (Claude Lévi-Strauss)

PARTE I

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioquímica
Universidade Federal do Pampa

EFEITO DA OXIMA (3Z)-5-CLORO-3-(HIDROXIIMINO)-INDOLIN-2-ONA SOBRE A HEPATOTOXICIDADE E OS DISTÚRBIOS NA HOMEOSTASE DA GLICOSE INDUZIDOS PELA INTOXICAÇÃO POR ORGANOFOSFORADO

Autor: Edina da Luz Abreu
Orientadora: Simone Pinton
Co-orientador: Cristiano Ricardo Jesse
Local e data da defesa: Uruguaiana-RS, 13 de fevereiro de 2019.

Os pesticidas organofosforados (OF's) são compostos químicos amplamente utilizados na agricultura, jardins, lar e veterinária. Esses compostos estão associados a vários efeitos adversos na saúde humana e animal. Os OF's apresentam como mecanismo primário de toxicidade a inibição na enzima acetilcolinesterase (AChE). Por isso, o estudo e síntese de moléculas denominadas oximas, com capacidade reativadora da atividade da AChE, ganharam destaque nas últimas décadas. Entretanto, os efeitos nocivos dos OF's vão além das alterações neurais. Esses compostos podem provocar danos sistêmicos. Diante disso, neste estudo, investigamos os efeitos da oxima, (3Z) -5-cloro-3- (hidroxiimino) indolin-2-ona (OXIMA), de síntese inédita, sob os níveis de glicose plasmática e glicogênio hepático, atividade hepática da AChE, além de marcadores de hepatotoxicidade e estresse oxidativo, induzidos pela exposição aguda ao organofosforado malation. Os ratos Wistar

machos receberam primeiramente malation, 250mg/kg, via intraperitoneal, e logo após a administração da OXIMA, 50mg/kg, via intragástrica. Após doze horas do tratamento o sangue foi retirado, via punção cardíaca e separado o plasma bem como o tecido para as análises bioquímicas futuras. A administração da OXIMA diminuiu a glicemia e o conteúdo de glicogênio hepático aumentados pelo malation. Além disso, a OXIME protegeu contra o aumento dos marcadores plasmáticos da função hepática (AST, ALT, ALP) e a inibição das enzimas catalase e da glutationa redutase no fígado de ratos tratados com malation. Além disso, a inibição da acetilcolinesterase hepática induzida pela exposição aguda ao malation, que consiste no mecanismo primário de toxicidade de organofosforados, foi suprimida pelo tratamento com a OXIMA. Assim, esses resultados indicam que a (3Z) -5-cloro-3- (hidroxiimino) indolin-2-ona é uma molécula promissora e pode ser considerada para outros estudos que buscam novas alternativas no tratamento de intoxicações por compostos organofosforados.

Palavras-chaves: acetilcolinesterase; aminotransferases; enzimas antioxidantes; metabolismo da glicose; organofosforados; oxima;

Federal University of Pampa

ABSTRACT

Dissertation of Master
Program of Post-Graduation in Biochemistry

EFEITO DA OXIMA (3Z)-5-CLORO-3-(HIDROXIIMINO)-INDOLIN-2-ONA SOBRE A HEPATOTOXICIDADE E OS DISTÚRBIOS NA HOMEOSTASE DA GLICOSE INDUZIDOS PELA INTOXICAÇÃO POR ORGANOFOSFORADO

Author: Edina da Luz Abreu
Advisor: Simone Pinton
Co-advisor: Cristiano Ricardo Jesse
Place and Date of the defense: Uruguaiana-RS, February 13, 2019.

In this study, the effects of oxime, (3Z) -5-chloro-3- (hydroxyimino) indolin-2-one (OXIMA), of unprecedented syndrome, on disorders of glucose metabolism and non-dental malation, a organophosphate insecticide in rats. Adult males were divided into four groups: Control; Malation; OXIMA; and Malation + OXIMA. Free pain after concomitant treatment with malathion (250mg / kg, i.p.) and / or oxyme (50mg / kg, i.g.), plasma and fox were collected. A single dose of OXIMA decreased glycemia and hepatic glycogen content increased by malathion. In addition, OXIMA attenuated the increase in plasma markers of liver function (AST, ALT, ALP) and inhibited the enzyme catalase and glutathione reductase in the liver of rats with the association. In addition, inhibition of hepatic acetylcholinesterase induced by long-term treatment has been one of the main toxicity factors of organophosphates, was suppressed by treatment with OXIMA. Thus, results indicate that (3Z) -5-chloro-3- (hydroxyimino) indolin-2-one is a promising

molecule and may be considered for other studies looking for novel alternatives without treatment of organophosphorus poisoning.

Keywords: acetylcholinesterase; aminotransferases; antioxidant enzymes; glucose metabolism; organophosphate; oxime.

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LISTA DE SIGLAS E ABREVIATURAS

AChE - Acetilcolinesterase

ALP - Fosfatase alacalina

ALT – Alanina aminotransferases

ANVISA – Agência Nacional de Vigilância Sanitária

AST – Aspartato aminotransferase

CAT - Catalase

DTNB - 5,5′-ditiobis-2-ácido nitrobenzóico

EDTA - Ácido etilenodiamino tetra-acético

GPx - Glutationa Peroxidase

GR - Glutationa Redutase

GSSG - Glutationa oxidada

i.p - intraperitoneal

LDH – Lactato desidrogenase

MAPA - Ministério da Agricultura Pecuária e Abastecimento

NADPH - Fosfato de dinucleotídeo de nicotinamida e adenina

NPSH - Tios não proteicos

OF – Organofosforado

SNC – Sistema Nervoso Central

TBARS - Espécies reativas ao ácido tiobarbitúrico

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1. INTRODUÇÃO

O Brasil é um grande consumidor de pesticidas, incluindo os OF's, em 2009, passou a ocupar a posição de maior consumidor mundial, atingindo a marca de 1 milhão de toneladas de substâncias tóxicas consumidas (FERREIRA et al., 2017). Por isso, devido à alta toxicidade apresentada por esses compostos OF's, a intoxicação aguda ou crônica é considerada um problema de saúde pública (BERTOLOTE et al., 2006; BRASIL, 2016; CHOWDHARY et al., 2014).

Os pesticidas organofosforados (OF's) são compostos químicos amplamente utilizados na agricultura, jardins, lar e veterinária (ULLAH et al., 2018). Esses compostos estão associados a vários efeitos adversos na saúde humana e animal (DEVAULT et al., 2018; FERRÉ et al., 2018; STROBEL et al., 2018). Apresenta como mecanismo primário de toxicidade a inibição da atividade da enzima acetilcolinesterase (AChE). Esta enzima catalisa a hidrólise da acetilcolina em colina e acetato no sistema nervoso central (SNC) e periférico, nos gânglios autônomos e na placa motora (SURESHA; KUMAR, 2017). Mas, os efeitos da exposição aos OF's vão além das alterações neurais. Diversos estudos com animais relatam efeitos sistêmicos, como por exemplo, na homeostase da glicose após exposição aguda e crônica a OF's (KAMATH et al., 2007; LASRAM et al., 2008). Outros, relatam que os OF's, como o clorpirifós e o acefato causaram toxicidade através do estresse oxidativo e alterações nos níveis de glicose plasmática e glicogênio hepático (ACKER; NOGUEIRA, 2012b; EVERETT; MATHESON, 2018). Ainda, há relatos na literatura que sugerem que os OF's influenciam as vias da glicogenólise e da gliconeogênese bem como superestimulam o eixo hipotálamo-hipófise-adrenal (JOSHI; RAJINI, 2009, 2012).

Dentre os OF's comumente utilizados, o malation [2-dimetoxifosfinotioilsulfanilbutanodioato de dietilo] se destaca devido à sua alta toxicidade (REBECHI et al., 2014; COLOVIC et al., 2013). De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA, 2012) é altamente tóxico. O malation é um dos compostos OF's mais lipofílicos, assim é facilmente distribuído por todo o corpo, atingindo concentrações maiores, principalmente no fígado, rim, intestino delgado, trato urinário e pulmões. Sua bioativação é mediada primariamente por enzimas hepáticas do citocromo P450 (MUTCH; WILLIAMS, 2007), gerando um metabolito ativo,

denominado malaoxon, este apresenta efeito tóxico cinco vezes maior que o composto primário (HUANG et al., 2015, SELMI et al., 2018). As enzimas presentes no fígado são sensíveis a ação do malation. Por isso, a literatura sugere que a exposição aguda a este OP pode induzir a hepatotoxicidade (SELMI et al., 2018; ABDEL- SALAM et al., 2017; LASRAM et al., 2015) afetando o metabolismo das enzimas aspartato e alanina aminotransferases, fosfatase alcalina e lactato desidrogenase (ACKER; NOGUEIRA, 2012b; al. 2017)

A estratégia terapêutica padrão adotada para a exposição aguda aos OFs inclui o uso de atropina (anticolinérgica) e oximas (reativadores da AChE). Derivados de oximas (pralidoxima e obidoxima) têm sido utilizados como antídoto na etapa de desintoxicação, porém a efetividade dessas terapias ainda é controvérsia, pois depende do composto OP que causa a intoxicação, sua dose e tempo de exposição (MASSON; NACHON, 2017). O mecanismo de ação dessas moléculas é por ataque nucleofílico ao átomo de fosforo do OF. Entretanto, essas drogas apresentam baixa penetração na barreira hematoencefálica e ação nucleofílica (capacidade de receber elétrons) variada (CAVALCANTI et al., 2016). Assim, nas últimas décadas, o desenvolvimento e síntese de novas moléculas oxima ganharam destaque (LORKE; PETROIANU, 2018).

Diante disso, uma molécula de síntese inédita, a (3Z) -5-cloro-3- (hidroxiimino) indolin-2-ona (Figura 1), pode ser uma opção promissora no tratamento da intoxicação por organofosforado, pois, em estudos preliminares realizados pelo nosso grupo, não apresentou efeitos tóxicos em ratos e *Artemia salina*, bem como mostrou ter potencial farmacológico em reverter a inibição da AChE induzida pelo organofosforado malation em *Artemia salina* (dados não publicados).

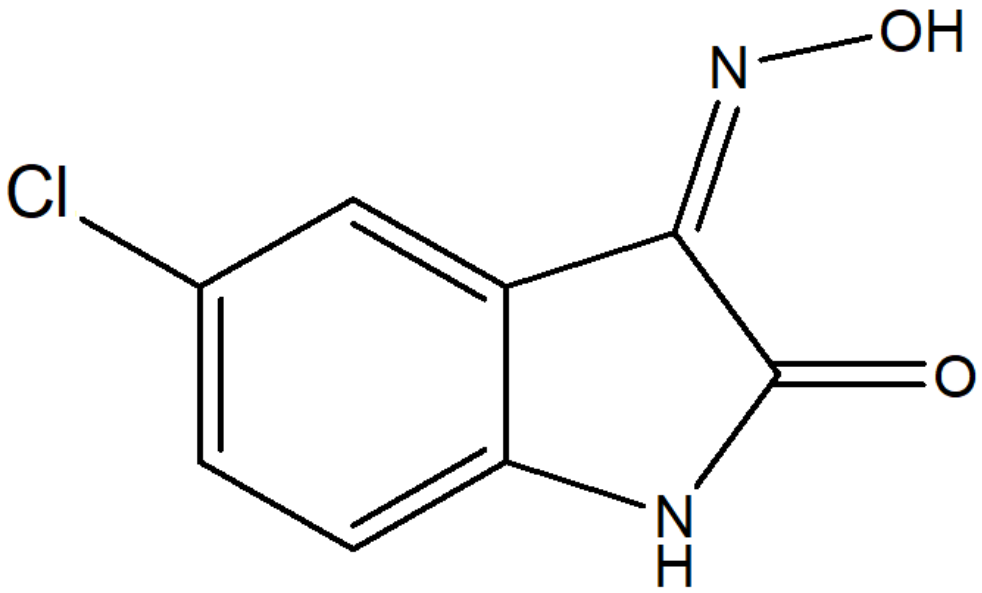


Figura 1 – Estrutura química da a (3Z) -5-cloro-3- (hidroxiimino) indolin-2-ona.

Fonte: Martins et al., 2016.

2. REVISÃO BIBLIOGRÁFICA

2.1 Agroquímicos

Os agroquímicos são produtos e agentes de processos físicos, químicos ou biológicos, utilizados nos setores de produção, armazenamento e beneficiamento de produtos agrícolas, pastagens, proteção de florestas, nativas ou plantadas, e de outros ecossistemas e de ambientes urbanos, hídricos e industriais. Visa alterar a composição da flora ou da fauna, a fim de preservá-las da ação danosa de seres vivos considerados nocivos. Também são considerados agrotóxicos as substâncias e produtos empregados como desfolhantes, dessecantes, estimuladores e inibidores de crescimento (BRASIL, 2002).

2.2 Organofosforados (OF's)

Agroquímicos OF's são os pesticidas mais utilizados no mundo, com mais de 100 compostos em uso (SURATMAN et al., 2018). São caracterizados como ésteres, amidas ou derivados tiol dos ácidos de fósforo, contendo várias combinações de carbono, hidrogênio, oxigênio, fósforo, enxofre e nitrogênio (PEREZGASGA et al., 2012). Os OF's possuem alta lipossolubilidade assim distribuem-se de forma rápida pelos tecidos orgânicos com fácil passagem pela barreira placentária e hematoencefálica podendo iniber permanentemente a enzima AChE. A inibição ocorre através da fosforilação da enzima, isso leva a acúmulo de acetilcolina e consequentemente superestimulação das terminações nervosas, tornando inadequadas a transmissão de seus estímulos às células musculares, gandulares, ganglionares e do SNC (MILESON et al., 2008; EDDLESTON; HILLIPS, 2004). O mecanismo de inibição da AChE é por ação do OF no sítio esterático (ativo) da enzima, por meio de uma ligação covalente do átomo de fósforo do OF com o átomo de oxigênio do aminoácido serina (Figura 2). A inibição da AChE pelo OF pode conduzir ao envelhecimento da enzima devido a uma reação de desalquilação do OF ligado a enzima. Neste momento resulta na formação de uma forte interação por ligação de hidrogênio entre o resíduo da histidina da tríade catalítica protonado e o átomo de oxigênio do inibidor produzindo uma inibição irreversível (CAVALCANTI et al., 2016).

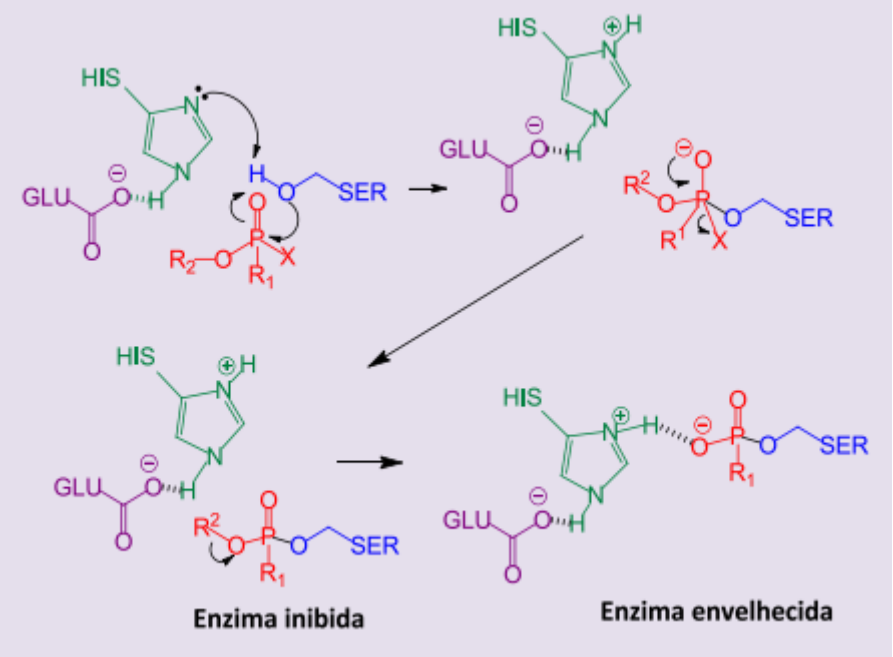


Figura 2 - Mecanismo de inibição e envelhecimento da AChE por OF.

Fonte: Cavalcanti et al., 2016.

Os OF's podem ser absorvidos através das vias, oral, inalatória, dérmica e mucosas. Os efeitos da exposição podem ocorrer minutos ou horas depois dependendo do tipo de composto OF. Sendo, as principais manifestações clínicas agudas:

a) Muscarínicas: vômito, diarreia, cólicas abdominais, broncoespasmos, bradicardia, cefaleia, incontinência urinária, visão borrada, hipovolemia;
b) Nicotínicas: hipertensão arterial, tremores, fraqueza;
c) SNC: ansiedade, agitação, confusão mental, ataxia, depressão de centros cardio-respiratório, convulsões e coma.

Mesmo que o mecanismo primário de toxicidade dos OF's seja a inibição da AChE, diversos autores têm relatados outros efeitos adversos em vários sistemas e órgãos, como por exemplo, fígado, rim, músculo, coração, testículos, dentre outros (ACKER,2012; ABDOLLAHI et al., 2009; KALENDER et al., 2007; NAHID et al., 2016). Muitos desses efeitos tóxicos são associados a diminuição das defesas antioxidantes enzimáticas e não-enzimáticas e elevação da peroxidação lipídica (VANONA et al., 2018). Por isso, o aumento do estresse oxidativo (EO) pode ser um dos principais mecanismos de toxicidade dos OF's (ACKR, 2012). Sendo caracterizado como

um aumento de espécies reativas e diminuição das defesas antioxidantes, o EO pode levar a danos às macromoléculas e ao DNA desencadeando diversas patologias (BIRBEN et al., 2012).

Além disso, nas últimas anos, alterações no metabolismo da glicose estão sendo associadas a exposição aguda ou crônica aos OF's. Relatos na literatura utilizando animais experimentais demonstraram que a exposição a acetato, malation e paration, provocaram aumento da glicemia diante da exposição (RUCKMANI et al., 2011; ACKER, 2012; RATHISH et al., 2016 RAMIREZ-VARGAS et al., 2018;). Os mecanismos envolvidos nesse quadro hiperglicêmico, são relatos pela capacidade dos OF's em ativar algumas enzimas das vias metabólicas da glicogenólise e da gliconeogênese (REZG et al., 2006; JOSHI et al., 2012) e ainda serem capazes de inibir as enzimas da glicólise (ABDOLLAHI et al., 2009).

2.3 Malation

O malation [2-dimetoxifosfinotioilsulfanilbutanodioato de dietilo] (figura 3) é um dos pesticidas organofosforados da classe toxicológica II, descrito como altamente tóxico, apresentando uma LD50 oral de 5-50 mg/Kg (ANVISA, 2012). A exposição pode provocar diversos efeitos maléficos na saúde humana e animal. Devido a sua natureza lipofílica, possui assimilação intestinal simples e rápida e, também consegue atingir todos os tecidos do corpo humano e animal.

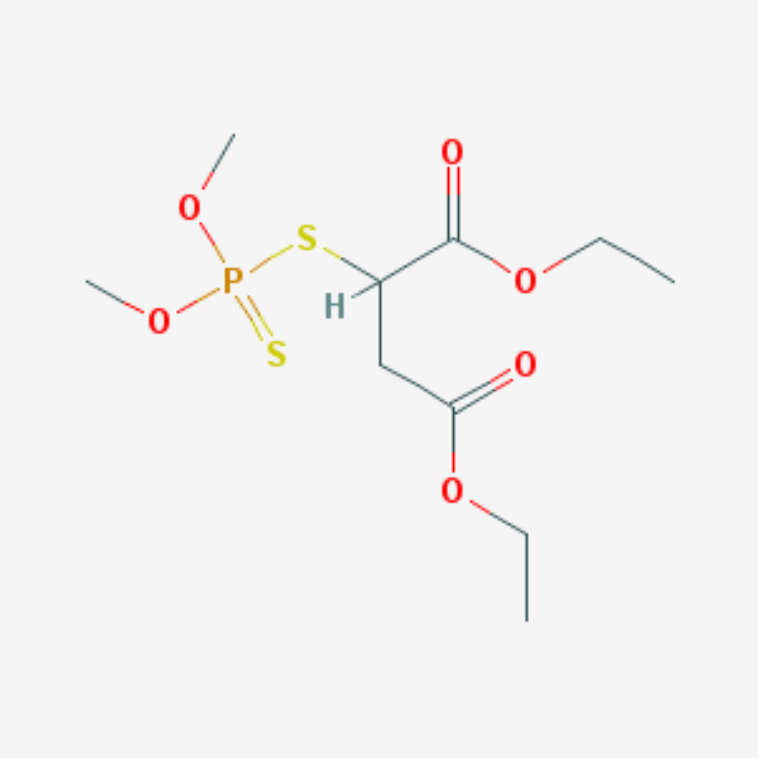


Figura 3 – Estrutura química do malation.

Fonte: Open Chemistry Data Base https://pubchem.ncbi.nlm.nih.gov/compound/malathion

Relatos na literatura associam a exposição ao malation ao desenvolvimento de patologias tais como insuficiência do sistema imunológico, pancreatite, doença hepática, hematológica, lesão renal, diminuição da fertilidade (DELGADO et al., 2006; REUS et al., 2008; FRANCO et al., 2009; SELMI et al., 2015). Sendo o fígado o principal local de biotransformação xenobiótica, este pode desempenhar um papel central na resposta a alterações provocadas pelo malation, pois a estrutura hepática bem como as enzimas hepáticas são sensíveis ao malation. O malation quando metabolizado pelo fígado, através das enzimas do CYP 450, produz o metabólito, malaxon, que é cinco vezes mais tóxico e provocou aumento da peroxidação lipídica e diminuição das defesas antioxidantes enzimáticas de ratos expostos (HUANG et al., 2015; SELMI et al., 2018).

Atualmente, um dos efeitos metabólicos da exposição aguda ao malation refere-se as alterações na homeostase na glicose. Na figura 4 pode-se observar uma das vias que OF's podem aumentar a glicemia de ratos expostos. O malation possui a capacidade de ativar a enzima glicogênio fosforilase, esta enzima catalisa a clivagem fosforolítica do glicogênio iniciando a sua rota de degradação. Outra enzima sensível a ativação pelo

malation é a PEPCK, enzima responsável por dar início a via da gliconeogênese. Uma vez atividade essas duas vias metabólicas, o organismo pode apresentar um quadro de hiperglicemia.

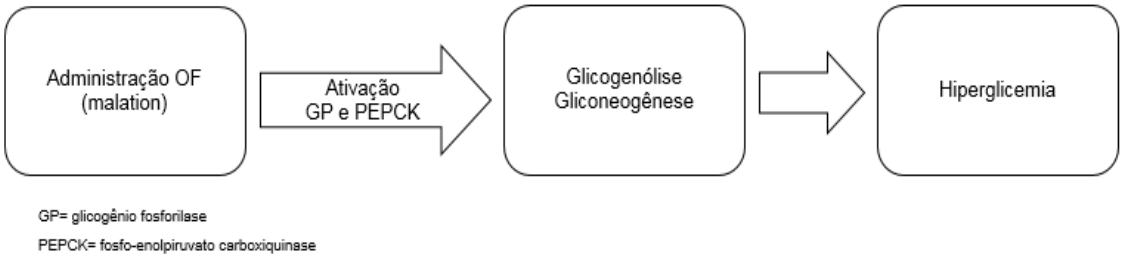
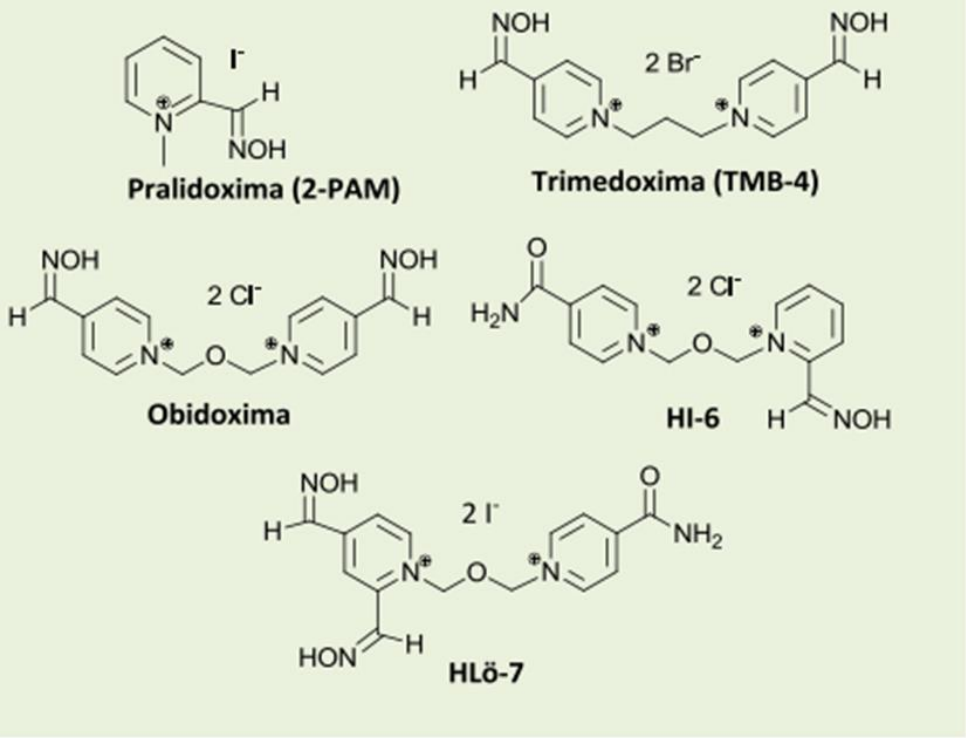


Figura 4 – Mecanismo de ação do OF (malation) no metabolismo da glicose de ratos expostos. (Adaptado de Rezg et al., 2008.)

2.4 Oximas

O interesse nas moléculas oximas surgiu após 1905, quando Lev Tschugaeff descreveu a alta seletividade e sensibilidade da dimetiglioxime. Mas foi apartir das décadas de 40 e 60 que elas ganharam destaque. E, desde de então iniciou-se pesquisas para aplicação em síntese de novas moléculas de uso na indústria e produtos farmacêuticos (VESSALLY; ABDOLI, 2016). As oximas são moléculas orgânicas que apresentam como fórmula geral RRC=N-OH, ganharam destaque devido a sua capacidade de reativar a atividade da enzima acetilcolinesterase de forma mais rápida.

Na clínica as oximas são utilizadas no tratamento padrão da intoxicação por OF’s, especialmente, a pralidoxima e a obidoxima. No quadro 1, são apresentadas as estruturas químicas de algumas dessas moléculas em uso clínico.



Quadro 1 – Exemplos de oximas e suas estruturas químicas.

Fonte: Google imagens

Na estrutura da AChE existe um centro ativo para inativação da enzima, que contém um sítio aniônico e um esterásico. A inibição da AChE por compostos OF se dá através da fosforilação do sítio esterásico (sitio ativo). As oximas agem diretamente na enzima fosforilada (Figura 5), pois conseguem restabelecer as condições do sítio ativo, devido a capacidade de doarem prótons, isso contribui para o deslocamento do radical fosfato da ligação com o sítio esterásico da enzima e usa vez quebrada a ligação a atividade da enzima é restabelecida (GARCIA et al.,2010; MUSILEK et al., 2011).

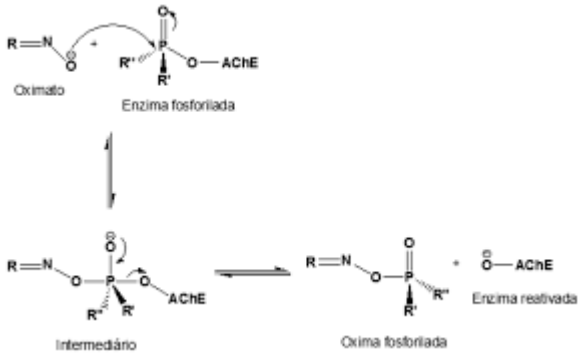


Figura 5 – Ação das oximas na reativação da AChE.

Fonte: Petronilho et al., 2011.

A inibição da AChE pode levar a um processo chamado de “envelhecimento”, ocasionado pela reação de desalquilação do OF ligado a enzima. Quando esse processo é completado a inibição se torna irreversível devido à forte ligação por pontes de hidrogênio entre a enzima e o OF. Assim, a ação efetiva das moléculas reativadoras acontecesse somente durante o período denominado de “intervalo crítico” que vai desde a inibição até antes da conclusão do ciclo de envelhecimento. Portanto a efetividade das oximas é, proporcional à precocidade de sua administração. Pela diversidade de compostos OF’s existentes e as diferenças de absorção, distribuição e toxicidade deles, ainda não há uma oxima universal e totalmente eficaz contra a intoxicação destes compostos. (MASSON; NACHON, 2017).

A síntese de novas moléculas oximas ocorre pela adição de cloridato de hidroxilamina a um aldeído ou cetona utilizando meio alcoólico aquoso como solvente (SOARAES et al., 2013). E a capacidade de ação das oximas depende do grau de nucleofilicidade que varia de acordo com a composição da molécula bem como suas características físico-químicas, tais como: presença de anel piridínico quaternizado, o número de anéis piridínicos, a posição do grupo oxima na estrutura (orto ou para) e o tamanho do espaçador entre os anéis de piridina nos derivados bipiridínicos ARAÚJO; GONSALVES, 2015). Assim, nos últimos anos, várias moléculas de síntese inédita foram estudadas e demonstraram efeitos como capacidade antioxidante e anti-inflamatória (Puntel et al., 2008; Mohassab et al., 2017). Ainda, de acordo com Freitas (2012) a oxima butano-2,3-dionatiossemicarbazona apresentou efeitos farmacológicos contra o dano testicular provocado pela exposição crônica ao cloreto de cádmio. Conforme, Choi (2014) a indirubina-3-oxima foi capaz de diminuir a adipogênese via ativação da Wnt-β catenina no fígado. Ainda reduziu os níveis plasmáticos e hepáticos de triglicerídeos, colesterol total e fosfatase alcalina. E atenuou a resistência à insulina, sendo sugerido como alternativa terapêutica suggests a possibility to develop an anti-obesity therapy without frente os efeitos deletérios da obesidade.

Embora, que a literatura relate a grande capacidade reativadora das oximas, maior que 60 % contra a ação de OF’s, o amplo espectro desses compostos, especificidades de ação e toxicidade têm sido fatores limitantes nesse processo. Por isso, o estudo de novas moléculas e suas possíveis ações farmacológicas são de suma importância.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar o possível efeito da oxima (3z)-5-cloro-3-(hidroxiimino)-indolin-2-ona sobre a hepatotoxicidade e os distúrbios na homeostase da glicose induzidos pela intoxicação aguda induzida pelo organofosforado malation em ratos.

3.2 Objetivos Específicos

- Verificar possíveis alterações nos níveis de glicose plasmática e glicogênio hepático;
- Analisar marcadores de hepatoxicidade;
- Investigar parâmetros de estresse oxidativo no fígado;
- Avaliar o efeito do malation na atividade hepática da AChE e o possível papel protetor da (3z)-5-cloro-3-(hidroxiimino)-indolin-2-ona;

PARTE II

Manuscrito

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de manuscrito. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio manuscrito e representam a íntegra deste estudo. O manuscrito está disposto da mesma forma que foi submetido para a Revista "Chemico-Biological Interactions" que apresenta Qualis B1 na área CBII .

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A single dose of (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one attenuates hyperglycemia and hepatic damage caused by malathion in rats

Edina da Luz Abreu[a]; Anne Suély Pinto Savall[a]; Allyson Ardais Boneberg[a]; Bianca Barreto Martins[b], Vanessa Carratú Gervini[b]; Tuane Bazanella Sampaio[c]; André Ricardo Fajardo[d]; Natália Paroul[e]; Daniel Henrique Roos[a]; Simone Pinton[a]*

[a]Universidade Federal do Pampa (UNIPAMPA) - Campus Uruguaiana, Uruguaiana, CEP 97500-970, RS, Brazil.

[b]Universidade Federal do Rio Grande - Campus Carreiros, Rio Grande, CEP 96201-900, RS, Brazil.

[c]Instituto Federal de Educação, Ciência e Tecnologia Farroupilha – Campus Santo Ângelo, Santo Ângelo, CEP 98806-700, RS, Brazil.

[d]Universidade Federal de Pelotas (UFPel) - Campus Capão do Leão s/n, Pelotas CEP 96010-900, RS, Brazil.

[e]Universidade Regional Integrada, Campus Erechim, CEP 99700-000, RS, Brazil

*Corresponding author:

Simone Pinton,

Universidade Federal do Pampa - Campus Uruguaiana, Uruguaiana, CEP 97500-970, RS, Brazil

Phone: 55-55-3911-0200

E-mail: sipinton@gmail.com

Abstract

In this study, we investigated the effects of a single dose of a new oxime, (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one (OXIME), in the hyperglycemia and hepatic damage induced by malathion exposure, an organophosphate insecticide, in rats model. For this, adult male Wistar rats were divided into four groups: control, malathion, OXIME, and malathion+OXIME. Twelve hours after the treatments, the plasma and liver were collected for biochemical analyses. As assessed, a single dose of OXIME lowered the glycemia levels and hepatic glycogen content enhanced by malathion. Also, the OXIME blocked the increase of plasma markers of hepatic function and the enzymatic inhibition of catalase and glutathione reductase in the liver of malathion-treated rats. Moreover, the hepatic acetylcholinesterase inhibition induced by malathion acute exposure was suppressed by OXIME treatment. Overall, these findings indicate that a single dose of (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one could be a new alternative in the treatment of poisoning by organophosphates compounds.

Keywords: acetylcholinesterase; aminotransferases; antioxidant enzymes; glucose metabolism; organophosphate; oxime.

1. Introduction

Organophosphates pesticides (OP) are associated with adverse effects on human and animal health (Devault et al., 2018; Ferré et al., 2018; Strobel et al., 2018). The OP are chemicals compounds widely used in agriculture, gardens, household and veterinary (Ullah et al., 2018). Their effects are mainly linked to the inhibition of acetylcholinesterase (AChE) activity. The AChE catalyzes the acetylcholine hydrolysis to choline and acetate in the central and peripheral nervous system, autonomic ganglia, and motor end-plate (Suresha & Kumar, 2017). Furthermore, OP cause toxicity through oxidative stress and glucose homeostasis alterations such as chlorpyrifos and acephate (Acker & Nogueira, 2012b; Everett & Matheson, 2018).

Considering the numerous toxic effects induced by OP, glucose homeostasis changes and hepatic damage have been largely investigated. Studies with animals have shown altered glucose homeostasis following acute and chronic exposure to OP's (Kamath et al., 2007; Lasram et al., 2008). Glycogenolysis, gluconeogenesis, and hypothalamus-pituitary-adrenal axis stimulation seem to be altered by OP, resulting in hyperglycemia (Joshi and Rajini, 2009, 2012).

Among the OP, malathion [S-1,2(bis-ethoxycarbonyl)ethyl-O,O-dimethyl phosphorodithioate] stands out due to its high toxicity (Rebechi et al., 2014; Colovic et al., 2013). Malathion is distributed mainly to the liver, kidney, small intestine, urinary tract, and lungs. The bioactivation of malathion is mediated mainly by enzymes of cytochrome P450 in the liver (Mutch & Williams, 2007), generating the active metabolite malaoxon (Huang et al., 2015; Selmi et al., 2018). Further, the acute exposition to OP induce hepatotoxicity (Selmi et al., 2018; Abdel-Salam et al., 2017; Lasram et al., 2015),

and they disrupted the hepatic enzymes metabolism (Acker & Nogueira, 2012b; Xu et al., 2017)

The standard therapeutic strategy for acute exposure to OP includes the use of atropine (anticholinergic) and oximes (AChE reactivators). Oxime derivatives (pralidoxime and obidoxime) have been used as the antidote in detoxification step, however the results remain unsatisfactory. These drugs display low penetration into the blood-brain barrier and inefficient nucleophilic action against organophosphorus (Cavalcanti et al., 2016). Moreover, based on the mechanism of OP-mediated AChE inhibition, new drugs or alternative approaches in therapy of OP poisoning are under consideration (Lorke & Petroianu, 2018).

Thus, unpublished synthesis molecules oxime can be a promising option. The (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one (Fig. 1), did not present toxic effects in the preliminary studies carried out by our group (unpublished data). In this study, in order to evaluate a novel therapeutic approach, we investigated the effects of a single dose of (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one on alterations in the glucose homeostasis and hepatic damage induced by malathion acute exposure in rats.

FIGURE 1

2. Materials and Methods

2.1. Drugs and chemicals

Malathion 500 CE (5.0%) (Biocarb Chemical Industry LTDA, Curitiba, PR, Brazil) was obtained from commercial grade. (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one (OXIME) was synthesized at the School of Chemistry and Food of Federal University of Rio Grande (FURG), RS, Brazil as described by Martins et al. (2016). For this, an equimolar mixture of 5-chloroisatin and hydroxylamine chlorhydrate in ethanol was used. The reaction medium was acidified using acetic acid and placed under reflux (135 °C) for 5 h. The reaction was vacuum filtered and the compound was isolated as a golden yellow precipitate. After that, the isolated compound was washed in cold distilled water (F.P. 252-274 °C). Analysis of the ^{1}H ^{13}C nuclear magnetic resonance spectroscopy analysis showed that the compound obtained presented analytical and spectroscopic data in full agreement with its assigned structure. The chemical purity of OXIME (99.9%) was determined by gas chromatography–mass spectrometry. All other chemicals were obtained from analytical grade and standard commercial suppliers.

2.2. Animals

Adult male Wistar rats (n = 28), 2 months of age (250 - 350 g) were purchased from Federal University of Santa Maria, RS, Brazil. The animals were provided with food (commercial diet) and water *ad libitum* and maintained in the animal house at controlled conditions: temperature (22 ± 2 °C) and 12 h light-dark cycle (lights on at 7:00 a.m.). All experiments were performed according to the local ethics committee of the Federal University of Pampa, Brazil (CEUA/UNIPAMPA 024/2016). All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

2.3. Experimental design

In order to access the effects of an acute exposure to malathion and the putative beneficial effects of OXIME on hepatic markers, glucose homeostasis and oxidant-antioxidant system, rats were randomized into four groups: (i) control - canola oil by intragastric gavage (i.g.; 3 mL/kg) plus distilled water by intraperitoneal route (i.p.; 5 mL/kg) (n=7); (ii) malathion - canola oil (i.g.) plus malathion (250 mg/kg; i.p.) (n=7); (iii) OXIME - [(3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one, 50 mg/kg; i.g.] plus distilled water (i.p.) (n=7); (i.v) malathion + OXIME - [(3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one, 50 mg/kg; i.g.] plus malathion (250 mg/kg; i.p.) (n=7). The dose regime of OXIME and malathion was established based on a previous study performed by our research group (unpublished data).

Twelve hours after treatment with malathion and/or oxime and/or vehicles, all rats, in fasting, were anesthetized for blood collection by heart puncture (Karami-Mohajer et al., 2017). Plasma was extracted by centrifugation at 2500 g for 10 min and conserved at - 20 °C for posterior analyses. In the sequence, the animals were killed and their livers were removed. The liver samples were homogenized in 50 mM Tris-HCl (pH 7.4; 1:10 *w/v*). Except for protein carbonyl content assay, the homogenates were centrifuged at 2500 g for 10 min at 4 °C. The low-speed supernatants (S_1) or the homogenates without centrifugation were used to the biochemical assays.

2.4. Glucose homeostasis evaluation

Plasma glucose and hepatic glycogen levels were measured as indicators of the glucose homeostasis. Plasma glucose levels were established by an enzymatic method

based on the oxidase/peroxidase system using a commercial kit (Bioclin, Belo Horizonte, Minas Gerais, Brazil. Hepatic glycogen content was performed according to the method described by Good et al. (1933). Briefly, 0.3 g of the liver was digested in 3 mL of KOH 30%, incubated for 10 min at 90 °C, cooled and brought to acid pH by addition of trichloroacetic acid 20%. Precipitated protein was removed by centrifugation at 3000 g for 10 min. Glycogen was precipitated by ethanol and then weighted. The results are expressed in mg/dL for glucose levels and in g of glycogen/100 g of liver for glycogen determination.

2.5. Hepatic function markers

The plasma samples were used to determine the aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities as a parameter of the hepatic function. The colorimetric method was carried out to measure both AST and ALT activities (Reitman and Frankel, 1957). Whereas, ALP and LDH activities were accessed using a commercial kit (Bioclin, Belo Horizonte, Minas Gerais, Brazil). The values were expressed as U/dL.

2.6. Oxidative stress markers

2.6.1. Thiobarbituric acid reactive species (TBARS) levels

Hepatic TBARS levels, a measure of lipid peroxidation, were performed using an aliquot (200 µL) of S_1, 500 µL thiobarbituric acid (0.8%), 500 µL acetic acid buffer, 200 µL sodium dodecyl sulfate (SDS, 8.1%) and 100 µL distilled water. The mixture was

incubated at 95 °C for 2 h. The absorbance was measured at 532 nm. The results were expressed as nmol MDA/mg protein as described by Ohkawa et al. (1979).

2.6.2. Protein carbonyl content

Protein carbonyl content in the liver was determined through of the reaction between protein carbonyls and 2,4-dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound (Reznick & Packer,1994). Briefly, hepatic homogenates without centrifugation were diluted 1:10 (*v/v*) and an aliquot of 1 mL was added to the reaction mixture containing 200 µL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). All tubes were incubated at room temperature in the dark for 1 h and shaken with a vortex mixer each 15 min. After that, 500 µL of denaturation buffer, 1.5 mL of ethanol and 1.5 mL of hexane were added to each tube. The tubes were shaken with a vortex mixer for 40 s and centrifuged at 3000 g for 15 min. The supernatants obtained were discarded. The pellets were washed twice with 1 mL ethanol: ethyl acetate (1:1, *v/v*) and resuspended in 1 mL of denaturation buffer. Absorbance was determined at 370 nm. Data were expressed as nmol carbonyl content/mg protein.

2.7. Enzymatic antioxidant defenses

Catalase (CAT) activity was spectrophotometrically assayed by the method of Aebi (1984), which involves monitoring the consumption of H_2O_2 in the S_1 at 240 nm. The enzymatic reaction was initiated by adding an aliquot of 40 µL of the S_1 from livers and the substrate (H_2O_2) to a concentration of 0.3 M in a medium containing 50 mM

phosphate buffer, pH 7.0. The enzymatic activity was expressed in Units (1U decomposes 1 µmol H_2O_2/min at pH 7 at 25 °C)/mg protein.

Glutathione peroxidase (GPx) activity in the liver was measured by the NADPH oxidation rate at 340 nm using H_2O_2 as substrate according to Wendel (1981). The reaction mixture consisted of EDTA, nicotinamide adenine dinucleotide phosphate (NADPH), GSH, sodium azide, and glutathione reductase (GR). The reaction was initiated by the addition of H_2O_2. The disappearance of NADPH at 340 nm was recorded at room temperature. Enzyme activity was defined as nmol NADPH/min/mg protein.

Glutathione reductase (GR) activity in the liver was estimated by the method of Carlberg and Mannervik (1985). The reagent mixture was composed of 150 mM potassium phosphate buffer (pH 7.0), 1.5 mM EDTA, 0.15 mM NADPH. Oxidized glutathione (GSSG) was used as the substrate. GR activity is proportional to NADPH decay at 340 nm. The enzymatic activity was expressed as nmol NADPH/min/mg protein.

2.8. Non-protein sulfhydryl (NPSH) content

To determine hepatic NPSH content, S_1 was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and the free SH-groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB (5,5′-dithiobis-2-nitrobenzoic acid). The color reaction was measured at 412 nm. NPSH levels were expressed as nmol NPSH/g tissue (Ellman, 1959).

2.9. Hepatic AChE activity

Samples of liver were homogenized in 0.25 M sucrose buffer (1:10, w/v) and centrifuged at 2400 g for 15 min at 4 ºC. AChE activity was carried out according to the method of Ellman et al. (1961), using acetylthiocholine as substrate. The activity of AChE was spectrophotometrically measured at 412 nm and expressed as nmol/min/mg protein.

2.10. Protein determination

The protein content was quantified by the Bradford (1976) method using Coomassie blue. The mixture was incubated at room temperature for 10 min and the developed color was spectrophotometrically determined at 595 nm. Bovine serum albumin (1 mg/mL) was used as the standard.

2.11. Statistical analysis

The normal distribution of the data was tested with D'Agostino and Pearson normality test. Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by the Tukey's multiple range test when appropriated (GraphPad Prism 6 software, San Diego, CA, USA). Data were expressed as the mean ± standard error of mean (S.E.M.) of 7 animals/group. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Glucose homeostasis determination

Plasma glucose levels were substantially increased by malathion acute exposure ($F_{(1,24)}$ = 6.36, p = 0.0187). The Tukey's test indicated that a single dose of OXIME prevented the hyperglycemia caused by malathion in rats (p = 0.0355) (Fig. 2A). Furthermore, the two-way ANOVA of hepatic glycogen data showed a significant main effect of the malathion treatment ($F_{(1,24)}$ = 5.26, p = 0.0309) (Fig. 2B). Malathion significantly raised the glycogen contents in rats' liver (p = 0.0174) and a single dose of OXIME was able to block this effect (p = 0.0134)(Fig. 2B).

FIGURE 2

3.2 . Hepatic function markers

ALT, AST, ALP, and LDH are plasma indicators of the function hepatic. Data from ALT, AST and ALP activities demonstrated a significant malathion × OXIME interaction (ALT [$F_{(1,24)}$= 7.27, p = 0.0126]; AST [$F_{(1,24)}$= 14.79, p = 0.0008]; ALP [$F_{(1,24)}$= 9.52 p = 0.0051). Malathion acute exposure increased ALT, AST and ALP activities in the rats' plasma when compared to those of the control group. In addition, OXIME treatment decreased AST and ALP activities increased by malathion. LDH activity did not alter by both OXIME and malathion treatments (Table 1).

TABLE 1

3.3. Oxidative stress markers

According to two-way ANOVA, the animals exposed both malathion and OXIME treatments did not change the hepatic TBARS and protein carbonyl levels rats (data not shown).

3.4. Enzymatic antioxidant defenses

The two-way ANOVA of CAT and GR data indicated a significant malathion × OXIME interaction [CAT: ($F_{(1,24)}$= 7.58, p = 0.0111); GR: ($F_{(1,24)}$ = 5.22, p = 0.0315)]. In addition, Tukey's test revealed that acute exposure to malathion decreased the CAT and GR activity in the liver, which were protected by the OXIME treatment (Fig. 3A and 3C). GPx activities in the liver present no significant differences in this protocol (Fig. 3B).

FIGURE 3

3.5. Non-protein sulfhydryl (NPSH) content

As noticed from the two-way ANOVA analysis, no significant difference regarding the NPSH content was found on the rat´s livers exposed to malathion and/or treated with OXIME (data not shown).

3.6. Hepatic Cholinesterase (ChE) activity

Data from hepatic ChE activity demonstrated a significant malathion × OXIME interaction ($F_{(1,24)}$= 11.59, p = 0.0023). Tukey's post hoc test comparisons revealed that the malathion acute exposure inhibited the ChE activity in the rats' liver when compared to those of the control group. Moreover, a single dose of OXIME blocked the malathion-induced ChE inhibition (Fig. 4).

FIGURE 3

4. Discussion

The present findings clearly demonstrate the beneficial effects of (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one against to hyperglycemia and hepatic damage induced by malathion acute exposure in rats. A single intragastric dose (50 mg/kg) of OXIME demonstrated a hypoglycemic action in relation to the increase of plasma glucose and hepatic glycogen levels caused by malathion. Regarding the hepatoprotective effect, the OXIME blocked the increase of AST and ALP activities in the plasma and the inhibition of CAT and GR activities in the liver of malathion-treated rats. Moreover, the hepatic AChE inhibition induced by malathion acute exposure was prevented by OXIME treatment.

The OP are the biggest and the most varied group of insecticides. The long application of OP in public health and agricultural programs was accompanied by a potentially hazardous impact on humans, animals, plants, and the environment (water,

air, soil, and food) and causes severe acute and chronic poisoning (Baiomy et al., 2015). In fact, the toxicity of OP results in adverse effects on many organs and systems such as the liver, kidney, nervous system, immune system, and reproductive system (Mansour & Mossa, 2010). In this way, Acker et al. (2012a,b) demonstrated that both chlorpyrifos and acephate acute exposures induce metabolic disorder in rats.

Among the metabolic disorders induced by OP, the alterations in the glucose homeostasis have been highlighted. Abdollahim et al (2004) suggest that the hyperglycemic effect of malathion is linked to stimulation of glycogenolysis and gluconeogenesis pathways. Also, malathion overestimates the glycogen synthetase activity (Lasram et al., 2015). Herein, the hyperglycemic action of malathion was observed through an increase in the plasma glucose and hepatic glycogen levels after the exposure. Importantly, these alterations were suppressed by the treatment with a single dose of OXIME.

The liver is one of the target organs of the malathion toxicity. This OP causes degenerative changes and disrupts the hepatic architecture (Xu et al., 2017). Hepatocellular necrosis is commonly investigated through of determination of plasma activities of aminotransferases, AST and ALT that are localized in the hepatocytes (Sookoian & Pirola, 2015). In this way, our results corroborate with the literature data that show increased levels of AST, ALT, and ALP after malathion exposure (Abdel-Salam et al., 2017; Rezg et al., 2008). In addition, we demonstrated that a single dose of OXIME modulated the activities these plasma markers of hepatotoxicity, evidencing its hepatoprotective effect.

Glutathione system represents the main antioxidant defense. Glutathione has a crucial role in cellular signaling and antioxidant defenses either by reacting directly with

reactive oxygen or nitrogen species. In concert with its dependent enzymes, denoted as the glutathione system, glutathione is responsible for the detoxification of reactive oxygen and nitrogen species (ROS/RNS) and electrophiles produced by xenobiotics (Morris et al., 2014). In fact, the malathion acute exposure has induced a decrease in the GR and CAT activities in the liver, which was suppressed by a single dose of OXIME.

The treatment with oximes is established for malathion poisoning. However, the oximes clinically available display many side effects, such as low penetration into the blood-brain barrier and inefficient nucleophilic action (Cavalcanti et al., 2016). In this context, there is a clear demand for new reactivators of malathion-inhibited ChE activity with a higher efficacy than those currently available. A single dose of OXIME tested in the present study showed a remarkable effect on the ChE activity inhibition caused by malathion acute exposure, indicating its putative application as a novel therapeutic option for malathion poisoning.

5. Conclusion

In summary, acute exposure to malathion induced changes on glucose and glycogen metabolism, hepatoxicity, reduced activities of enzymatic antioxidant defenses and AChE in the liver. Importantly, a single dose of (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one attenuates the malathion-induced toxicity in rats. Notwithstanding more studies are needed, these findings indicate the promising effects of (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one against the hyperglycemia and hepatic

damage induced by malathion acute exposure, as well as an AChE activity reactivator, emerging as a possible novel therapeutic option for OP poisoning.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Figure Footnotes

Fig. 1. Chemical structure of (3Z)-5-Chloro-3-(Hydroxyimino)indolin-2-one.

Fig. 2. Effects of malathion (250 mg/kg) and/or OXIME (50 mg/kg) treatments on plasma glucose (A) and hepatic glycogen (B) levels of rats. Data are reported as mean ± S.E.M. of 7 animals/group. $^{*}p < 0.05$ as compared to the control group; $^{\#}p < 0.05$ as compared to the malathion group (two-way ANOVA/Tukey's test).

Fig. 3. Effect of malathion (250 mg/kg) and OXIME (50 mg/kg) treatments on the hepatic antioxidant enzymes – catalase (A), glutathione peroxidase (B), and glutathione reductase (C) – in rats. Data are reported as mean ± S.E.M. of 7 animals/group. $^{*}p < 0.05$ as compared to the control group; $^{\#}p < 0.05$, as compared to the malathion group (two-way ANOVA/Tukey's test).

Fig. 4. Effects of malathion (250 mg/kg) and/or OXIME (50 mg/kg) treatments on hepatic cholinesterase activity in rats. Data are reported as mean ± S.E.M. of 7 animals/group. $^{**}p < 0.01$ as compared to the control group; $^{\#}p < 0.05$ as compared to the malathion group (two-way ANOVA/Tukey's test).

Table Footnotes

Table 1. Effects of OXIME (50 mg/Kg; i.g.) on ALT, AST, LDH, and ALP activities in the plasma of rats exposed to malathion (250 mg/Kg, i.p.).

Figures

Figure 1

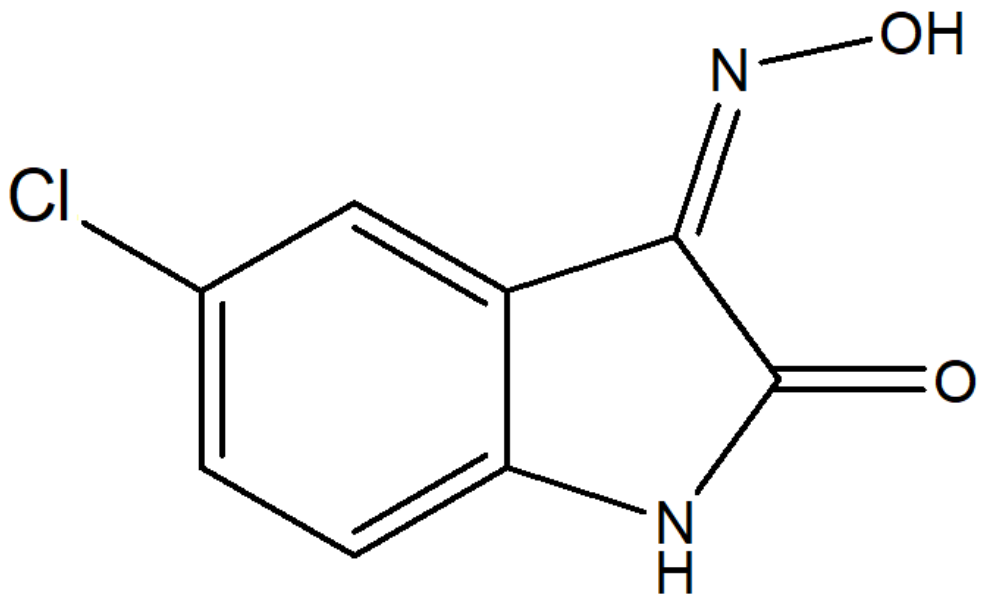
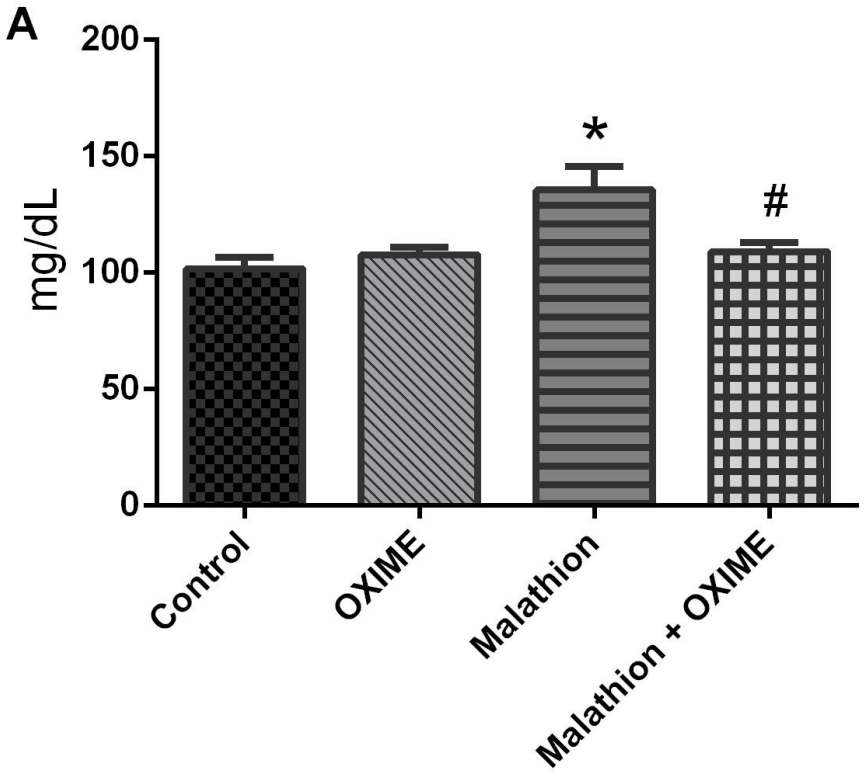


Figure 2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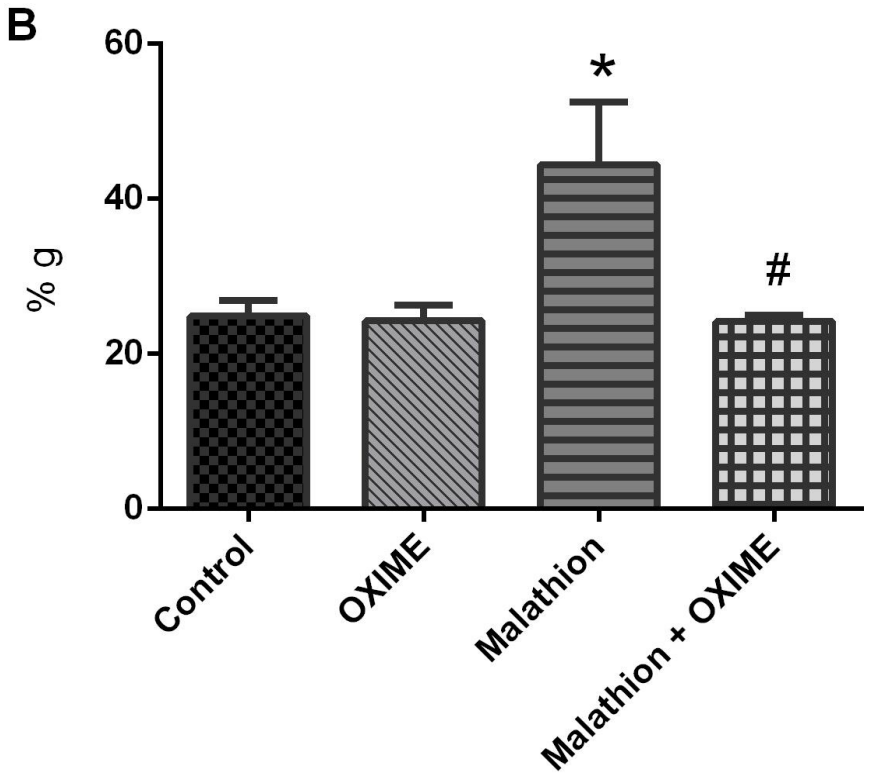
OXIME
Malathion
Malathion + OXIME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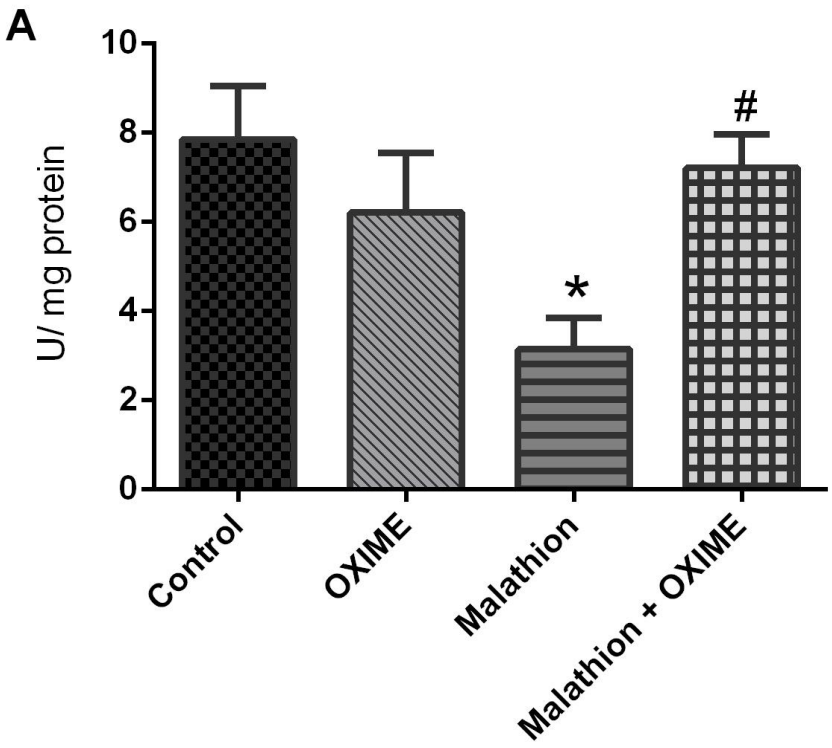
Control
OXIME
Malathion
Malathion + OXIME

Figure 3


A
U/ mg protein
10
8
6
4
2
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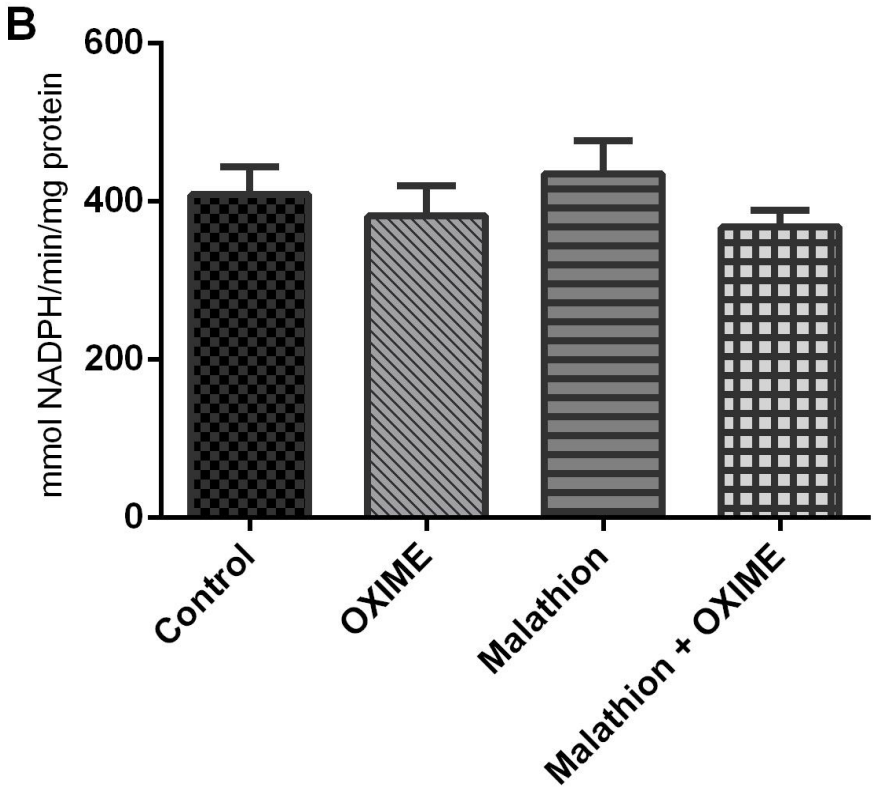
Control
OXIME
Malathion
Malathion + OXIME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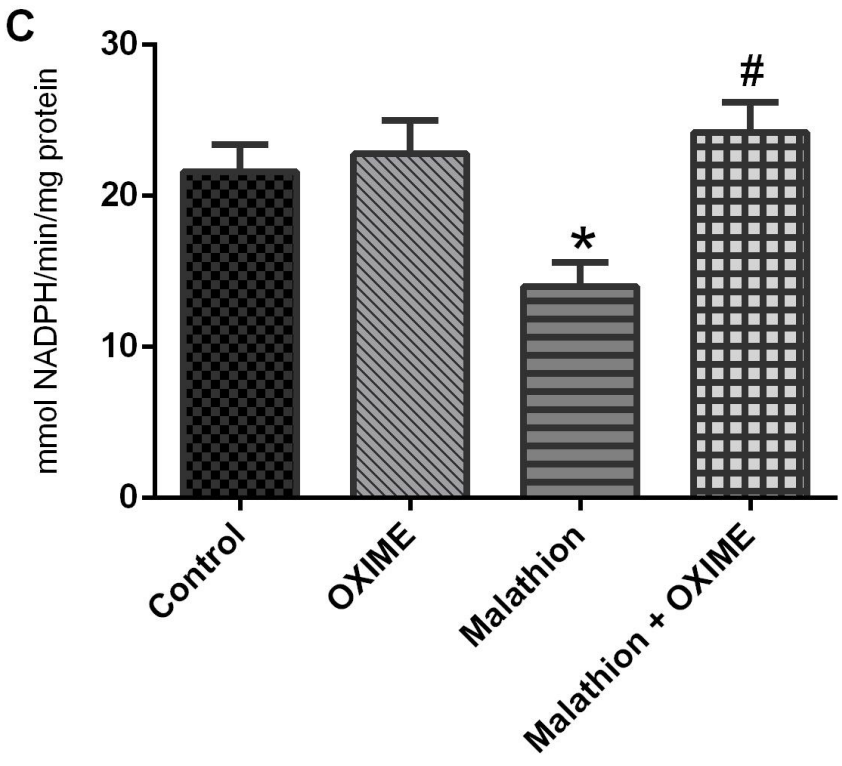
mmol NADPH/min/mg protein
Control
OXIME
Malathion
Malathion + OXIME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Control
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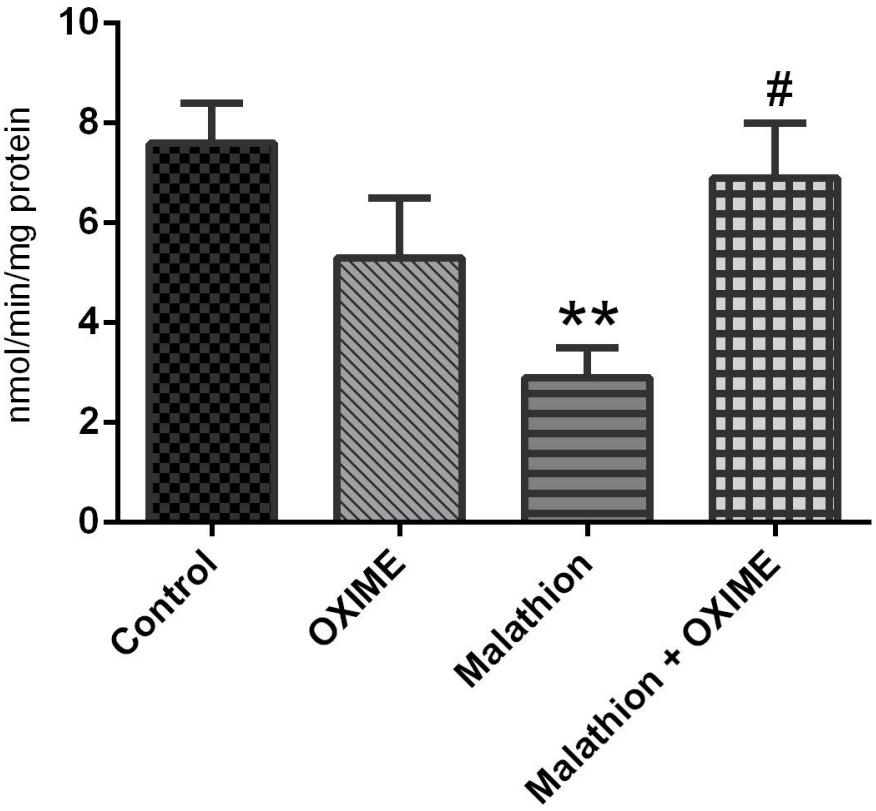
Malathion
Malathion + OXIME

Figure 4


nmol/min/mg protein
10
8
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Control
OXIME
Malathion
Malathion + OXIME

Table

Table 1

Group	ALT	AST	LDH	ALP
Control	43.69 ± 13.72	106.68 ± 12.95	393.20 ± 41.00	60.84 ± 16.67
OXIME	68.95 ± 21.35	115.78 ± 7.32	466.00 ± 59.40	119.76 ± 25.20
Malathion	172.83 ± 51.03*	201.01 ± 10.78*	373.60 ± 77.60	189.53 ± 39.96*
OXIME + Malathion	118.02 ± 26.95	117.39 ± 15.58$^{\#}$	395.40 ± 107.00	81.50 ± 20.34$^{\#}$

Data are reported as the mean ± S.E.M. of 7 animals per group and expressed as U/dL. *$p < 0.05$ as compared to the control group, $^{\#}p < 0.05$ as compared to the malathion group (two-way ANOVA/Tukey's multiple range test).

PARTE III

4. CONCLUSÃO

O presente estudo demonstrou que a exposição aguda ao organofosforado malation altera a homeostase da glicose. Além de afetar o metabolismo de enzimas hepáticas de ratos machos Wistar. Foi verificado que a administração aguda da oxima (3Z) -5-cloro-3- (hidroxiimino) indolin-2-ona foi capaz de atenuar os seguintes danos:

- Aumento da glicemia em jejum;
- Alterações na atividade das enzimas hepáticas AST, ALT e ALP;
- Inibição da atividade da Catalase e GR no fígado;

Em conclusão, sugerimos que há a necessidade de um maior controle e monitoramento no uso do pesticida malation. E que a oxima (3Z) -5-cloro-3-(hidroxiimino) indolin-2-ona pode ser uma alternativa promissora para o tratamento da intoxicação deste organofosforado. Porém, é preciso mais estudos sobre vias de administração, doses, absorção, distribuição, interações fármaco-alvo, visto que essa é uma oxima de síntese inédita.

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6. PERSPECTIVAS

Espera-se a continuação deste trabalho no doutorado. Entendemos que a exposição aguda ao malation ocorre, na maioria das vezes, de forma acidental ou propositada, por isso seria interessante investigar um período maior de exposição bem como, diferentes doses do tratamento com a oxima estudada. A intoxicação a um organofosforado pode desencadear efeitos sistêmicos nos humanos e animais, assim, pretende-se investigar outros tecidos alvos da exposição. Para isso, almeja-se ampliar os protocolos experimentais de análise, avaliando, marcadores de apoptose como a Caspase-3 e marcadores inflamatórios como IL-6 e 1-Beta, dentre outros.

Por fim, pelo entendimento de que a ciência, principalmente a oriunda da Academia Pública, têm como principal missão contribuir com a sociedade espera-se que nossos estudos sejam publicados e expostos em eventos nacionais e internacionais. E os trabalhos desenvolvidos possam servir para o desenvolvimento de estratégias terapêuticas mais eficazes contra a intoxicação por organofosforados.

ANEXO I - Protocolo Comitê de Ética em Uso de Animais

MINISTÉRIO DA EDUCAÇÃO
FUNDAÇÃO UNIVERSIDADE FEDERAL DO PAMPA
(Lei nº 11.640, de 11 de janeiro de 2008)

Pró-Reitoria de Pesquisa

COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

Fone: (55) 3413 4321, E-mail: ceua@unipampa.edu.br

CERTIFICADO DE APROVAÇÃO DE PROTOCOLO PARA USO DE ANIMAIS EM PESQUISA

Número de protocolo da CEUA: **024/2016**

Título: **Efeito Protetor da 5-Cloro-isatina-3-oxima contra a Toxicidade Induzida pelo Organofosforado Malation**

Data da aprovação: **18.08.2016**

Período de vigência do projeto: 01.02.2018

Pesquisador: Simone Pinton

Campus: **Uruguaiana**

Telefone: 55 81373931

E-mail: simonepinton@unipampa.edu.br

Prof. Dr. Vanusa Manfredini
Coordenadora CEUA/UNIPAMPA