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EFEITOS DO DISSELENETO DE DIFENILA EM CARPAS EXPOSTAS A UMA FORMULAÇÃO COMERCIAL CONTENDO CIPERMETRINA E CLORPIRIFÓS

Santa Maria, RS
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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientadora: Profª. Drª. Vania Lucia Loro

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Santa Maria, RS
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DEDICATÓRIA

Aos meus pais e irmãos que sempre me apoiaram em todas minhas escolhas e decisões e que fazem isto ser real.

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RESUMO

EFEITOS DO DISSELENETO DE DIFENILA EM CARPAS EXPOSTAS A UMA FORMULAÇÃO COMERCIAL CONTENDO CIPERMETRINA E CLORPIRIFÓS

AUTORA: Cíntia Corte Real Rodrigues

ORIENTADORA: Profª. Drª. Vania Lucia Loro

A atividade agrícola é um dos principais fatores que levam ao aumento de contaminantes nos ecossistemas aquáticos podendo causar estresse oxidativo em peixes. O objetivo do trabalho foi verificar os efeitos protetores de uma dieta suplementada com 3,0 mg/kg de disseleneto de difenila $(PhSe)_2$ em peixes expostos ao Colosso®, uma formulação comercial contendo 15% de cipermetrina e 25% de clorpirifós. Foram utilizadas 32 carpas divididas em quatro grupos: (1) controle, (2) $(PhSe)_2$, (3) Colosso® (4) Colosso® + $(PhSe)_2$. Os peixes foram expostos durante 60 dias. Os níveis de peroxidação lipídica em fígado foram maiores nos peixes expostos à formulação comercial. Entretanto os peixes alimentados com selênio tiveram os níveis de peroxidação lipídica reduzidos no mesmo tecido. Em brânquias não ocorreu alteração nos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), mas os peixes alimentados com selênio tiveram os níveis de proteína carbonil menores ou semelhantes ao controle. A dieta contendo selênio preveniu a formação de proteínas carboniladas no grupo exposto a formulação comercial. Em fígado, a atividade da catalase (CAT) diminui em todos os grupos comparada ao grupo controle. Por outro lado, a atividade da SOD aumentou no grupo exposto ao pesticida, retornando aos valores do controle quando os peixes foram expostos ao Colosso® + $(PhSe)_2$. A atividade da enzima glutationa peroxidase (GPx) em fígado e brânquias foi maior nos peixes alimentados com ração suplementada com $(PhSe)_2$. O conjunto de resultados sugerem que o selênio apresentou ação protetora frente aos danos oxidativos causados pela exposição ao pesticida. Sua ação foi importante, aumentando a atividade da GPx, visto que o mesmo faz parte da formação estrutural dessa enzima.

Palavras-chave: Peixe. Selênio. Antioxidante.

ABSTRACT

EFFECTS OF DIPHENYL DISELENIDE IN CARPS EXPOSED TO A COMMERCIAL FORMULATION CONTAINING CYPERMETHRIN AND CHLORPYRIFOS

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ADVISOR: Vania Lucia Loro

Agricultural activity is one of the main factors that lead to the increase of contaminants in aquatic ecosystems and may cause oxidative stress in fish. The objective of this work was to verify the protective effects of diet supplemented with 3.0 mg/kg of diphenyl diselenide $(PhSe)_2$ in fish exposed to Colosso®, a commercial formulation containing 15% of cypermethrin and 25% of chlorpyrifos. Thirty-two carps were divided into four groups: (1) control, (2) $(PhSe)_2$, (3) Colosso®, (4) Colosso® + $(PhSe)_2$. The fish were exposed during 60 days. The levels of lipid peroxidation in liver were higher in fish exposed to the commercial formulation. However, the fish fed selenium had levels reduced of lipid peroxidation in the same tissue. In gills not occur change in levels of thiobarbituric acid reactive substances (TBARS), but fish fed selenium protein carbonyl levels had less than or similar to the control. The diet containing selenium prevented the formation of protein carbonyls in the group exposed to the commercial formulation. In liver catalase (CAT) activity decreases in all groups compared to control. On the other hand, SOD activity increased in the group exposed to the pesticide, returning to the values of the control when the fish were exposed to Colosso® + $(PhSe)_2$. The activity of the enzyme glutathione peroxidase (GPx) in liver and gill was greater in fish fed with $(PhSe)_2$ supplemented diet. The set of results suggest that selenium presented protective action against the oxidative damage caused by exposure to the pesticide. Its action was important, increasing the activity of GPx, since the same is part of the structural formation of this enzyme.

Keywords: Fish. Selenium. Antioxidant.

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INTRODUÇÃO

MANUSCRITO

LISTA DE ABREVIAÇÕES

$(PhSe)_2$ - Disseleneto de difenila
CAT- Catalase
COL- Colosso®
CP- Cipermetrina
CPO- Clorpirifós
EROS - Espécies Reativas ao Oxigênio
GPx- Glutationa Peroxidase
H_2O_2- Peróxido de Hidrogênio
IDA- Ingestão diária aceitável
PC- Proteína carbonil
PL- Peroxidação lipídica
SOD- Superóxido Dismutase
TBARS- Substâncias Reativas ao Ácido Tiobarbitúrico

SUMÁRIO

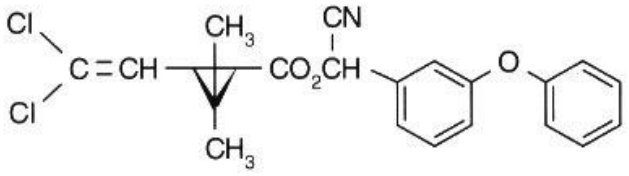
1. INTRODUÇÃO

A atividade agrícola é um dos principais fatores que levam ao aumento de contaminantes nos ecossistemas aquáticos. Os resíduos agroquímicos oriundos dessa atividade podem facilmente contaminar corpos de água afetando adversamente organismos não-alvo, incluindo os peixes (BRETAUD et al. 2000; SENGER et al. 2005; TONI et al. 2010).

A formulação comercial Colosso®, contendo 15% de cipermetrina (Figura 1) mais 25% de clorpirifós (Figura 2) e 1% de citronela é usada para muitas práticas agrícolas especialmente contra carrapatos. Usualmente, a fim de minimizar a resistência a inseticidas e outros produtos já utilizados no ramo agrícola, uma técnica utilizada é a combinação de vários produtos incluindo inseticidas, no entanto, tendo como consequência os riscos de toxicidade e poluição ambiental (IDRIS et al., 2012).

A cipermetrina é um agroquímico que possui uma classificação toxicológica do tipo II (toxicidade moderada) e pertence ao grupo químico dos piretróides da classe inseticida e formicida, com ingestão diária aceitável (IDA) de 0,05 mg/kg p.c. (ANVISA, 2010), utilizada no controle de pragas e no controle de vetores de doenças (KHAZRI et al., 2015). A ampla utilização de piretróides sintéticos ocasionou uma descarga desses compostos no meio aquático, aumentando as possibilidades de intoxicação de organismos aquáticos, uma vez que esses são mais sucetíveis à intoxicação por esses compostos que outros animais (YONAR, 2011). Segundo Yang et al. (2014), os peixes são mais suseptíveis à intoxicação por piretróides por apresentarem alta taxa de absorção branquial. A mesma ocorre devido a lipofilicidade dos piretróides que contribui para a sensibilidade dos peixes a exposições a esse composto (BORGES et al., 2007).

Figura 1: Estrutura química da cipermetrina

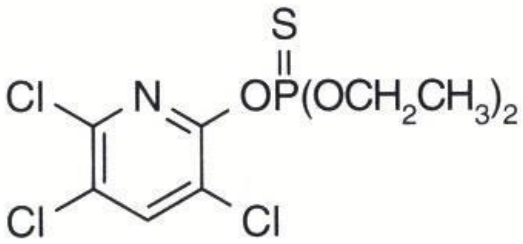


Fonte: (ANVISA, 2010)

Alguns estudos têm mostrado que a exposição à cipermetrina é tóxica para peixes e outros organismos aquáticos, como mexilhões, uma vez que aumenta os níveis de espécies reativas ao oxigênio (ERO), induz peroxidação lipídica (PL), formação de proteina carbonil (PC), altera a atividade de enzimas antioxidantes e propicia demais danos em peixes, como dano ao DNA e apoptose celular (JIN et al., 2011; YONAR, 2011a; SHI et al., 2011; KHAZRI et al, 2015; WEI et al., 2015).

O clorpirifós é um organofosforado da classe inseticida, formicida e acaricida, de classificação toxicológica II, com IDA de 0,01 mg/kg p.c. (ANVISA, 2015), utilizado nas atividades agrícolas para controlar pragas e vetores de doenças (ACKER et al.2012). Um dos principais danos da intoxicação aguda e crônica à organofosforados é a inibição da atividade da enzima acetilcolinesterase (SCHLENK, 2005, IDRIS et al., 2012; RODRÍGUEZ-FUENTES et al., 2015). No entanto, a exposição ao clorpirifós pode acarretar outros danos, como induzir dano oxidativo em diferentes modelos animais, por meio do aumento da peroxidação lipídica e carbonilação de proteínas, bem como pela diminuição de algumas defesas antioxidantes, como as enzimas superóxido dismutase e catalase (ACKER et al., 2012; URAL, 2013; ADEADARA at al., 2015) .

Figura 2: Estrutura química do clorpirifós



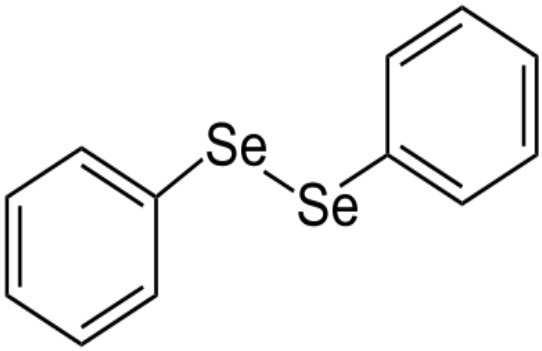
Fonte: (ANVISA, 2015)

Em toxicologia aquática, a fim de analisar os possíveis danos causados aos organismos aquáticos expostos a diferentes agroquímicos, diferentes biomarcadores têm sido validados. A análise do dano oxidativo, entre outros parâmetros bioquímicos pode ser importante ferramenta para verificar quais tecidos ou vias são mais afetadas pelos diferentes agroquímicos (MENEZES et al., 2011; MORAES et al., 2011; CLASEN et al., 2014; MURUSSI et al., 2014). A exposição de peixes aos diferentes produtos lançados na água pode ser responsável pela indução de dano oxidativo em diferentes órgãos, uma vez que esses

produtos tendem a induzir a formação de EROS, como por exemplo, peróxido de hidrogênio, superóxido, ânion superóxido e radical hidroxila (JIN et al., 2011; SHI et al., 2011). Na literatura, a produção de EROS na presença desses compostos é vista como um possível mecanismo de toxicidade em organismos aquáticos (CLASEN et al., 2014; MURUSSI et al., 2014). Nessa situação, observa-se alterações nos níveis de substâncias reativas ao ácido tiobarbitúrico, do mesmo modo que se observa carbonilação de proteínas (CATTANEO et al., 2012; TONI et al., 2011). Alterações no perfil das enzimas antioxidantes como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), e no sistema antioxidante não enzimático, como tióis não-protéicos (NPSH) e ácido ascórbico, em diferentes espécies de peixes também são observadas após exposição a diferentes agroquímicos (CLASEN et al. 2014; MENEZES et al. 2013a; TONI et al. 2013). Estando as EROS em acúmulo excessivo associado à diminuição das defesas antioxidantes, ocorre estresse oxidativo, indicando toxicidade desses compostos (URAL, 2013).

Nesse sentido, vários compostos vêm sendo estudados com o objetivo de prevenir e/ou reverter as alterações causadas por contaminantes em organismos aquáticos, entre os quais, destaca-se o papel protetor de vitaminas e antioxidantes (SASHA et al. 2009; YONAR e SAKIN, 2011; YONAR et al.,2012), incluindo compostos de selênio (MENEZES et al. 2012; MENEZES et al. 2016). Assim destacamos o disseleneto de difenila $(PhSe)_2$ (Figura 3), que é um composto sintético orgânico de selênio reconhecido por seus efeitos antioxidantes em diferentes modelos animais (ACKER et al. 2012; MENEZES et al. 2012; CECHELLA et al. 2014; ZAMBERLAN et al. 2014; ADEADARA et al. 2015).

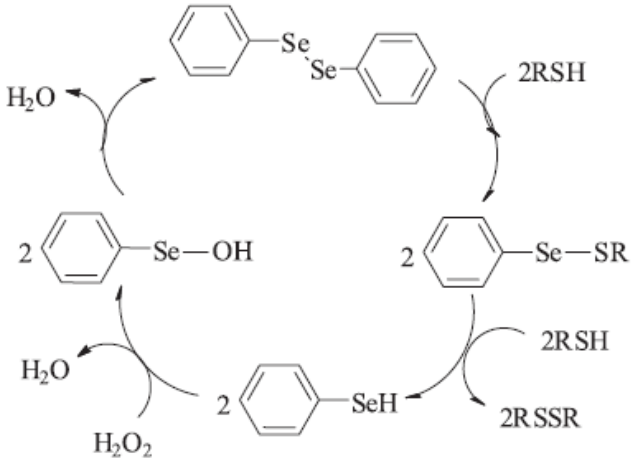
Figura 3: Estrutura química do disseleneto de difenila $(PhSe)_2$



Fonte: Nogueira e Rocha (2011).

Os efeitos protetores do $(PhSe)_2$ são devido a sua capacidade de degradar H_2O_2. Esse efeito se associa à ação antioxidante e mimética da GPx (Figura 4), degradando o H_2O_2 (NOGUEIRA et al. 2004). Contudo, os efeitos do $(PhSe)_2$ dependem da dose estudada, visto que em altas concentrações, apresenta efeitos tóxicos já reportados para mamíferos e peixes (NOGUEIRA e ROCHA, 2010; MENEZES et al. 2013b).

Figura 4: Ciclo catalítico do $(PhSe)_2$ na degradação de H_2O_2, ação mimética à GPx.



Fonte: Nogueira e Rocha (2010).

Os compostos sintéticos orgânicos podem ser facilmente adicionados como um suplemento dietético para peixes. A adição de antioxidantes, tais como $(PhSe)_2$ na dieta de carpas vem sendo utilizada para prevenir o dano oxidativo e melhorar as defesas antioxidantes, como observado por Menezes (2012). A suplementação com $(PhSe)_2$ tem propriedades antioxidantes, reduzindo o estresse oxidativo e aumentando a atividade de enzimas antioxidantes em peixes expostos a diferentes agroquímicos como quinclorac (MENEZES et al. 2012; MENEZES et al. 2014) e clomazone (MENEZES et al. 2013), bem como em outros modelos animais como mamíferos (RIBEIRO et al. 2013) e *Drosophila Melanogaster* (ADEADARA et al. 2015).

Nesse sentido, estudos avaliando a toxicidade de agroquímicos e conjuntamente as propriedades antioxidantes do $(PhSe)_2$ são importantes para oferecer alternativas que possam minimizar os efeitos tóxicos causados pelos diferentes agroquímicos que podem afetar peixes de interesse comercial e utilizados para o consumo humano, como as carpas. A carpa húngara (*Cyprinus carpio*) (Figura 5) é originária da Europa Oriental e da Ásia Ocidental. Devido a

sua rusticidade, resistência a diferentes temperaturas e facilidade de criação seu cultivo ocorre em todos os continentes. É uma espécie onívora que se alimenta de invertebrados, plantas, algas, consome larvas de insetos e crustáceos, podendo alimentar-se também de pequenos peixes (QUEROL et al. 2005; MABUCHI et al. 2006)

Figura 5: Exemplar de carpa húngara (*Cyprinus carpio*).

Fonte: Arquivo pessoal.

Considerando os danos causados pela exposição à cipermetrina e ao clorpirofós, e as propriedades antioxidantes do $(PhSe)_2$ já descritas na literatura, o estudo teve como objetivos:

- Verificar parâmetros do estresse oxidativo e do sistema antioxidante;
- Analisar a possível bioacumulação do composto tóxico nos filés;
- Verificar se a dieta contendo selênio pode proteger os peixes contra os efeitos causados por esse composto.

2. MANUSCRITO

Diphenyl diselenide diet in *Cyprinus carpio* against cypermetrin plus chlorpyrifos exposure: protective role of selenium

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Diphenyl diselenide diet in *Cyprinus carpio* against cypermetrin plus chlorpyrifos exposure: protective role of selenium

Cíntia Corte Real Rodrigues, Aline Teixeira Marins, Jeane de Lima Costa Gomes , Maiara Dorneles Costa, Charlene Cavalheiro de Menezes, Jossiele Leitemperger, Martha Bohrer Adaime, Renato Zanella, Osmar Damian Prestes, and Vania Lucia Loro*

Abstract

Pesticides can cause oxidative damage in aquatic organisms. The present study investigated if dietary diphenyl diselenide $(PhSe)_2$ could provide protection against the detrimental effects induced by commercial formulation Colosso® containing cypermethrin (CP) plus chlorpyrifos (CPF) in fish. Juveniles of carp (*Cyprinus carpio*) with an average weight of 28 ± 0.92 g were divided in four experimental groups for 60 days: (1) control, (2) $(PhSe)_2$, (3) Colosso® and (4) Colosso® + $(PhSe)_2$. Thiobarbituric acid reactive substances (TBARS) levels were reduced in liver of fish fed with a diet containing $(PhSe)_2$. Diet containing $(PhSe)_2$ promoted *per se* a decrease in protein carbonyl levels in gills of carps. The enzyme catalase (CAT) in liver showed reduced activity for all groups compared to control and superoxide dismutase (SOD) activity increased in pesticide exposed fish,but not in those fed with selenium in the feed. The enzyme GPx exhibited protective effects in fish exposed to Colosso® and treated with selenium at same time. In conclusion, the data obtained in this study show that supplementation with $(PhSe)_2$ can prevent the damage caused by Colosso®, as it reduces oxidative damage and increases the antioxidant defenses in fish.

Keywords: Pesticide; Oxidative damage; *Cyprinus carpio*; Diphenyl diselenide; Antioxidant; Selenium

Introduction

In order to improve agricultural production, pesticide use is very common, leading to increased contaminants in aquatic ecosystems (Menezes et al. 2011; Toni et al. 2013; Clasen et al. 2014). A formulation containing cypermethrin (CP) 15% plus chlorpyrifos (CPF) 25% and citronella 1% is used for many agriculture practices specially against ticks using the commercial formulation Colosso® (COL) (Cruz et al. 2015).

In the context of toxic effects to fish many laboratory studies have been conducted to verify toxicity of variety of compounds found in water. Cypermethrin at low concentrations is not lethal to fish, but is toxic and capable of inducing damage and stress to animals (Saha et al. 2009). Recent studies showed that CP exposure cause oxidative damage in *Cyprinus carpio* (Yonar et al. 2011). Similarly, chlorpyrifos (CPF) studies indicate that this compound is capable of causing oxidative damage and inhibition of antioxidant enzymes in fish (Yonar et al. 2012; Ural et al. 2013; Khalil et al. 2015).

The exposure of fish to different types of chemicals can induce changes in the oxidative profile, increased reactive oxygen species, lipid peroxidation, protein carbonylation and changes in antioxidants enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Clasen et al. 2012; Menezes et al. 2012; Toni et al. 2013).

Thus, studies have suggested a protective role for vitamins and antioxidants containing selenium in order to ameliorate these alterations caused by pesticides in fish species (Saha et al. 2009; Yonar et al. 2011; Menezes et al. 2013a; Ural et al. 2013). The interest in organoselenium compounds has intensified because of their pharmacological potential, such as observed in diphenyl diselenide $(PhSe)_2$, a organoselenium compound known for its antioxidant potential in different animal models (Menezes et al. 2012; Zamberlan et al. 2014; Cechella et al. 2014; Fuiza et al. 2015; Adeadara et al. 2015), when used in low doses. On the

ther hand, when used in high doses it is a pro-oxidant molecule (Nogueira and Rocha 2010; Menezes et al. 2013b).

The common carp is rustic, resistant to different temperatures, has high productivity, widely distributed geographically and is one of the main species used in aquaculture (Querol et al. 2005; Mabuchi et al. 2006). Considering that pesticides frequently induce oxidative damage in fish tissues, and also due to the extensive use of commercial formulations containing CP plus CPF, the present study was delineated to verify if dietary supplemented with $(PhSe)_2$ could afford protection against the detrimental effects induced by commercial formulation.

Materials and methods

Chemicals

Insecticide was obtained commercially following the formulation containing: 15% Cypermethrin + 25% Chlorpyriphos + 1% Citronellal (Colosso®). Diphenyl diselenide ($(PhSe)_2$), malondialdehyde (MDA), 2-thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), 2,4-dinitrophenylhydrazine (DNPH), bovine serum albumin, hydrogen peroxide (H_2O_2) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Fish and experimental design

Juvenile carp with an average weight of 28 ± 0.92 g and measuring 11 ± 0.21 cm length were obtained from a fish farm (RS, Brazil). Fish were acclimated to laboratory conditions for 10 days, they allocated in 40 L boxes into four experimental groups of 8 fish each group. They were kept in continuously aerated tap water with a static system and with a natural photoperiod (12h light/12h dark). Water conditions were: temperature 22 ± 1.0^{o}C, pH 7.2 ± 0.2 units, dissolved oxygen 7.2 ± 1.0 mg/L, non ionized ammonia 0.3 ± 0.01 μg/L,

nitrite 0.05 ± 0.01 mg/L. During acclimation period the fish were fed twice a day with commercial fish pellets (Supra, Brazil). Feces and pellet residues were removed each other day by suction. After acclimation time, the fish were fed with control diet (without $(PhSe)_2$)) or 3.0 mg/kg of $(PhSe)_2$ for 60 days. Experimental diets were formulated according to Menezes et al. (2013b). The fish were divided in four experimental groups as follows: (1) control group (fed with a control diet), (2) $(PhSe)_2$ group (fed with a diet supplemented with 3.0 mg/kg of $PhSe)_2$), (3) Colosso® group (fed with a control diet and exposed to) Colosso®), (4) Colosso®+ $(PhSe)_2$ group (fed with a diet supplemented with 3.0 mg/kg of $(PhSe)_2$ and exposed to) Colosso®). At the end of the trial period, the fish were anesthetized with benzocain 0.1 % and euthanized to collect liver, gills and white muscle. Feces and pellet residues were removed by suction every other day. The Colosso® was replaced every other day to maintain the concentration of 0.5 µg/L of CP and 0.8 µg/L of CPF. The concentration of CP were choose according to Saha et al. (2009). Water quality parameters were monitored daily and were maintained equal those of the acclimation period. The concentration of CP and CPF were monitored on alternate days by high-performance liquid chromatography (HPLC), according to Zanella et al. (2002), but were not detected by *Limit of detection*. The concentration of CP and CPF in muscle of fish was determined in finish of experimental period according to Munaretto et al. (2013) (Table 1).

Tissue preparation

After the end of the trial period, the fish from each group were anesthetized with benzocain 0.1 % and euthanized for tissue collection. Samples of liver, muscle and gills removed and kept at -4 ° C until analysis. The experimental protocol was authorized by the board in animal experimentation of the Federal University of Santa Maria by number: 030/2014 .

Lipid peroxidation determination

Lipid peroxidation was estimated by TBARS (thiobarbituric acid reactive substances) production, performed by malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured. In liver and gills homogenates (100 and 150 µL) were added 8.1% SDS, acetic acid and 0.67% thiobarbituric acid, totaling a final volume of 2.0 mL. The reaction was incubated for 90 min at 100ºC and optical density was measured in a spectrophotometer at 532 nm. TBARS levels were expressed as nmol MDA/mg protein according to Buege and Aust (1978)..

Protein Carbonyl assay

Liver and gills were homogenized in 10 volumes (w/v) of 10 mM Tris-HCl buffer pH 7.4 using a glass homogenizer. Protein carbonyl content was assayed by the method described by Yan et al. (1995) with some modifications. Briefly, soluble protein (1.0 mL) was reacted with 10 mM DNPH in 2N hydrochloric acid. After incubation at room temperature for one hour in dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3.0%), 2.0 mL of heptane (99.5%) and 2.0 mL of ethanol (99.8%) were added sequentially, vortexed for 30s and centrifuged for 15 min. Then, the protein isolated from the interface was washed twice by resuspension in ethanol/ethyl acetate (1:1), and suspended in 1.0 mL of denaturing buffer and the carbonyl content was measured spectrophotometrically at 370 nm. Assay was performed in duplicate and two tubes blank incubated with 2N HCl without DNPH was included for each sample. The total carbonylation was calculated using a molar extinction coefficient of 22.000 M/cm and expressed as nmol carbonyl/mg protein.

Antioxidant enzymes: Superoxide dismutase (SOD) and catalase (CAT) activity

The SOD and CAT activities were analyzed only in liver. The tissue was homogenized in a Potter-Elvejhem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.5 (1:20 dilution), centrifuged at 10.000 X *g* for 10 min at 4°C. SOD activity was performed based on inhibition of the radical superoxide reaction with adrenalin as described by Misra and Fridovich (1972) by measuring the speed of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50 mM, pH 10) and adrenalin (1 mM). The SOD activity was expressed as UI SOD/ mg protein. The CAT activity was assayed by ultraviolet spectrophotometry according to Nelson and Kiesow (1972). The assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M) and 0.05 mL homogenate. Change of H_2O_2 absorbance in 60 s was measured by spectrophotometry at 240 nm. CAT activity was expressed in µmol/min/mg protein.

Glutathione peroxidase (GPx)

GPx activity was assayed by ultraviolet spectrophotometry following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase (Paglia and Valentine, 1987). The tissue (liver and gills) was homogenized in a Potter- Elvejhem glass/Teflon homogenizer with 150 mM NaCl (1:10 dilution), centrifuged at 10,000 × *g* for 10 min at 4 ◦C. The assay mixture consisted of potassium phosphate buffer (100 mM, pH 7.0), 1 mM NaN_3, 1 mM reduced glutathione (GSH), 0.15 mM NADPH, 10 µL glutathione reductase and homogenized tissue (liver or gills) (20 µL). The reaction was initiated by the addition of 100 µl 0.4 mM H_2O_2. The specific activity was determined using the extinction coefficient of 6.22 mM/cm and expressed as µmol/min/mg protein.

Non enzyme antioxidants: Non-protein thiols (NPSH) and Ascorbic acid (AA) levels

NPSH and ascorbic acid levels were determined in liver and gills by the method of Ellman (1959) and Roe (1954) respectively. The preparation of homogenate for the two determinations was the same. The tissues were homogenized with 1.5 mL Tris HCl 50 mM (pH 7.5) followed by centrifugation at 3.000 X *g* for 10 min. An aliquot of supernatants (1.0 mL) was mixed (1:1) with 10 % trichloroacetic acid and then centrifuged. To determine NPSH of tissues an aliquot (500 µL) of supernatant was added in a phosphate buffer 0.5 mM (pH 6.8), 10mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), cysteine 0.5 mM. The color reaction was measured at 412 nm. NPSH levels were expressed as µmol NPSH/g of tissue. For determination of ascorbic acid, an aliquot of the supernatants (300 µL) was mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL), 0.6 mg/mL thiourea, $CuSO_4$ (0.075 mg/mL), and trichloroacetic acid 13.3%; and incubated for 3 h at 37^0C. After, H_2SO_4 65% (v/v) was added to the medium. Ascorbic acid levels were expressed as µg ascorbic acid/g tissue.

Protein determination

Protein was determined by the Comassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford, 1976).

Statistical analysis

Statistical analyses were performed using a two-way analysis of variance (ANOVA) followed by Newman-Keuls's test. Data exhibited homogeneous variance and were expressed as means ± standard error (S.E.M.). The value of $p \leq 0.05$ was considered statistically significant for all analyses. Analysis was performed using GraphPad Prism 6.01.

Results

Oxidative damage parameters

TBARS and Protein Carbonyl

Carps exposed only to Colosso® in water did not show any alterations on TBARS levels in liver and gills as compared to control (Figure 1A and 1B). However, TBARS levels were reduced in liver of fish fed with a diet containing $(PhSe)_2$. This decrease was also observed on fish exposed to pesticide and fed with diet containing $(PhSe)_2$ at same time (Figure 1A). In gills no changes were observed on fish fed with diet containing $(PhSe)_2$ in relation to control or between exposed groups (Figure 1B).

Diet containing $(PhSe)_2$ in this experimental model promoted *per se* a decrease in protein carbonyl content in gills of carps fed for 60 days (Figure 2B). On the contrary, liver did not show any in selenium group compared to control value, but showed protein carbonyl reduction in the group exposed to commercial formulation and fed with diet containing selenium as compared to control and with fish not fed with selenium diet (Figure 2A). The same pattern was observed in gills, fish receiving diet containing $(PhSe)_2$ showed reduced levels of protein carbonyl as compared to values recorded in fish not fed with this diet. However, the values of protein carbonyl did not recover to control values after Colosso® exposure (Figure 2B).

Antioxidant enzymes

CAT, SOD and GPx activities

Liver CAT activity was reduced for all treatment compared to control groups.The group that showed the highest inhibition was the association between Colosso® and $(PhSe)_2$

(Figure 3A). The commercial formulation increased liver SOD activity. In addition fish fed with $(PhSe)_2$ diet showed reduced liver SOD activity . However, the treatment with $(PhSe)_2$ plus Colosso® provided a normalisation of the liver SOD activity (Figure 3B). The enzyme GPx in liver increased in $(PhSe)_2$ group. In Colosso® exposed group as well in Colosso® + $(PhSe)_2$ the enzyme showed reduced activity as compared with control and selenium group. The group fed selenium show higher GPx activity compared to control (Figure 4A). Similarly, the gills of carp showed higher GPx enzyme activity in the selenium group than in the control group (Figure 4B).

Ascorbic Acid levels (AA) and non protein thiols (NPSH)

In liver of carps fed with $(PhSe)_2$ were observed an increase in ascorbic acid levels compared to the control group. In Colosso® group there was no change in relation to the control. In gills, ascorbic acid levels increased in all groups compared to the control, with no statistical difference between groups (Table 2). NPSH levels in liver increased in all groups compared to the control. In gills the groups Colosso® and Colosso® +$(PhSe)_2$ showed increased levels while in group $(PhSe)_2$ there was no change compared to control (Table 2).

Discussion

The results obtained at the present study showed that residues of CP no were found in muscle, suggesting that this compound was completely metabolized and excreted by the animal, however studies on the detoxification mechanism of this compound associated to CPF are needed to confirm this hypothesis. However, in both groups exposed to this commercial formulation were found CPF residues in the tissue of animals and can thus justify the other results of the study.

Fish exposed to Colosso® and fed with selenium reduced lipid peroxidation and protein carbonyl formation in liver when compared to control group. The reduced oxidative damage for proteins was also recorded in gills, where protein carbonyl exhibited reduced levels in fish fed with $(PhSe)_2$ when compared to pesticide exposed group. Menezes et al. (2013) working with silver catfish (*Rhamdia* sp.) showed that diet containing selenium (3.0 mg/kg $(PhSe)_2$) reverted oxidative damage after clomazone exposure. Gills of carps showed different response concerning lipid peroxidation whereas the selenium in diet did not change this parameter. The sum of results concerning biomarkers of oxidative damage recorded at present study pointed out the protective role of $(PhSe)_2$ on carps fed and poisoned with Colosso® simultaneously.

Many studies showed that $(PhSe)_2$ exhibited antioxidant properties in animals fed with diets containing the compound (Barbosa et al. 2008; Ribeiro et al. 2013; Menezes et al. 2014; Dias et al. 2014). The role atributted to selenium was reducing the oxidative damage and at same time increasing the antioxidant capacity of tissues against toxic substances (Acker et al. 2012; Menezes et al. 2012; Adedara et al. 2015). Besides the protective effects of $(PhSe)_2$, some authors related the toxic effects of $(PhSe)_2$ on mammals and fish according to concentration of $(PhSe)_2$ tested (Nogueira et al. 2004; Nogueira and Rocha 2010; Menezes et al. 2013). For the same species tested at present study, Menezes et al. (2012) proved that preventive treatment with $(PhSe)_2$ reduced TBARS levels in liver and gills in *Cyprinus carpio* exposed to quinclorac herbicide.

The protective role of $(PhSe)_2$ changes frequently according to the concentration of tested toxic compound and also due to specific tissue responses (Menezes et al. 2013b). The reduction of the activity of CAT enzyme and SOD in the liver suggests that the combination of dietary selenium together with simultaneous poisoning could inhibit the protective effect of selenium, reducing the activity of antioxidant enzymes, since fish studies of the same species

exposed to quinclorac and fed with the same concentration of $(PhSe)_2$ showed that selenium protects tissues against the toxicity of the pesticide enhancing the antioxidant properties (Menezes et al. 2012). The same authors verified that when *Rhamdia quelen* were fed with 3 mg/kg of $(PhSe)_2$ these enzymes maintained their antioxidant properties besides intoxication with herbicide quinclorac (Menezes et al. 2014).

Our results show increased SOD activity in the liver of the fish exposed to Colosso®, showing an induction of activity due to formulation exposure. The results suggests that pesticide poisoning induced the increased of liver SOD activity and the diet containing $(PhSe)_2$ was able to reduced SOD activity to control values. Similar results were reported by Yonar et al. (2011) and Ural (2013) that observed SOD activity increased when exposed to CP. Increased activity of this enzyme in fish exposed to Colosso® may occur in order to reverse the excess hydrogen peroxide produced by exposure to toxic compound.

Ural (2013) observed a decrease in CAT activity in fish exposed to CPF and attributes this decline to increased lipid peroxidation. Whereas the oxidative damage caused by exposure to Colosso®, our results show that fish exposed to this formulation presents carbonyl protein increased in the liver, which may explain the decrease in the catalase activity in the same tissue of these animals, since ROS can convert the groups amino group of the protein by altering its primary function (Almroth et al. 2005).

The increased activity of GPX enzyme assayed in liver and gills, demonstrates the protective effect of selenium in these fish tissues fed diet supplemented with $(PhSe)_2$ compared to the control group, as noted by Menezes et al. (2012; 2013a) in fish tissues such as catfish and silver carp exposed to different toxic compounds. It is known that inorganic and organic types including selenium $(PhSe)_2$ can induce synthesis of antioxidants and selenium selenoenzymes is a structural component of glutathione peroxidase (Nogueira et al 2004;.

Nogueira and Rocha 2011). This fact could explain the increase of this enzyme in fish fed with $(PhSe)_2$ at present study. The induction of gill GPx activity could be an important tool to control pesticide poisoning, considering that the gills are the first organ of contact between toxic compounds and fish.

Increased levels of NPSH in fish fed selenium are also observed in other studies using 3.0 m⁄kg of $(PhSe)_2$, suggesting selenium's protective effects on the maintenance of these antioxidants (Menezes et al. 2013a; 2014; 2016). Other studies with different animal models corroborate the results found in this study (Barbosa et al. 2008; Ribeiro et al. 2013). Ascorbic acid is an antioxidant that has the ability to neutralize certain ROS, especially hydroxyl (Namik 1990), radical initiator known as lipid peroxidation. In the liver of animals fed $(PhSe)_2$ was observed decrease lipid peroxidation and increased levels of ascorbic acid. In the gills where all groups showed high levels of ascorbic acid no lipid peroxidation, thus indicating that ascorbic acid would neutralize the hydroxyl radical.

In conclusion the data obtained in this study show that exposure to Colosso® is capable of inducing oxidative damage and alter the profile of some antioxidant enzymes in fish exposed to this compound. Supplementation with selenium showed antioxidant properties, since improved the non-enzymatic and enzymatic antioxidant response and was able to reverse protein damage in animals exposed to Colosso® simultaneous supplementation with $(PhSe)_2$.

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Legends

Fig. 1 TBARS levels in liver (A) and gills (B) of *Cyprinus carpio* fed for 60 days with diets containing 3.0 mg/kg of $(PhSe)_2$ and exposed to Colosso®. Different letters indicate differences between groups ($p< 0.05$). Data are reported as mean ± SEM (n=8).

Fig. 2 Protein carbonyl content in liver (A) and gills (B) of *Cyprinus carpio* exposed to Colosso® and fed with diets containing 3.0 mg/kg of $(PhSe)_2$. Different letters indicate differences between groups ($p< 0.05$). Data are reported as mean ± SEM (n=8).

Fig. 3 CAT activity (A) and SOD activity (B) in liver of *Cyprinus carpio* exposed exposed to Colosso® and fed with diets containing 3.0 mg/kg of $(PhSe)_2$. Different lettersindicate differences between groups ($p< 0.05$). Data are reported as mean ± SEM (n=8).

Fig. 4 GPx activity in liver (A) and gills (B) of *Cyprinus carpio* exposed exposed to Colosso® and fed with diets containing 3.0 mg/kg of $(PhSe)_2$. Different letters indicate differences between groups ($p< 0.05$). Data are reported as mean ± SEM (n=8).

Tables

Table 1: Cypermethrin and chlorpyrifos concentration (mg/kg) in muscle during the exposure period. nd = not detected. The results were expressed as mg/ kg of tissue.

Groups	0.5 µg/L of cypermethrin	0.8 µg/L of chlorpyrifos
Control	nd	nd
$(PhSe)_2$	nd	nd
Colosso®	nd	0,0145
Colosso® + $(PhSe)_2$	nd	0,0344

Table 2. Content of AA and NPSH in the liver and gills of *Cyprinus carpio* exposed to Colosso® and fed with diets containing $(PhSe)_2$.

	Control	$(PhSe)_2$	Colosso®	Colosso® + $(PhSe)_2$
Liver				
AA	3.60 ± 0.065^a	4.32 ± 0.057^b	3.64 ± 0.106^a	5.03 ± 0.178^c
NPSH	0.314 ± 0.013^a	0.375 ± 0.011^b	0.428 ± 0.010^c	0.474 ± 0.005^d
Gills				
AA	2.48 ± 0.047^a	2.74 ± 0.034^b	2.89 ± 0.033^b	2.79 ± 0.129^b
NPSH	0.108 ± 0.027^a	0.073 ± 0.016^a	0.384 ± 0.005^b	0.32 ± 0.012^c

Different letters indicate differences between groups ($p < 0.05$). Data are reported as mean ± SEM (n=8).

Figures

Fig. 1

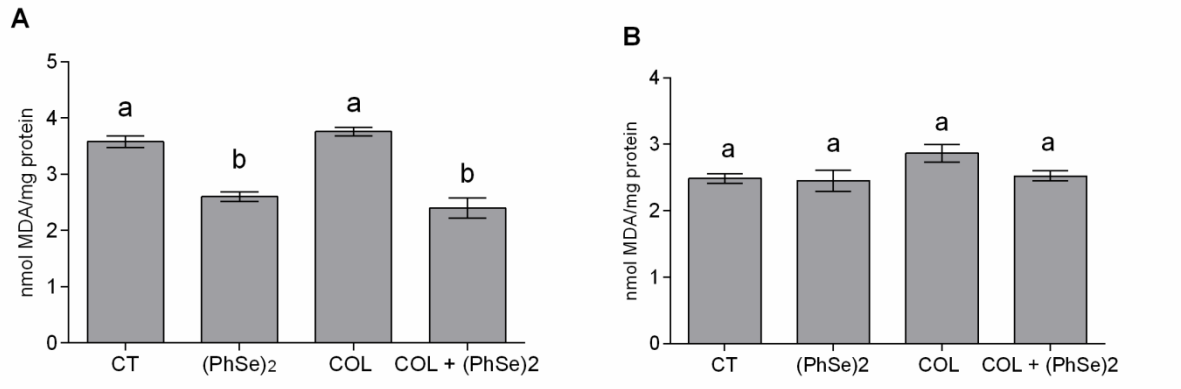

Fig. 2

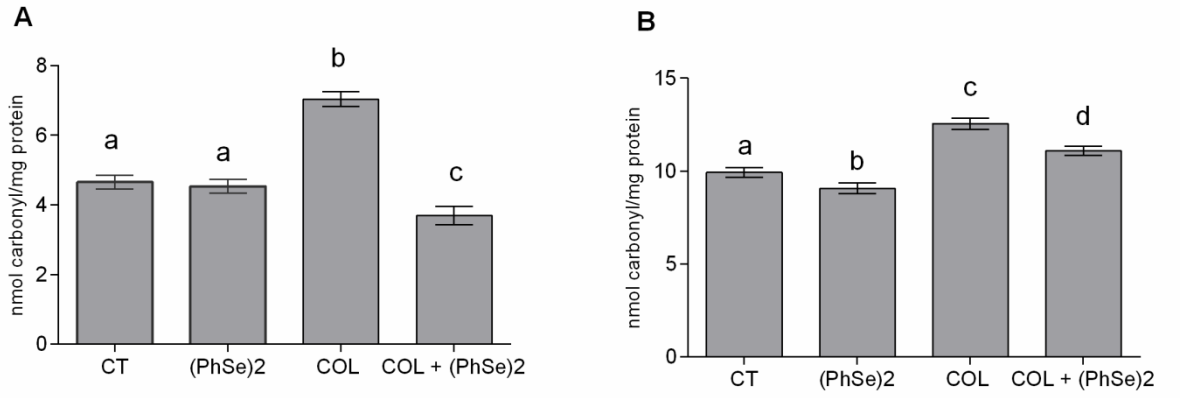

Fig. 3

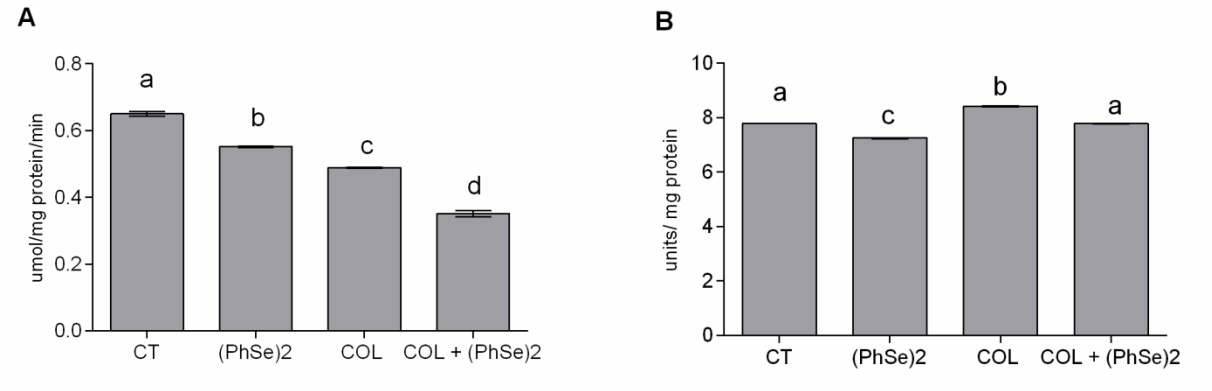
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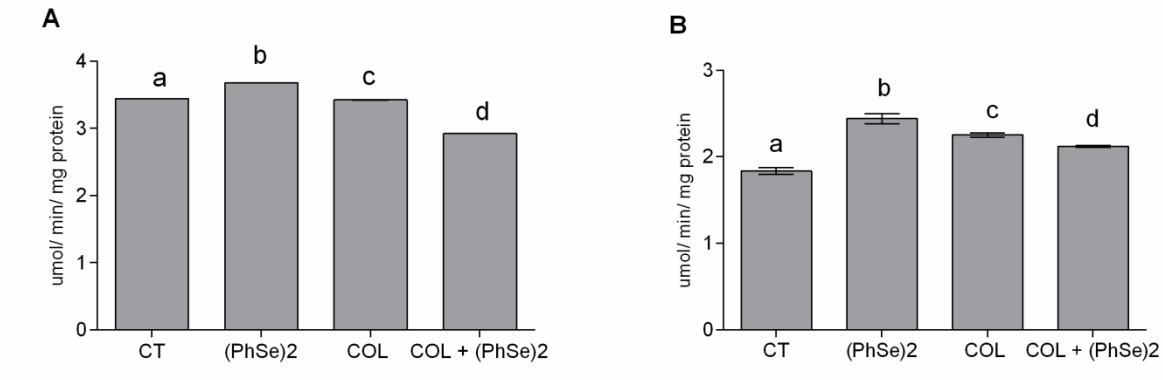
Fig. 4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3. CONCLUSÕES

- A exposição dos animais ao Colosso® é capaz de induzir estresse oxidativo nos animais.
- A ração suplementada com $(PhSe)_2$ é capaz de reverter os danos oxidativos causados pelo Colosso®.
- O $(PhSe)_2$ apresenta propriedades antioxidantes uma vez que aumenta a resposta de antioxidantes não enzimático e da enzima a atividade da glutationa peroxidase.
- O clorpirifós foi o composto que acumulou no músculo dos peixes, sendo maior nos peixes expostos ao composto e alimentados simultaneamente com $(PhSe)_2$.
- A exposição dos animais a compostos tóxicos não combinados é uma ferramenta para saber qual dos componentes da formulação está causando os danos oxidativos.
- A suplementação com selênio simultânea à exposição ao Colosso® consegue reverter os danos oxidatvos nos animais e manter os níveis dos antioxidantes, indicamdo assim os efeitos protetores do $(PhSe)_2$.

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