UNIVERSIDADE FEDERAL DO PAMPA

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EXPOSIÇÃO AO FUNGICIDA MANCOZEBE RESULTA EM ALTERAÇÕES NO EQUILÍBRIO REDOX DE PEIXES ADULTOS E EM DESENVOLVIMENTO EMBRIONÁRIO

São Gabriel
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RESUMO

Agrotóxicos têm sido amplamente utilizados no combate as pragas na atividade agrícola, no entanto, podem ser prejudiciais ao meio ambiente. O estresse oxidativo, caracterizado por um desbalanço entre pró-oxidantes e antioxidantes, tem sido apontado como um dos principais mecanismos de toxicidade induzida por agrotóxicos. Tendo em vista que organismos aquáticos são dependentes do equilíbrio redox para a homeostase celular, qualquer desequilíbrio neste processo pode resultar em neurotoxicidade, ou ainda, no comprometimento do desenvolvimento embrionário dos organismos. O fungicida agrícola multissítio mancozebe (Mz), da classe dos ditiocarbamatos, tem sido utilizado em diversas culturas há mais de 50 anos, devido a sua baixa toxicidade e persistência ambiental. Entretanto, esse composto já se demonstrou tóxico à biota aquática. Existem poucos estudos sobre os mecanismos de neurotoxicidade e a potencial indução de embriotoxicidade desse composto. A carpa comum (*Cyprinus carpio*) e o peixe-zebra (*Danio rerio*) tem se destacado como modelos experimentais relevantes para diversos campos de pesquisa, dentre eles a toxicologia ambiental. Com o presente estudo, tivemos como objetivo no capítulo I; investigar potenciais mecanismos de ação tóxica e respostas adaptativas relacionadas ao estresse oxidativo, em carpas expostas ao Mz. Já no capítulo II buscamos elucidar os efeitos toxicológicos do Mz sobre o desenvolvimento embrionário de peixe-zebra e o uso de um antioxidante clássico, como a N-acetilcisteína, com intuito de verificar o papel do desequilíbrio redox na embriotoxicidade induzida pelo Mz. Os resultados obtidos no capítulo I demonstraram um aumento significativo na concentração de manganês no sangue e cérebro de carpas expostas ao Mz. Observamos também alterações significativas na atividade de importantes enzimas antioxidantes e a ativação do fator de transcrição Nrf2, regulador mestre da resposta antioxidante em organismos, demonstrando o potencial neurotóxico deste fungicida em peixes. No capítulo II, a exposição de peixe-zebra ao Mz comprometeu a sobrevivência dos embriões tratados, induziu danos ao DNA, morte celular, anomalias morfológicas e alterações nas respostas sensório-motoras. Em paralelo, observamos alterações significativas nas defesas antioxidantes. Em contrapartida, o pré-tratamento com N-acetilcisteína foi capaz de bloquear os efeitos tóxicos induzidos pelo Mz em todos os parâmetros analisados, reforçando a hipótese do envolvimento do estresse oxidativo na embriotoxicidade

induzida pelo Mz, e, ainda, contribuindo na elucidação do papel fundamental do equilíbrio redox no desenvolvimento normal de embriões de peixe-zebra. Os dados aqui mostrados são o primeiro relato sobre a ativação de Nrf2 em peixes expostos ao Mz, assim como o efeito deletério do Mz sobre a embriogênese normal do peixe-zebra. Além disso, é possível salientar a importância desses estudos para o entendimento do estresse oxidativo dentro da toxicologia aquática.

Palavras-chave: Estresse oxidativo, fator de transcrição, neurotoxicidade, embriotoxicidade, mancozebe, antioxidante.

ABSTRACT

Agrochemicals have been widely used in the control of pests in agricultural activity, however, they can be harmful to the environment. Oxidative stress which is characterized by an imbalance between pro-oxidants and antioxidants has been identified as one of the main mechanisms of toxicity induced by pesticides. Considering that aquatic organisms are dependent on the redox equilibrium for cellular homeostasis, imbalance in such process may result in neurotoxicity, or even compromise the embryonic development of organisms. The manzozeb (Mz), a manganese/zinc-containing dithiocarbamate multi-site fungicide of the dithiocarbamate class has been used in several crops for over 50 years due to its low acute toxicity and environmental persistence. Though, this compound has already been shown to be toxic to aquatic biota. However, there are few studies on the mechanisms of neurotoxicity and the potential induction of embryotoxicity of this compound. The common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) have been highlighted as experimental models relevant to several fields of research, among them environmental toxicology. In the present study, our aim in chapter I was to investigate potential mechanisms of toxic action and adaptive responses related to oxidative stress in carps exposed to Mz. In chapter II, we sought to elucidate the toxic effects of Mz on the embryonic development of zebrafish and the use of a classical antioxidant, such as N-acetylcysteine, in order to verify the role of redox imbalance in Mz-induced embryotoxicity. The results obtained in Chapter I demonstrated a significant increase in the concentration of manganese in the blood and brain of carps exposed to Mz. We also observed significant changes in the activity of important antioxidant enzymes and the activation of the transcription factor Nrf2, the master regulator of the antioxidant response in organisms, indicating the potential neurotoxic effect of this fungicide in fish. In Chapter II, zebrafish exposure to Mz compromised the survival of treated embryos, induced DNA damage, cell death, morphological abnormalities and alterations in sensorimotor responses. In parallel, we observed significant changes in antioxidant defenses. In contrast, pre-treatment with N-acetylcysteine was able to block Mz-induced toxic effects in all analyzed parameters, reinforcing the hypothesis of the involvement of oxidative stress in embryotoxicity induced by Mz, and also contributing to the elucidation of the important role of redox balance in the normal development of zebrafish embryos. The data presented here are the first report on the activation of Nrf2 in fish exposed to Mz, as well as the deleterious effect of Mz on the normal

embryogenesis of zebrafish. In addition, it is possible to emphasize the importance of these studies for the understanding of stress within the aquatic toxicology area.

Key-words: Oxidative stress, transcription factor, neurotoxicity, embryotoxicity, mancozeb, antioxidant.

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LISTA DE ABREVIATURAS E SIGLAS

Mz – mancozebe

Mn – manganês

Zn – zinco

Fe – ferro

Cu - cobre

DTC - ditiocarbamatos

EBDC – etileno-bisditiocarbamato

PD – produto de degradação

IA – ingrediente ativo

mg L^{-1} – miligramas por litro

hec – hectare

EROS – espécies reativas de oxigênio

O_2^- - superóxido

$OH^{\cdot}$ – radical hidroxila

H_2O_2 – peróxido de hidrogênio

O_2 – oxigênio molecular NAC

NAC –N- acetilcisteína

RSH – radical tiol

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1 INTRODUÇÃO

A crescente produção e extensão das áreas agrícolas nos últimos anos demandam o uso intenso de agrotóxicos dos mais variados ingredientes ativos. No Brasil, essa demanda se torna ainda mais evidente, pelo fato do país ser um dos maiores produtores de soja, arroz e demais *commodities*, com isso, comercializando quantidades significativas dessas substâncias químicas (EMBRAPA, 2016).

Os agrotóxicos têm efeitos diretos no aumento da produção agrícola, pois combatem diversas fitopatologias. Contudo, o seu uso inadequado pode causar a contaminação do meio ambiente, por meio de processos naturais através da lixiviação, ação de ventos, não absorção pelas plantas, irrigação, escoamento superficial, ou ainda por descarte indevido dessas substâncias, movimentando seus resíduos químicos do seu alvo específico, dessa forma, causando a contaminação de compartimentos ambientais adjacentes, como o solo e água (ARIAS-ESTÉVEZ et al., 2008; KATAGI, 2006). Nesse contexto, seus ingredientes ativos (IA) ou produtos de degradação (PD) podem causar danos nos tecidos de plantas e animais, assim prejudicando o desenvolvimento e fisiologia de organismos não- alvo, mesmo em concentrações sub-letais (AKTAR; SENGUPTA; CHOWDHURY, 2009).

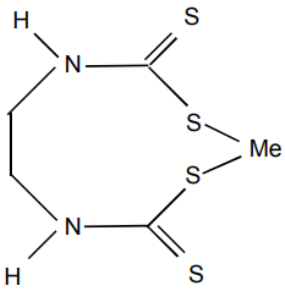
Dentre os diversos efeitos fisiológicos indiretos causados por esses contaminantes ambientais, é possível referir o desequilíbrio do estado redox celular dos organismos, que pode levar a danos em diferentes níveis da organização biológica, desde o nível molecular/bioquímico, fisiológico e comportamental, por fim, comprometendo o equilíbrio de um ecossistema (LUSHCHAK, 2011; VALAVANIDIS et al., 2006).

2 REFERENCIAL TEÓRICO

2.1 Fungicidas Etilenosbisditiocarbamatos

A origem dos etilenosbisditiocarbamatos (EBDCs) vem primeiramente dos ditiocarbamatos (DTCs) que são uma classe de fungicida, derivados do ácido bisditiocarbâmico, análogos dos carbamatos (CH_3NO_2), onde são substituídos os átomos de oxigênio por enxofre (KANCHI; SINGH; BISETTY, 2014). Sua síntese ocorre em condições alcalinas a partir da reação de aminas primárias e secundárias com dissulfeto de carbono.

Portanto, são compostos que se diferem pelo elemento, no caso metais (Me), que se liga ao seu esqueleto organossulfurado, com isso, formando um complexo (Fig. 1).

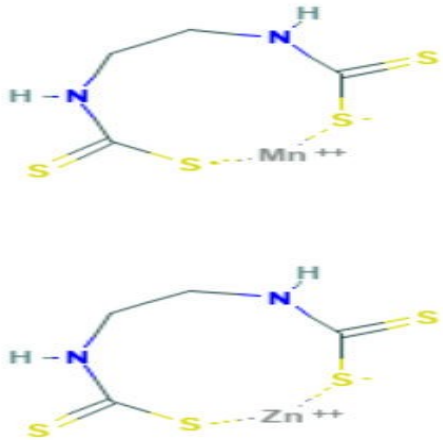


Fonte: Arcanjo, 2007.
Figura 1: Fórmula estrutural dos EBCDs. e os seus íons metálicos (Me).

2.2 Mancozebe e o seu potencial agrícola

O composto Mz, de fórmula molecular $(C_4H_6MnN_2S_4)_a(Zn)_y$ (Fig.2), é um EBCD que possui em sua formulação metais como manganês (Mn^{2+}) e zinco (Zn^{2+}), que confere estabilidade a molécula (RUNKLE et al., 2017). Dentre os fungicidas protetores multissítios, o mancozebe tem merecido destaque em função dos resultados obtidos a campo.

O efeito fungicida deste EBDC tem caráter protetor devido a sua aplicabilidade no início do plantio e permanência na superfície da folha (GULLINO et al., 2010). Esse composto atua por sua ação de contato quando exposto ao ambiente, que inativa os grupos sulfidrídricos nas enzimas de fungos. Se destaca também sua característica quelante, no qual, privam as células de metais (GUPTA, 2017).



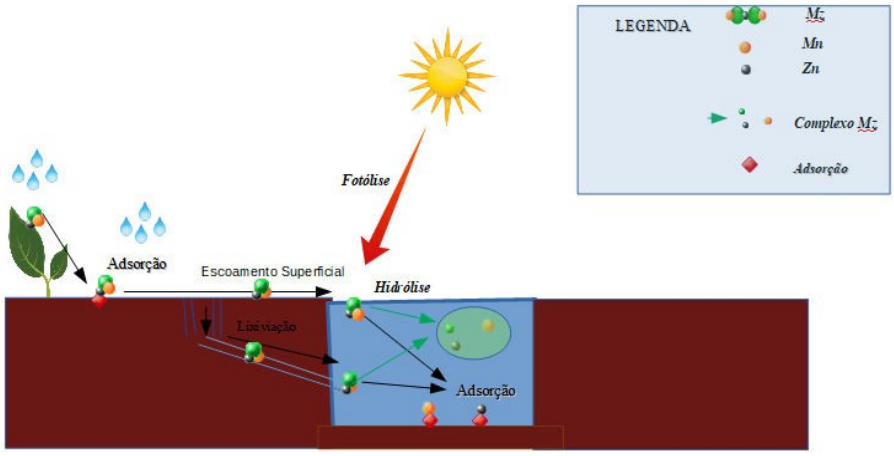
Fonte: https://pubchem.ncbi.nlm.nih.gov/compound/13307026
Figura 2: Fórmula estrutural do Mz.

De acordo, com o relatório de comercialização de agrotóxicos realizado pelo IBAMA (2016), Mz representa o terceiro princípio ativo mais comercializado no Brasil em número de toneladas, devido ao baixo custo de investimento e sua grande eficácia como protetor associado a outros fungicidas sítio-específicos (RODRIGUES, 2006). Segundo especificações recomendadas para a cultura de soja, principal *commoditie* no Brasil, são recomendadas múltiplas aplicações por safra (±2 kg. hec^{-1}) (SILVA et al., 2015).

2.3 Estabilidade ambiental de Mz

Esse fungicida possui um baixo potencial de volatilização no ar e meia-vida de 2 dias em solos aeróbicos e 8 dias em solos anaeróbicos com forte adsorção a partículas nessa matriz ambiental, sendo que nas matrizes aquáticas o composto é rapidamente hidrolisado, apresentando meia vida de 2 dias (LÓPEZ-FERNÁNDEZ et al., 2016).

Em vista dessas peculiaridades físico-químicas e risco ambiental, recomenda-se a avaliação toxicológica baseada em estimativas de exposição ao resíduo hidrolítico total de mancozebe, referido como o complexo mancozebe (Fig.3). Esse complexo consiste em espécies multi-químicas, dentre estes, os seus metais constituintes manganês (Mn) e zinco (Zn) que são produtos originados através de reações químicas como hidrólise, e ainda por fotólise. Os metais constituintes do mancozebe são os primeiros sítios de quebras na molécula liberando manganês e zinco no ambiente (HWANG; CASH; ZABIK, 2003). Esses metais em quantidades elevadas podem se concentrar em sistemas biológicos não alvo e matrizes ambientais.



Fonte: Editada pelo autor.
Figura 3: Movimento para sistemas aquáticos de superfície do complexo Mz.

Nessa linha, o complexo mancozebe, que possui em sua formulação cerca de 20 % de manganês e 2,5 % de zinco, tornam esse agrotóxico um importante aporte na contaminação de metais nos ecossistemas aquáticos. Além disso, esses íons metálicos podem contribuir para uma alteração fisiológica nos organismos aquáticos, dentre essas mecânicas de toxicidade, em um evento fisiopatológico conhecido como estresse oxidativo, no qual, se torna relevante na toxicologia ambiental em sistemas aquáticos (SEVCIKOVA et al., 2011; TUZUKI et al., 2017; VIEIRA et al., 2012).

2.4 Mz e o estresse oxidativo

Poluentes ambientais como agrotóxicos possuem a capacidade de causar dano oxidativo (SLANINOVA et al., 2009). Estes contaminantes com potencial pró-oxidativo podem afetar diferentes sistemas biológicos, dentre os quais, os organismos aquáticos, que são particularmente vulneráveis ao desequilíbrio redox (ČERMAK; PAVIČIĆ; ŽELJEŽIĆ, 2018; PAŠKOVÁ; HILSCHEROVÁ; BLÁHA, 2011).

O estresse oxidativo é entendido como um desequilíbrio entre a produção de espécies reativas de oxigênio (EROs) e a capacidade do organismo em neutralizá-las através do sistema de defesa antioxidante celular. Forman (2016) sugere uma definição mais abrangente, no qual as EROS passam de estado estacionário para uma concentração elevada de forma aguda ou crônica, dessa forma, podendo levar à modificação oxidativa de constituintes celulares, assim resultando na perturbação do metabolismo celular e vias regulatórias.

Os principais atores desse desequilíbrio redox e consequentemente, danos às biomoléculas, são derivados da parcial redução do oxigênio, levando à produção de radicais intermediários, como superóxido (O_2), peróxido de hidrogênio (H_2O_2) e radical hidroxila (OH^-) referidos como EROS. Dentro dos sistemas biológicos $O_2^{-\cdot}$ é geralmente a primeira espécie de EROS a se formar, podendo levar à formação de uma espécie reativa não radicalar como H_2O_2, e, ainda, por reação com metais suscetíveis a mudanças na sua camada de valência (RODRIGO-MORENO; POSCHENRIEDER; SHABALA, 2013), originar o OH^- considerado a EROS mais reativa, no qual, é capaz de extrair prótons e elétrons de biomoléculas (lipídeos, proteínas, ácidos nucleicos), consequentemente originando nessas moléculas mais elétrons desemparedados e espécies radicalares.

Na homeostase redox há um espectro de EROS onde níveis mais baixos podem ter consequências biologicamente importantes, como a proliferação e diferenciação celular, os quais, são eventos importantes na especialização biológica e geração da diversidade celular, portanto, contribuindo para a morfologia e fisiologia normal do organismo (SCHIEBER; CHANDEL, 2014). Entretanto, quando ocorre um aumento das EROS que ultrapassa os níveis usados para sinalização redox, pode haver danos diretos à célula, dessa maneira, ativando vias de sinalização envolvidas na cascata de morte celular (MITTLER, 2017).

O Mz pela sua composição metálica, se torna um relevante poluente dentro da toxicologia ambiental no advento do desequilíbrio redox, já que os íons metálicos são conhecidos como indutores do estresse oxidativo (VAN GENUCHTEN; PEÑA, 2017). Esses metais de transição podem aumentar a proporção de Fe (II), assim estimulando a geração de EROS via reação de Fenton, ou ainda, atuando na interferência de processos relacionados a complexos com biomoléculas (FARINA et al., 2013). Tais complexos podem inativar ou modular os sistemas enzimáticos críticos ao reparo e defesa celular, ou ainda estruturas proteicas, com isso, levando à disfunção celular e consequentemente morte celular.

Dentro das diversas perturbações fisiopatológicas, a neurotoxicidade exercida pelo Mz, pode estar relacionada ao aumento de EROS (DOMICO et al., 2006; TSANG; TROMBETTA, 2007), pelo seu constituinte metálico, manganês, pois esse metal é capaz de penetrar a barreira hematoencefálica, consequentemente, bioacumulando-se no cérebro dos organismos não-alvo (KRISHNA et al., 2014). Nessa linha, o Mn ainda pode ser relacionado a oxidação de neurotransmissores dopaminérgicos, nos quais podem originar compostos intermediários citotóxicos (ROTH et al., 2013).

Já no contexto do estresse oxidativo na vida aquática, particularmente em peixes, há evidências do envolvimento do estresse oxidativo na neurotoxicidade através de seus produtos de degradação do Mz. Atamaniuk et al. (2014) expondo Goldfish em concentrações correlacionadas à aplicação agrícola (3-10mgL^{-1}) demonstraram uma alteração nas atividades das enzimas antioxidantes no cérebro dos peixes expostos ao composto frente ao controle. Recentemente, Zizza et al. (2017) demonstraram efeitos neurodegenerativos e comportamentais em peixes-rei (*Thalassoma pavo*), sugerindo também a contribuição do Mn no estresse oxidativo, apontado com um dos possíveis percussores da neurotoxicidade nesse estudo.

Assim, dentre essas possíveis contribuições pró-oxidativas dos constituintes metálicos do complexo mancozebe, ganha importância a elucidação da adaptabilidade dos

organismos em sintetizar e controlar as defesas antioxidantes, que podem prevenir, atenuar, ou ainda, bloquear danos oxidativos frente à exposição a poluentes organometálicos como o Mz.

2.5 Antioxidantes

Os organismos através da evolução, desenvolveram respostas biológicas contra injúrias relacionadas ao dano oxidativo, denominadas de defesas antioxidantes enzimáticas e não enzimáticas, através de adaptações moleculares e bioquímicas, que tem um contexto importante dentro a ecologia redox, por serem consideradas alterações preventivas a níveis sub-organímos frente a exposição ao xenobiótico pró-oxidante (VAN DER OOST; BEYER; VERMEULEN, 2003).

Dentre os mecanismos adaptativos ao estado de estresse oxidativo tem destaque o fator de transcrição sistema Keap1 – Nrf2 (do inglês "Kelch ECH *associating protein 1- nuclear factor erythroid 2- related factor 2").* O Nrf2 tem papel crucial na ativação da região promotora de genes ligados de forma direta ou indireta na sobrevivência celular, e ainda na restauração da homeostase redox celular (VOMUND et al., 2017).

Segundo Ngyuen e demais autores (2009) (NGUYEN; NIOI; PICKETT, 2009), o mecanismo de ativação desse fator de transcrição pode ser resumido da seguinte forma; Keap1 inibe o Nrf2, levando o complexo à degradação, desde que o Keap1 seja ubiquitinado, portanto esse fator de inibição tem várias cisteínas, que em contato com as EROS, serão oxidadas, dessa forma a oxidação das cisteínas agirão como interruptor redox na liberarão do Nrf2, que no núcleo formará heterodímeros com outros fatores de transcrição (*small Malf*), permitindo a ativação de elementos de resposta antioxidante (ARE) (do inglês "*antioxidant response element"*).

Entre as defesas antioxidantes ativadas pelo Nrf2, com a finalidade de neutralizar e detoxificar as EROs, através de enzimas primárias que atuam na neutralização de EROS, encontram-se a superóxido dismutase (SOD) e catalase (CAT), além de biomoléculas antioxidantes não enzimáticas e enzimas pertencentes aos sistemas glutationa e tioredoxina. Dentre as enzimas antioxidantes de primeira linha de defesa contra as EROS, metaloenzima SOD que compreende tanto a sua isoforma contendo manganês (MnSOD), no qual tem maior concentração e localização na mitocôndria, quanto a cobre e zinco (CuZnSOD), onde se encontra em maior concentração no citosol, nos quais, são responsáveis pela conversão de $O_2^{\cdot-}$ para H_2O_2 e oxigênio molecular (O_2) (SHENG et al., 2014). Já a catalase (CAT), que atua na decomposição de H_2O_2 em H_2O e O_2, dessa forma, complementando a detoxificação iniciada pela SOD, tem sua atividade e ampla distribuição nas células dentro de sub-organelas chamadas perioxiossomos, também utiliza metais como

Fe e Mn como cofatores. (CHELIKANI; FITA; LOEWEN, 2004).

O sistema glutationa inclui o principal antioxidante não enzimático, o tripeptídeo γ-glutamilcisteínaglicina (GSH), o qual, é sintetizado pela maioria dos organismos aeróbicos e usado pelas células para controlar níveis de EROS, por interação direta com estas espécies químicas ou como cofatores para enzimas desintoxicantes (LU, 2009). Além disso, os níveis de tióis podem elucidar o estado de homeostase redox envolvidos nos mecanismos de diferenciação, crescimento e proliferação celular, e até mesmo na proteção de tecidos embrionários (ASHTIANI et al., 2011). Adicionalmente, podem atuar como cofatores de enzimas importantes na biotransformação de xenobióticos e neutralização de EROS, dentre essas enzimas, se destacam a glutationa S-transferase (GST) e a glutationa peroxidase (GPx) (DEPONTE, 2013).

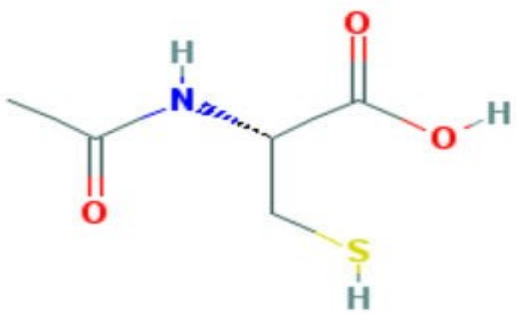
A GST atua na biotransformação de xenobióticos e também promove a conjugação do GSH com produtos oxidantes, contribuindo dessa forma para o sistema de defesa antioxidante (STRANGE et al., 2001). Já a GPx, é uma enzima redutora de hidroperóxidos orgânicos e inorgânicos utilizando o tripeptídeo GSH como doador de elétrons, além disso, contém o aminoácido selenocisteína no seu sítio ativo, sendo esse constantemente oxidado e reduzido durante o ciclo catalítico (IGHODARO; AKINLOYE, 2017). Por fim, a GR, enzima integrante do sistema glutationa, é responsável pela importante reconstituição da GSH, reduzindo a glutationa oxidada (GSSG) à glutationa reduzida (GSH) a custas de nicotinamida adenina dinucleótido fosfato (NADPH), e assim, regenerando o ciclo da GSH (COUTO; WOOD; BARBER, 2016).

O sistema tioredoxina composto pela tioredoxina redutase (TrxR), tioredoxina (Trx-$(SH)_2$) e NADPH está presente em todos organismos, tendo papel central na homeostase redox (LU; HOLMGREN, 2014). A (TrxR) é uma enzima citosólica responsável pela manutenção de tióis livres no "estado redutor", via elétrons do NADPH, sendo que o sistema glutationa também é responsável por manter esse estado redutor no interior das células, caracterizando dessa forma, uma interligação entre esses dois sistemas (CASAGRANDE et al., 2002).

2.5.1 Antioxidante N-acetilcisteína

Halliwell (2006) fornece uma descrição geral de um antioxidante como uma molécula que pode retardar, prevenir ou remover a modificação oxidativa de outra molécula. No entanto, uma distinção importante entre essas moléculas com efeito redutor, é a diferença entre antioxidantes endógenos enzimáticos e não-enzimáticos, assim como antioxidantes sintéticos utilizados como drogas farmacológicas ou ainda como ferramentas nas investigações experimentais na pesquisa básica.

Dentre os antioxidantes sintéticos a NAC (Fig.4) é um derivado da cisteína com um grupo acetil ligado ao seu átomo de nitrogênio e como a maioria dos tióis (RSH) pode ser oxidado por uma grande variedade de radicais e também servem como um nucleófilo (SAMUNI et al., 2013). NAC pode ainda proferir a ligação com metais de transição, principalmente ao seu grupamento tiol, dessa forma, causando um efeito quelante nos íons metálicos para que possam ser excretados do compartimento intracelular para o meio extracelular (ELBINI DHOUIB et al., 2016).



Fonte: https://pubchem.ncbi.nlm.nih.gov/compound/12035
Figura 4: Fórmula estrutural da NAC.

2.6 Peixes como modelos experimentais

Tendo em vista a importância das investigações sobre os efeitos pró-oxidantes dos agrotóxicos, esse trabalho se baseou na utilização de dois modelos aquáticos da família Cyprinidae, (*Cyprinus carpio e Danio rerio*), considerada uma das maiores famílias em termos quantitativos dentro dos peixes de água doce (NELSON, 2006). Os membros dessa

família possuem grande importância para a pesquisa biológica básica, além de poderem representar-se como modelos receptores ecológicos em ambientes contaminados.

2.6.1 Carpas

A carpa comum (*Cyprinus carpio*) (Fig.7) comumente utilizado na aquicultura comercial, foi proposto como organismo de teste em ensaios toxicológicos, devido à importância econômica global.

Fonte: http://fishbase.org/summary/Cyprinus-carpio+carpio.html
Figura 5: Carpa comum (*Cyprinus carpio*).

Na fase juvenil das carpas já se encontram desenvolvidos os principais órgãos e tecidos envolvidos. O aparelho circulatório desses peixes dessa espécie na fase juvenil se encontra desenvolvido (IUCN, 2008) , tornando esse sistema capaz de transportar qualquer substância química (xenobióticos ou nutrientes), no qual, possibilita que o modelo se torne relevante para a investigação experimental dentro do tema da neurotoxidade.

2.6.2 Peixe-Zebra

O peixe-zebra (*Danio rerio* HAMIlTON, 1822), é um teleósteo de água doce da família Cyprinidae já com grande prestígio e relevância na pesquisa básica nas fases embrionárias, larval, juvenil e adulta, como modelo para estudo em diversas áreas, como toxicologia, farmacologia e biologia do desenvolvimento (BAMBINO; CHU, 2017; GARCIA; NOYES; TANGUAY, 2016)

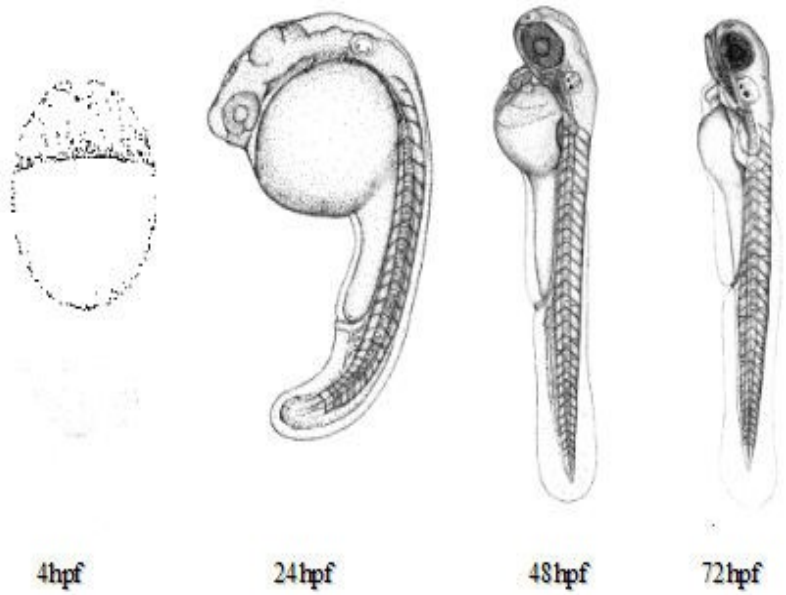
A fase embrio-larval se torna interessante pelo seu potencial de varredura de compostos em testes toxicológicos, devido ao seu desenvolvimento rápido e a transparência

dos embriões, o que possibilita observar o seu desenvolvimento desde as primeiras horas pós-fertilização (hpf) e posterior análise de “endpoints” morfológicos, fisiológicos e comportamentais (BRAUNBECK et al., 2015). O modelo também possibilita investigações toxicológicas e farmacológicas relacionados ao repertório comportamental, com gradual aumento na complexidade de respostas sensori-motoras nas fases embrio-larvais (KALUEFF et al., 2013; ORGER; DE POLAVIEJA, 2017), portanto, essas características, vem a reforçar o uso desse organismo como modelo alternativo em investigações neurocomportamentais frente a contaminantes ambientais.

Cada fase do desenvolvimento embrionário nos fornece “endpoints” essenciais no entendimento do mecanismo de toxicidade do composto testado. Dentre essas características é preferível separar a sua abordagem através de períodos de desenvolvimento, no qual foram elucidadas em trabalhos de grande magnitude e importância dentro do tema da embriologia. D esenvolvido de forma cuidadosa por Kimmel et al.(1995), tais períodos descritos por esse trabalho são apresentados na figura 6, e descritos de forma simplificada a seguir:

O Período de segmentação (10 – 24 hpf): órgãos começam a se desenvolver e se tornam visíveis. Nesse período, ocorre o desprendimento da cauda, formação de somitos, aumento do tamanho corporal e também adquirem a primeira movimentação dentro do córion, denominado de movimento espontâneo.

Durante o período de faríngula (24 – 48 hpf), ocorrem as seguintes modificações: organização bilateral, notocorda bem desenvolvida, formação das brânquias, desenvolvimento do cérebro e sistema nervoso, alongamento dos arcos faríngeos, pigmentação, formação do sistema circulatório e normalização dos batimentos cardíacos. Já o período de eclosão (48 - 72 hpf), ocorre o desenvolvimento das nadadeiras peitorais, bexiga natatória, neuromastos, brânquias e mandíbula. E, por fim, com 5 dias pós fertilização (dpf), onde ocorre a morfogênese completa, como também o desenvolvimento do nado ativo e exploratório.



Fonte: Adaptado e modificado de Kimmel et al., 1995.
Figura 6: Estágios do desenvolvimento embrio-larval do peixe-zebra (*Danio rerio*).

3 OBJETIVOS

3.1 Objetivo geral

Avaliar a potencial participação do estresse oxidativo sobre os efeitos neurotóxicos e embriotóxicos do MZ em peixes.

3.2 Objetivos específicos: CAPÍTULO I

- Avaliar os níveis de metais no sangue e cérebro de peixes expostos a concentrações sub-letais de Mz;

- Avaliar possíveis alterações em parâmetros pró-oxidantes em cérebro de peixes expostos ao Mz;

- Mensurar a atividade de enzimas antioxidantes e de detoxificação de xenobióticos no cérebro de peixes expostos a concentrações sub-letais de Mz;

- Verificar possíveis alterações na expressão e ativação do fator de transcrição Nrf2 em cérebro de peixes expostos a concentrações sub-letais de Mz;

- Avaliar possíveis alterações na viabilidade celular, assim como marcadores de morte celular no cérebro de peixes exposto a concentrações sub-letais de Mz.

3.3 Objetivos específicos: CAPÍTULO II

- Avaliar possíveis alterações morfológicas, fisiológicas e comportamentais em peixe-zebra expostos ao Mz durante o período de desenvolvimento embrionário;

- Mensurar possíveis alterações em parâmetros pró-oxidantes e atividade de enzimas antioxidantes em peixe-zebra expostos ao Mz durante o período de desenvolvimento embrionário;

- Avaliar potenciais alterações sensori-motoras em peixe-zebra expostos ao Mz durante o período de desenvolvimento embrionário;

- Analisar o potencial efeito protetor de *N*-acetilcisteína (NAC) contra os potenciais efeitos embriotóxicos induzidos por Mz em peixe-zebra.

4 CAPÍTULOS

4.1 Capítulo I - Mancozeb exposure results in manganese accumulation and Nrf2-related antioxidant responses in the brain of common carp *Cyprinus carpio*.

Artigo publicado na revista **Environment Science and Pollution Research.**

4.2Capítulo II - N-acetylcysteine inhibits Mancozeb-induced impairments to the normal development of zebrafish embryos.

Artigo publicado na revista **Neurotoxicology and Teratology**.

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RESEARCH ARTICLE

Mancozeb exposure results in manganese accumulation and Nrf2-related antioxidant responses in the brain of common carp *Cyprinus carpio*

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Abstract

Manganese (Mn)-containing dithiocarbamates such as Mancozeb (MZ) have been shown to induce oxidative stress-related toxicity in rodents and humans. However, little is known about the neurotoxic effects induced by MZ in fish. In this study, carp (*Cyprinus carpio*) were exposed to non-lethal waterborne concentrations of MZ, and oxidative stress parameters as well as metal accumulation in fish brains were evaluated. The experimental groups were as follows: control, MZ 5 mg/L, and MZ 10 mg/L. Fish were exposed for 7 days, and then brain was removed and prepared for subsequent analysis of antioxidant enzymes, reactive oxygen species (ROS), and expression of Nrf2 and phosphoNrf2. In parallel, manganese (Mn) levels were evaluated in blood and brain tissues. Mn levels were significantly increased in blood and brain of MZ-exposed carps. In addition, a concentration- dependent increase ($p < 0.05$) in ROS levels was observed in parallel to increments ($p < 0.05$) in the activity of major antioxidant enzymes, such as GPx, GR, and GST. On the other hand, significant decreases ($p < 0.05$) in CAT and SOD activities were observed. The expression of total and phosphorylated forms of Nrf2 was significantly ($p < 0.05$) upregulated in the brain of carps exposed to Mz when compared to the control, indicating an activation of the Nrf2 antioxidant pathway. Our study showed for the first time the activation of the Nrf2/ARE pathway and bioaccumulation of Mn induced by MZ exposure in fish species, highlighting important mechanisms of action and its toxicological impacts to aquatic organisms.

Keywords Mancozeb · Carp fish · *Cyprinus carpio* · Dithiocarbamate · Manganese · Bioaccumulation · Antioxidant responses

Introduction

Agrochemicals are widely used for the control of pests and pathogens that may compromise crop productivity. Although the use of pesticides has increased food production over decades, these substances can reach reservoirs, rivers, and streams leading to contamination of aquatic ecosystems and can cause potential ecological and public health problems (Grisolia 2005). When used improperly, even at non-lethal concentrations, these chemicals may induce deleterious physiological effects in exposed organisms, mainly aquatic biota (Grisolia 2005; Costa-Silva et al. 2015).

Commercial carbamate pesticides such as Mancozeb (MZ) belong to the subclass of dithiocarbamate pesticides containing manganese (Mn^{2+}) and zinc (Zn^{2+}) atoms coordinated with ethylene-bis-dithiocarbamate. MZ is a broad-spectrum fungicide and one of the most used agrochemicals on a global scale (Fitsanakis et al. 2002; Goldoni and Silva 2012). MZ has been claimed to present low acute toxicity to aquatic organisms, and to not persist in the environment, but there are concerns regarding the potential toxicological risks associated with its degradation by-products (US EPA 2005). In this regard, toxicity of MZ and other carbamates to fishes, including endocrine disruption (Bisson and Hontela 2002), impacts on olfactory neurophysiology (Jarrard et al. 2004), DNA, and

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chromosomal damage (Marques et al. 2016) and alterations of behavioral and neurodegenerative parameters (Zizza et al. 2017) have been shown in the literature. In addition, oxidative stress has been identified as a major mechanism by which MZ causes deleterious effects in fish (Kubrak et al. 2012; Atamaniuk et al. 2014). Despite this, the potential mecha- nisms involved in MZ-induced oxidative stress in fishes are not understood well to date.

MZ toxicity has been linked to its main degradation by-product: ethylenethiourea (ETU) (Xu 2000; US EPA 2005; Singh and Srivastava 2013), and manganese (Mn), zinc (Zn), and organic moieties (ethylene-bis-dithiocarbamate; EBDC) have been shown to be major contributors to its deleterious effects (Hoffman and Hardej 2012; Williams et al. 2013). Agricultural applications of dithiocarbamate fungicides containing Mn are commonly identified as potential sources of environmental Mn contamination (Geissen et al. 2010). Specifically, oxidative stress induced by MZ in fish has been linked to Mn-derived reactive oxygen species (ROS) formation mechanisms (Dolci et al. 2014; Gabriel et al. 2013; Vieira et al. 2012). However, Mn accumulation processes in fish following MZ exposures remain unclear.

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a key mediator of the cellular adaptive response to oxidative stress and xenobiotic exposure (Nguyen et al. 2009). Following redox signals, Nrf2 triggers the transcription of endogenous antioxidant/detoxifying enzymes through its binding to the antioxidant responsive element—ARE, thus regulating the antioxidant response of organisms (Tanito et al. 2007; Schülke et al. 2012). However, the participation of this transcription factor during oxidative stress related changes evoked by MZ exposure in fish has not been investi- gated to date.

Considering that little is known about oxidative-related changes in the brain of fish exposed to MZ, our goal with the present study was to evaluate potential changes in Nrf2 and antioxidant enzyme activity as well as Mn accumulation processes after exposure of juvenile common carp to non-lethal concentrations of MZ.

Materials and methods

Chemicals

Commercial Mancozeb (Emzeb 800 WP) was purchased from Sabero Organics, America S.A. Nitric acid (HNO_3), polypro- pylene, hydrogen peroxide (H_2O_2), 2,7-dichlorofluorescein diacetate (DCFH-DA), tert-butylhydroperoxide (tBOOH), glutathione reductase (GR), reduced glutathione (GSH), oxi- dized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate—reduced (NADPH), 5,5-dithio-bis(2-nitrobenzoic)acid (DTNB), 1- chloro-2,4-dinitrobenzene (CDNB), acetylthiocholine iodide, quercetin, N,N,N′,N′-Tetramethylethylenediamine (TEMED), β-actin-HRP conjugated antibody, and anti-rabbit-HRP secondary antibodies were purchased from Sigma-Aldrich (São Paulo, Brazil). Rabbit Anti-Nrf2 primary antibody was purchased from Santa Cruz Biotechnologies (Dallas, TX, USA). Phospho-Nrf2 primary antibody from rabbit was purchased from Abcam (Cambridge, UK).

Animals and treatments

Juvenile carp fish (*Cyprinus carpio*) (weight, 9.0 ± 0.5 g; length, 7.0 ± 1.0 cm) were obtained from a fish farm (Santa Maria, RS, Brazil). Fish were acclimated to laboratory condi- tions for 15 days in a static 250-L fiber-glass container system prior to the experiments and kept in continuously aerated wa- ter under a controlled photoperiod (12-h light/12-h dark). Water conditions were monitored on a daily basis and param- eters were as follows: temperature 22.5 ± 1.0 °C, pH 7.5 ± 0.2 units, dissolved oxygen 7.21 ± 1.0 mg/L, non-ionized am- monia 0.3 ± 0.01 mg/L, and nitrite 0.05 ± 0.01 mg/L. During the acclimation period, the carp were fed once a day with commercial fish pellets (Supra, Brazil). Feces and pellet resi- dues were removed by suction. A mechanical filtration system was constantly used to maintain water quality. A total of 90 healthy animals were used in this study. Juvenile carp (*C. carpio*) were obtained from a local aquaculture center as described above. The average body length and weight of in- dividuals were 6.0 ± 1.0 cm and 2.50 ± 0.5 g, respectively. Fish were acclimated in a 300-L container for 12 days under laboratory conditions and supplied with continuously aerated tap water and light/dark (12/12 h) photoperiod. Animals were divided into three groups: control, MZ 5 mg/L, and MZ 10 mg/L. The concentrations used were adapted from previ- ous studies undertaken in another fish species (Kubrak et al. 2012; Atamaniuk et al. 2014). Animals were exposed for 7 days in experimental aquaria (6 animals per tank; 30 animals per group totalizing 5 replicates of 6 animals) containing 10 L of de-chlorinated tap water and MZ solutions. The water was changed every 48 h. After exposure, fish were anesthetized in ice-cold water and euthanized by cervical rupture. Brain and blood samples were removed and kept in ultra-freezer (− 80 °C) for further biochemical analyses. Brain tissue was used for the determination of enzyme activities, western blot anal- ysis, and metals concentrations, while the blood was used for quantification of metals. Animal experimentation in this study fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Pampa under process number 043/2013.

Stability of MZ in fish water

Chromatographic determinations of MZ in exposure water were performed on a Shimadzu Prominence UFLC high-performance liquid chromatography equipped with an LC-6 AD pump and a UV SPD-20AV detector and a manual sample injector Rheodyne 7725 (Tokyo, Japan). LC Solution software was employed to record retention times and chromatograms. A Kromasil® C18 reversed-phase column (250 × 4.6 mm, i.d.; 5.0 μm) was used at ambient temperature. Separation was done at 0.8 mL/min with acetonitrile/water (40:60, *v*/*v*) for 20 min. Injection volume was 20 μL and the detection was done at 272 nm. The method was adapted from Gustafsson and Thompson (1981) and Al-Alam et al. (2017). Stock solu- tion of Mancozeb (Sigma-Aldrich, purity 97%) was prepared at 10 mg/L in System water Zebtec™, pH 7.2. Standard solutions (0.62, 1.25, 2.5, and 5.0 mg/L) were prepared by further dilution of the stock solution. HPLC method validation is described in Table 1.

Determination of metals in fish blood and brain

The determination of Mn, Zn, Cu, and Fe content in brain and Mancozeb samples was carried out using an inductively coupled plasma optical emission spectrometer with axial view configura- tion (Optima 4300 DV, Perkin Elmer, USA). For Mn and Zn determination in blood samples, an inductively coupled plasma mass spectrometer (Elan DRC II, PerkinElmer-SCIEX, Canada) was used (Table 2). Instrumental parameters, including nebulizer gas flow-rate and RF power for both instruments, are described in Table 3. Argon of 99.996% purity (White Martins, Praxair, Brazil) was used for plasma generation, nebulization, and as auxiliary gas. A multi-element certified stock reference solution (SCP33MS, SCP Science, Canada) containing 10 mg L^{-1} of all analytes was used to prepare the calibration solutions.

Samples were analyzed after previous digestion in open vessels, using microwave heating. About 20 and 50 mg of blood and brain, respectively, were digested with 300 μL of high-purity double distilled nitric acid (65%, d = 1.39 g cm^{-3}, Merck, Germany). The solution was irradiated for 30 s and manually shaken. This procedure was repeated 4 times. In sequence, 300 μL of H2O2 (30%, d = 1.11 g cm^{-3}, Merck, Germany) was added to digests and the procedure of heating was repeated. After cooling, digests were diluted with water up to 5 mL. For Mancozeb digestion, about 200 mg of sample were decomposed using 6 mL of double distilled nitric acid using conventional heating and open vessels. The heating pro- gram was (i) 80 °C for 1 h and (ii) 130 °C for 1 h. After cooling, the digests were diluted with water up to 25 mL in a polypropylene vessel (Table 4).

Table 1 HPLC method parameters determined for Mancozeb

Parameters determined	Values obtained
Retention time	10.017 min
Equation (y = ax + b)	$y = 5.74^{-5} + 4.41^{-2}$
Regression coefficient (R^2)	0.9988
Linearity	0.039–10 mg/L
LOD	0.073 mg/L
LOQ	0.155 mg/L
Recovery rate	103.4
% RSD	4.38

Table 2 Quantification of Mancozeb in fish water. Samples were quantified compared to standard curve

Sample	Retention time (min)	Area	Height	Concentration (mg/L)
Control	–	–	–	–
1.0 mg/L	10.153	5888	315	0.428
5.0 mg/L	9.939	31,832	1762	2.210
10 mg/L	10.072	64,771	3587	4.459

Determination of lipid peroxidation and ROS generation

Lipid peroxidation was quantified as thiobarbituric acid reac- tive substances (TBARS) following the method of Ohkawa et al. (1979). We also quantified 2,7-dichlorofluorescein diacetate (H_2DCF-DA) oxidation as a general index of ROS production in brain homogenates according to Pérez- Severiano et al. (2004). The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored at regular

Parameter	ICP-OES	ICP-MS
RF power (W)	1400	1400
Plasma gas flow rate (L min^{-1})	14.0	15.0
Auxiliary gas flow rate (L min^{-1})	1.00	1.20
Nebulizer gas flow rate (L min^{-1})	1.00	1.09
Spray chamber	Cyclonic	Cyclonic
Nebulizer	GemCone™	Concentric
Isotopes (m/z)		^{55}Mn
		^{68}Zn
Emission lines (nm)	Cu (327.393) Fe (213.857)	
	Mn (257.610)	
	Zn (213.857)	

Table 4 Quantification of metals on commercial MZ sample (EMZEB 800). Results ($n = 3$, the results are expressed as mg g^{-1}. *SD* standard deviation, *CV* coefficient of variation

Element	Concentration (mg g^{-1})	SD (mg g^{-1})	CV (%)
Mn	178.3	0.7	0.37
Zn	19.2	0.4	2.1
Cu	< 0.029		
Fe	0.203	0.01	4.9

10-min intervals during 1 h at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Mitochondrial viability

Mitochondrial viability was measured by resazurin assay (fluorescence) in brain homogenates. The assay is based on the ability of viable mitochondria to convert resazurin into a fluorescent compound resorufin (O'Brien et al. 2000). The fluorescence was acquired in a fluorescence plate reader (Perkin Elmer Enspire 2300) at 544 nm of excitation and 590 nm emission after 1 h of incubation at ambient temperature.

Western blot analysis

Western blotting was performed according to Costa-Silva et al. (2015). The whole fish brains were homogenized at 4 °C in buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM so- dium fluoride, and protease inhibitor cocktail (Sigma, MO). The homogenate was then centrifuged (1000*g*) for 10 min at 4 °C, and the supernatants collected. After total protein deter- mination using BSA as standard, β-mercaptoethanol was added to samples to a final concentration of 8%. Hereafter, 40% glycerol, 25 mM Tris, and bromophenol blue (pH 6.8) were added to samples. Then, samples were frozen at − 80 °C for further analysis. The proteins (30 μg per well) were sepa- rated by SDS-PAGE and electrotransferred to nitrocellulose membranes for approximately 3 h using a GE Health Care TE22 Mini Tank Transfer System at 4 °C. The membranes were blocked with 5% skimmed milk for 1 h. Then, mem- branes were washed in Tris-buffered saline with Tween (TBST) containing 100 mM Tris–HCl, 0.9% NaCl, and 0.1% Tween-20, pH 7.5, and incubated overnight (4 °C) with prima- ry antibodies anti-rabbit Nrf2 (1:1.000), anti-rabbit phospho NRF2 (1:1000) and anti β-Actin HRP conjugated (1:10.000). Finally, membranes were incubated with specific secondary antibodies anti-rabbit IgG (1:10.000) during 1 h at room tem- perature (except for β-Actin HPR conjugated). The immuno- blots were visualized on a Bruker IS4000MM Pro imaging system using ECL Western Blotting substrate Kit (Promega) and densitometric analysis of immunoreactive bands was per- formed using Scion Image® software.

Enzyme activities

All spectrophotometric enzymatic assays were performed in an Agilent Cary 60 UV/VIS spectrophotometer with an 18 cell holder accessory coupled to a Peltier Water System tempera- ture controller set at 25 ± 1 °C. Fish brain was homogenized in 20 mM HEPES pH 7.4 and centrifuged at 20,000*g* for 30 min at 4 °C The supernatant was isolated in aliquots for determi- nation of antioxidant enzymes activity.

Glutathione reductase

For the measurement activity of glutathione reductase (GR) activity, brain homogenate supernatant was added to medium containing phosphate buffer (0.0025 M EDTA, 0.25 M K2HPO4, 0.25 M KH2PO4, pH 7.0) and 100 mM NADPH.

The measurements were made by continuously monitoring NADPH decreases of absorbance for 2 min at 340 nm at 25 °C in a spectrophotometer. Reaction was initiated with addition of 10 mM GSSG (Carlberg and Mannervik 1985). One unit will cause the oxidation of 1.0 μmol of NADPH to NADP+ per minute ($\varepsilon = 6.22\ M^{-1}\ cm^{-1}$).

Glutathione peroxidase

Glutathione peroxidase (GPx) activity was determined spec- trophotometrically by the indirect method described by Wendel (1981). The assay consists in the continuously moni- toring NADPH decreases of absorbance for 5 min at 340 nm at 25 °C, at pH 7.0 as a reflection of GPx activity in the presence or GR and NADPH. The brain homogenate supernatant was added to medium containing phosphate buffer (0.0025 M EDTA, 0.25 M K2HPO4, 0.25 M KH2PO4, pH 7.0), 100 mM GSH, 100 mM NADPH, and 5 U/mL GR. The re-action was initiated by adding tBOOH at final concentration of 10 mM. One unit of GPx will consume 1.0 μmol of NADP+ from NADPH per minute ($\varepsilon = 6.22\ M^{-1}\ cm^{-1}$).

Glutathione S-transferase

The glutathione S-transferase (GST) activity was measured in brain homogenate supernatant as described by Habig and Jakoby (1981) using 1-chloro-2.4-dinitrobenzene (CDNB) as a substrate, which is suitable for the broadest range of GST isozymes. The reaction medium contains phosphate buffer (0.0025 M EDTA, 0.25 M K2HPO4, 0.25 M KH2PO4, pH 7.0) and GSH 100 mM. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is a linear increase in the absorbance at 340 nm, which can be monitored.

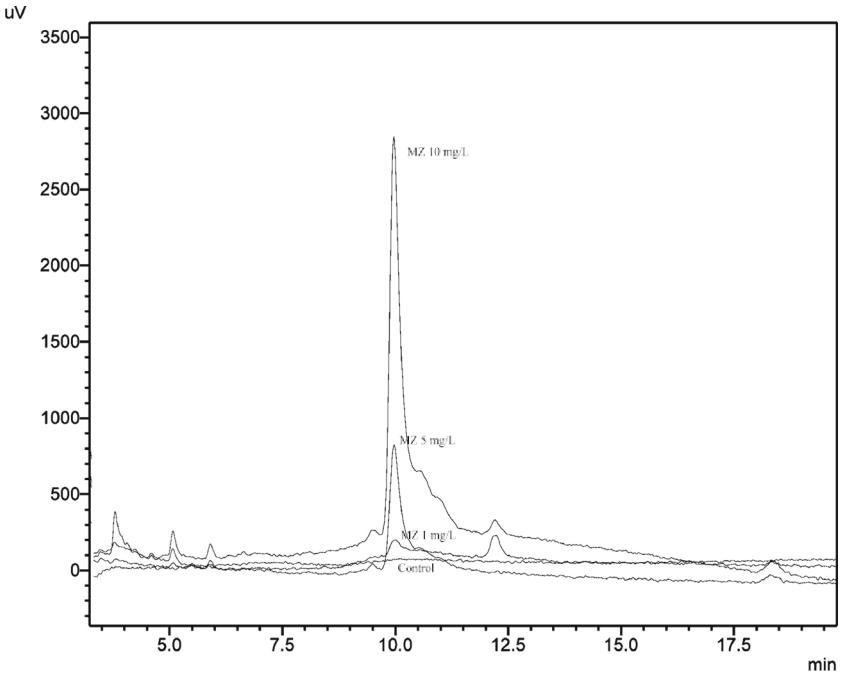

Fig. 1 Stability of MZ in fish water. Chromatographic determinations of MZ in fish water were performed by high-performance liquid chromatography. LC Solution software was employed to record retention times and chromatograms. A Kromasil® C18 reversed-phase column (250 × 4.6 mm, i.d.; 5.0 μm) was used at room temperature. Separation was done at 0.8 mL/min with acetonitrile/water (40:60, *v*/*v*) for 20 min. Injection volume was 20 μL and the detection was done at 272 nm (UV detector). Peaks were compared to a standard curve made with Mancozeb (Sigma-Aldrich, purity 97%) in fish system water Zebtec™, pH 7.2 (equation curve at Table 2)

spectrophotometrically at 25 °C during 2 min. One unit of GST activity is defined as the amount of enzyme producing
1.0 μmol of GS-DNB conjugate/min under the conditions of the assay ($\varepsilon = 9.60\ M^{-1}\ cm^{-1}$).

Catalase

Catalase (CAT) activity was assayed following the clearance of H2O2 at 240 nm in a reaction media containing 0.05 M phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H2O2, and 0.012% TRITON × 100 according to described elsewhere (Aebi 1984). One unit of CAT activity is defined as the amount of enzyme degrading 1.0 μmol of H2O2 per min under the conditions of the assay ($\varepsilon = 40.0\ M^{-1}\ cm^{-1}$).

Superoxide dismutase (SOD)

SOD was measured following the procedures established by Kostyuk and Potapovich 1989. In this assay, quercetin
0.49 mM is used as a superoxide radical sensor in the presence of 7.55 M N,N,N′,N′-Tetramethylethane-1,2-diamine (TEMED). The assay medium consisted of 0.025 M phos- phate buffer (EDTA 0.1 mM) at pH 10. Tissue SOD activity is expressed in units SOD/mg of total protein, where 1 unit is the amount of SOD required to give 50% maximal inhibition of the initial rate of quercetin reduction.

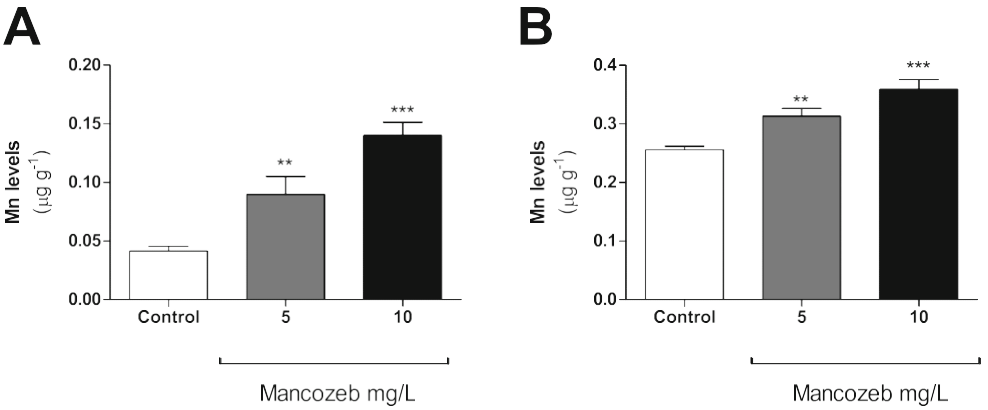

Fig. 2 MZ exposure increases manganese levels in fish blood and brain. a Levels of Mn in fish blood. b Levels of Mn in fish brains after MZ treatment. Results are expressed as mean ± SEM ($n = 6$). **$p \leq 0.01$ and ***0.001 compared to control

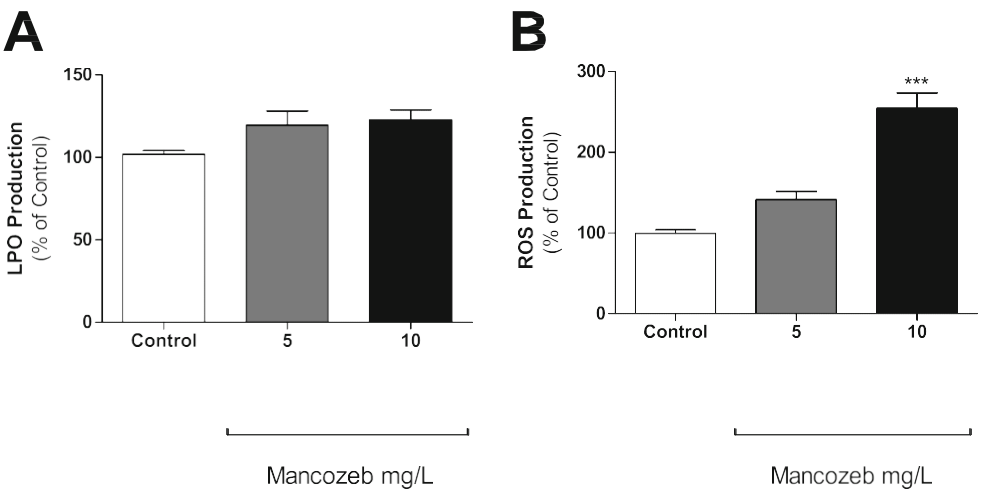

Fig. 3 MZ exposure increases ROS levels in fish brain. a Lipid peroxidation (TBARS levels). b ROS formation in fish brains after MZ treatment. Results are expressed as mean ± SEM ($n = 6$). ***$p \leq 0.001$ compared to control

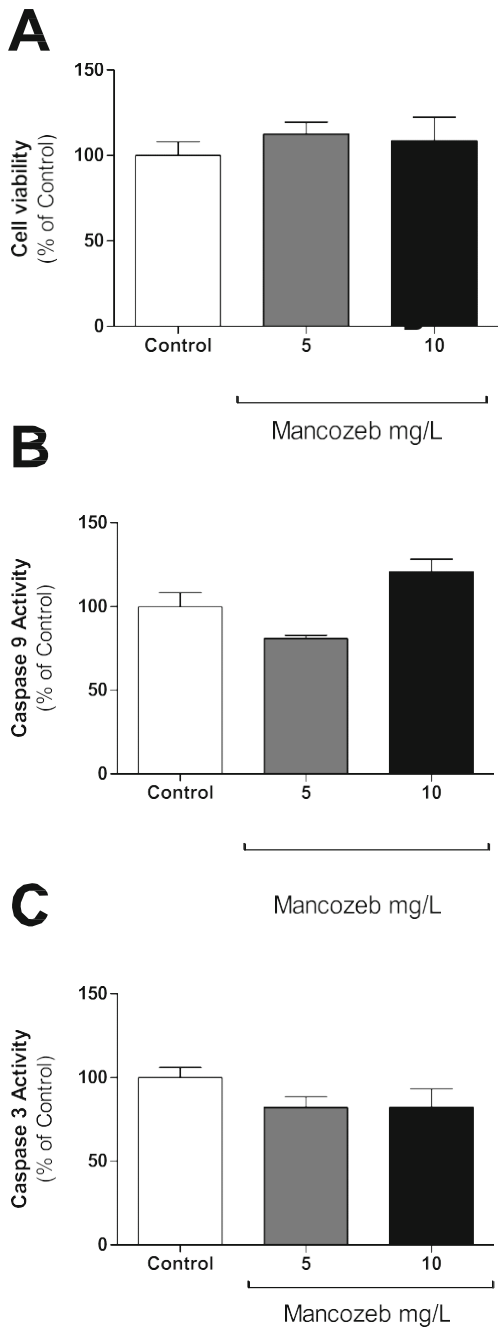

Fig. 4 MZ exposure induces no changes in mitochondrial viability and apoptosis in fish brain. a Cell viability, b caspase 9 activity, and (c) caspase 3/7 activity in fish brains after MZ treatment. Results are expressed as mean ± SEM (n = 6). No statistically significant changes were found

levels of manganese (Mn) were significantly increased in the blood and brain of exposed carps, indicating its accumulation in fish tissues, as compared to control (Fig. 2a, b). For instance, fish exposed to nominal con- centrations of 5 and 10 mg/L showed increases of 1.81 and 3.38 fold, respectively, in blood, while increases of 0.22 and 0.40 fold were observed for the brain. Zinc, Cu, and Fe levels were also evaluated in the blood and brain of carps. However, no significant changes were observed for these metals (data not shown).

MZ exposure increases ROS levels in fish brain

While there was a slightly increasing trend in TBARS levels (Fig. 3a), no statistically significant changes (p =0.0584; F = 4.378) in lipid peroxidation were apparent brains of carp exposed to MZ. In contrast, the production of reactive oxygen species, measured as oxidation of the fluorescent dye DCF-DA, was significantly increased ($p < 0.05$) in fish exposed to 10 mg/L MZ when compared to controls (Fig. 3b).

MZ exposure induces no changes in mitochondrial viability and apoptosis in fish brain

Exposure of fish to MZ did not cause significant changes on mitochondrial viability as compared to controls (Fig. 4a). Also, no significant alterations in the activity of caspase 3 and caspase 9 were observed (Fig. 4b, c).

MZ exposure induces increases in the expression of total and phosphorylated forms of Nrf2 in fish brain

Densitometry of immunoreactive bands revealed that ex- pression of total (Fig. 5b) and phosphorylated forms (Fig. 5c) of Nrf2 were significantly ($p < 0.05$) increased in the brain of carps exposed to both concentrations of Mz. The pNrf2/Nrf2 ratio was significantly increased only at MZ 10 mg/L (Fig. 5d).

Significant ($p < 0.05$) decreases of CAT and SOD activity were observed in brains of carps exposed to 5 and 10 mg/L MZ (Fig. 6a, b, respectively). GPx and GST activities were significantly increased in the brain of carps after exposure to MZ 5 and 10 mg/L, as compared to controls (Fig. 6c, e, re- spectively), whereas the activity of GR was significantly in- creased only at 10 mg/L MZ (Fig. 6d). TrxR activity was not changed by any of the MZ concentrations tested (Fig. 6g).

Discussion

Many studies have demonstrated the toxic effects of agro- chemicals to non-target organisms. This is particularly impor- tant when considering aquatic organisms, which are constant- ly subjected to substantial amounts of pollutants (Coronado et al. 2004; Fuentes-Rios et al. 2005). According to published studies, concentrations of MZ ranging around 10 mg/L, as used here, may be of environmental

relevance considering agricultural runoff, in which dosages recommended for crop

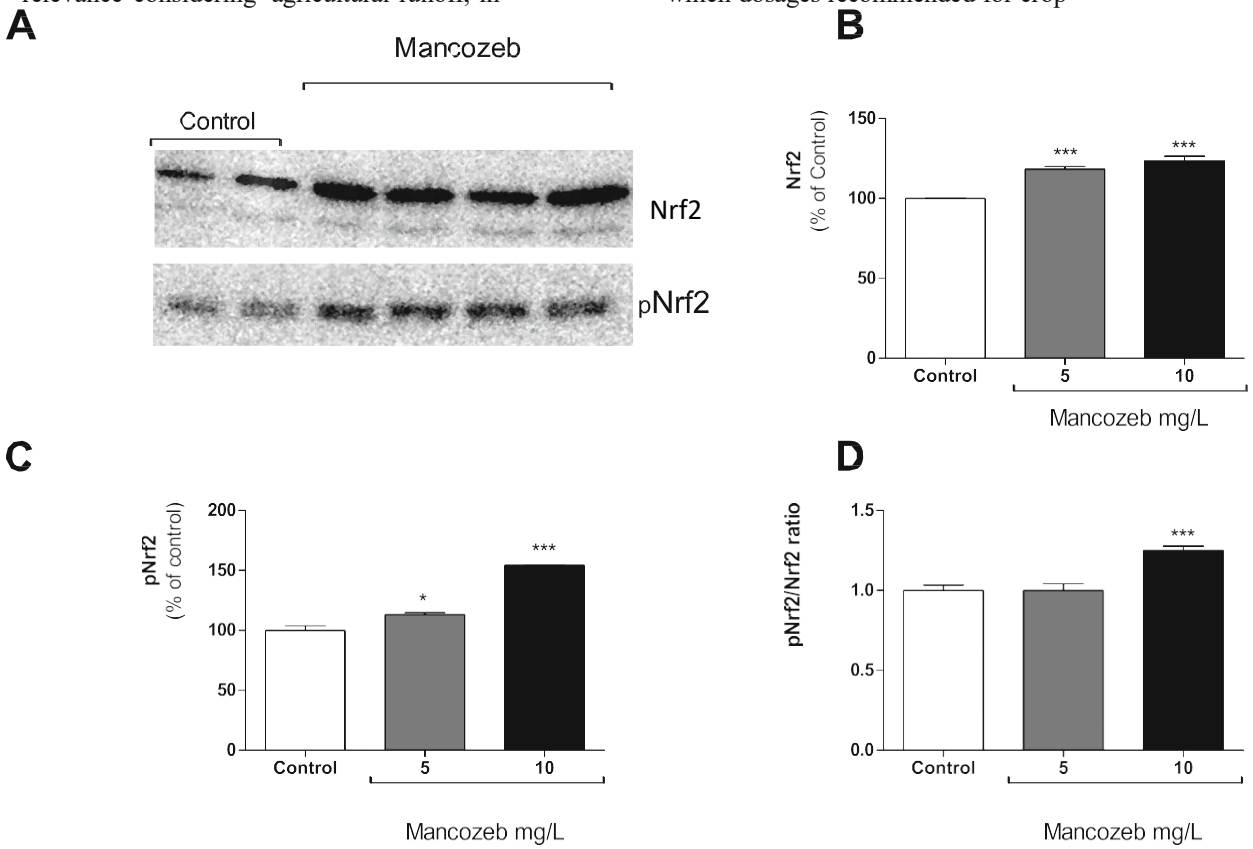

Fig. 5 MZ exposure induces increases in the expression of total and phosphorylated forms of Nrf2 in fish brain. a Representative immunoblots of total and phosphorylated forms of Nrf2. b Densitometric analysis of immunoreactive bands of total Nrf2. c Densitometric analysis of immunoreactive bands of phosphoNrf2. d PhosphoNrf2/Nrf2 ration in fish brains after MZ treatment. Results are expressed as mean ± SEM (n = 3). *p ≤ 0.05 and ***p ≤ 0.05 when compared to control

treatment are thousand times greater (Atamaniuk et al. 2014). The ecotoxicological effects of MZ have been investigated in different organisms (US EPA 2005); however, studies involv- ing biomarkers of oxidative stress and detoxification in fish are scarce to date. In the present study, we report increased Mn levels and the potential participation of Nrf2 signaling as im- portant mechanisms of toxicity and adaptive responses to non-lethal concentrations of MZ in the brain of common carp, highlighting the potential neurotoxic effect elicited by this fungicide in aquatic organisms.

Our results from ICP-OES analysis revealed augmented levels of Mn in blood and brain of MZ-exposed carps, in a dose-dependent manner, indicating its accumulation in fish tissues. In contrast, Zn, Cu, and Fe levels were not changed; highlighting the potential participation of Mn in the effects observed in MZ-treated carps. This hypothesis has been also suggested in studies using *Caenorhabditis elegans* (Harrison Brody et al. 2013) and fish (Zizza et al. 2017). In addition, it has been demonstrated that ETU, a major MZ metabolite, has minimal toxicity towards *C. elegans* (Easton et al. 2001). However, our data do not allow us to affirm whether the ob- served increased Mn accumulated in carp brains represents metal-EBDC complexes or free Mn ions, thus leading to the need of further studies to elucidate the potential Mn accumulation processes in fish exposed to MZ-containing fungicides.

According to results from stability of MZ in fish water, some unidentified peaks were present together with MZ, indi- cating the possible presence of MZ degradation products, in- cluding ETU. In addition, we found an approximately 50% drop in MZ concentrations in water at 48 h, which either indicates significant degradation/elimination of the fungicide in solution. Our findings are in agreement with literature, which reports the low stability of MZ in water solutions. For instance, a recent study (López-Fernández et al. 2017) has shown a MZ degradation rate of almost 80% at pH 8 (25 °C) at the first 50 h in solution. Other literature reports have demonstrated that MZ is strongly subject to hydrolysis and photolysis (Kontou et al. 2001; Garcinuño et al. 2004). For that reason, our experimental design included renewal of MZ solutions every 48 h during the course of carp exposure along 7 days. Therefore, the actual role of MZ metabolites, including metals and ETU, in the observed effects induced by MZ in carp brains needs further elucidation.

We also observed a significant increase in brain ROS levels after exposure of carps to MZ. The nervous system is partic- ularly vulnerable to ROS due to its high oxygen demand and neuronal membranes rich in polyunsaturated fatty acids (Halliwell 2006; Chen et al. 2008). Moreover, carp, as most fishes, naturally contain high amounts of polyunsaturated fatty acids (PUFAs) making fish particularly susceptible to lipid

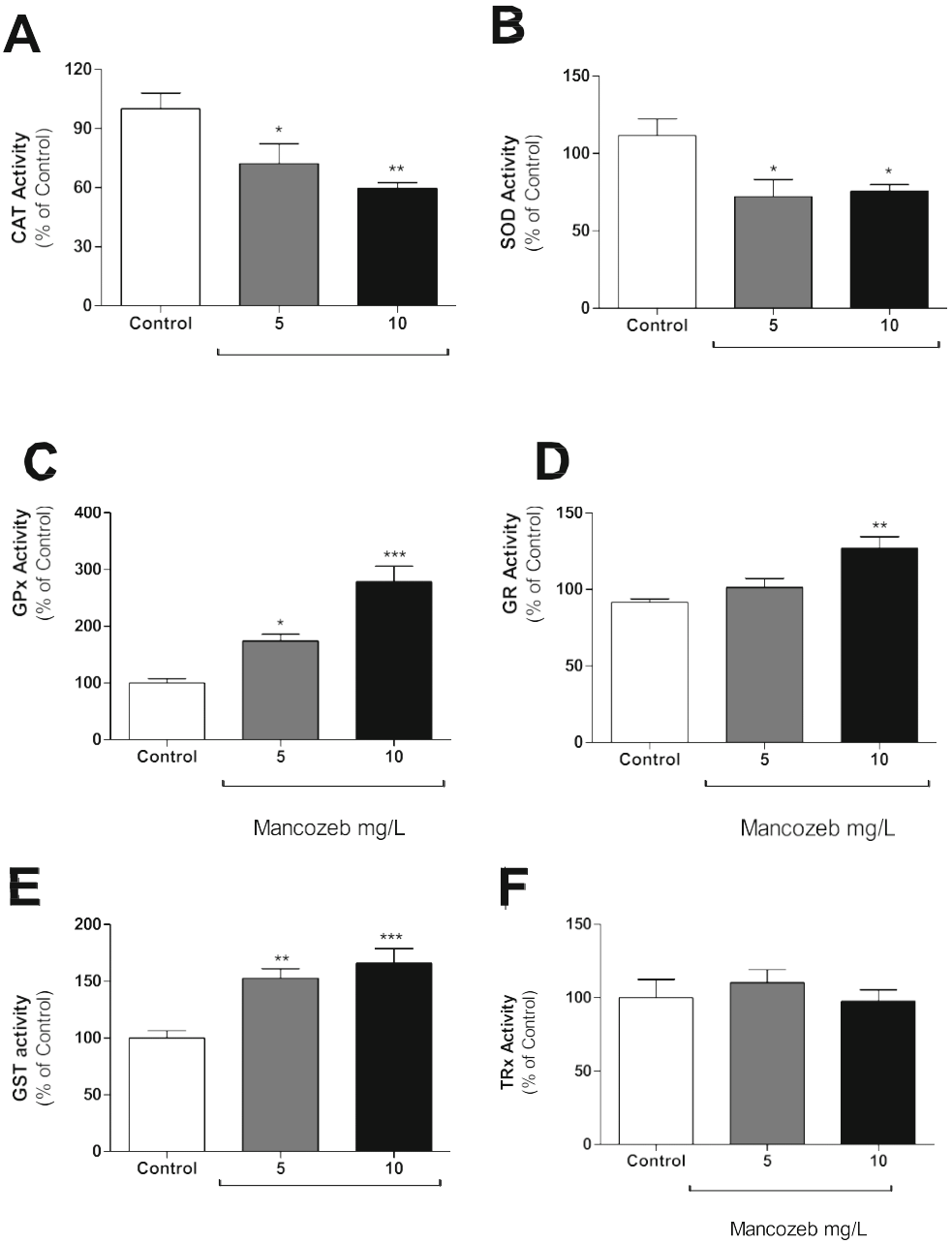

Fig. 6 MZ exposure induces changes in the activity of antioxidant enzymes in fish brain. a CAT, b SOD, c GPx, d GR, e GST, and f TRx activity in fish brains after MZ treatment. Data are expressed as mean ± SEM ($n = 6$). *$p < 0.05$, **$p \leq 0.01$, and ***0.001 when compared to control

peroxidation (Storey 1996; Steffens and Wirth 2004). Thereby, it was expected that carp exposed to MZ would show increased lipid peroxidation. However, no statistically signif- icant changes in lipid peroxidation were apparent even after exposure to the greatest MZ concentration of 10 mg/L. Furthermore, we did not observe significant changes in cell viability and apoptosis parameters in carps exposed to MZ. In fact, this result was expected since the concentrations of MZ tested (5 and 10 mg/L) were in the range of 10% or less the LC_{50} in fish (EPA 2005). Therefore, it is possible to suggest that the MZ concentrations tested in this study triggered adap- tive responses that may have ameliorated MZ-induced toxic- ity. In line with this, the observed increase in GST activity found here could be related to the elimination of lipid perox- ides via GSH conjugated products (Keppler 1999). In mam- mals, the GST-pi isoform (conserved between chordates) conjugates unsaturated aldehydes produced during the lipid peroxidation process and this regulation is coordinated with other antioxidant defenses (Hayes et al. 2005; Glisic et al. 2015). On the other hand, the similar increase of GPx and GR observed in our study could be acting together with GST against oxidative alterations induced by MZ (Nunes et al. 2016). Altogether, these observations are strong indica- tives that MZ-exposed carps have successfully mounted an antioxidant response that ameliorated potential adverse effects.

The cellular adaptive response to oxidative stress and xe-nobiotic exposure is primarily mediated by the nuclear factor erythroid 2-related factor 2 (Nrf2) (Nguyen et al. 2009). Once redox signals are generated, Nrf2 triggers the transcription of endogenous antioxidant enzymes such as GST, GPx, GR, SOD, CAT, and thioredoxin system through its binding to the antioxidant responsive element—ARE (Tanito et al. 2007; Schülke et al. 2012). We observed a significant increase in the protein levels of Nrf2 and its phosphorylated form in carp brains exposed MZ. Nuclear translocation of Nrf2 involves at least two known mechanisms: (i) redox modulation of re- active cysteines in a cytosolic Nrf2 inhibitory protein, known as Keap1, causing Nrf2 to dissociates from Keap1 and then moving to nucleus; and (ii) phosphorylation of Nrf2 at a serine 40 (Ser40) residue, leading dissociation from its inhibitory protein (Bloom and Jaiswal 2003). To our knowledge, this study is the first to demonstrate changes on Nrf2 protein in fish exposed to MZ. The observed activation (phosphorylation) of this transcription factor in the brain of exposed carps might be responsible for the changes induced in the activity of major antioxidant enzymes (GSTs, GPx, and GR), demonstrating an adaptive mechanism to mild oxidative stress and/or xenobiotic detoxification.

CAT and SOD are often considered the first line of defense against the deleterious effects of reactive oxygen species (Asagba et al. 2008). Under moderate stress, these enzymes can be upregulated; however, under high ROS conditions,their activities may decrease (Rodriguez et al. 2004). Apparently, this is not the case for the observed decrease in CAT and SOD activities in carp brains found in our study, since the increased ROS levels found were not sufficient to induce lipid peroxidation. Alternatively, the chelating ability of Mn-containing pesticides can lead to formation of com- plexes with proteins, acting as an inhibitory mechanism to- wards metal-containing enzymes (Larsson et al. 1976; Rath et al. 2011; Hogarth 2012). Furthermore, Mn ions can be re- leased when MZ is catabolized (Houeto et al. 1995; Calviello et al. 2006), suggesting that Mn can interact with the active centers of metal-containing enzymes like SOD and CAT. In this context, Chtourou et al. (2010) described a decrease in the antioxidant enzymes in cerebral cortex of rats that received Mn^{2+} in drinking water. It has also been showed that Mn^{2+} inhibits CAT activity in the brain of fish (Jena et al. 1998). Despite the potential inhibitory effects of Mn towards metal- containing enzymes, a potential inhibitory effect of MZ on antioxidant enzymes as SOD and CAT needs further elucida-ion. As previously discussed, significant increases were found in the activity of GST and GPx in our experimental animals, a fact that may be interpreted as a compensatory mechanism towards the observed negative impacts of MZ on CAT and SOD.

In conclusion, this study demonstrated the potential neurotoxicological impacts of the pesticide MZ in carp. Our results pointed to potential bioaccumulation of Mn in the brain of carps exposed to sublethal MZ concentrations: disturbances on redox balance, culminating in activation of the Nrf2 sig- naling pathway, which in turn resulted in adaptive responses involving antioxidant enzyme systems. Our findings may con- tribute to a better understanding of the adaptive mechanisms.

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Full length article

N-acetylcysteine inhibits Mancozeb-induced impairments to the normal development of zebrafish embryos

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ABSTRACT

Mancozeb (MZ), a manganese/zinc-containing ethylene-bis-dithiocarbamate (EBCD) fungicide has been claimed to present low acute toxicity and short environmental persistence, however, its effects on embryogenesis in non-target organisms is unclear. Here, we used zebrafish embryos (5 hpf) to assess the potential embryotoxic effects induced by MZ (up to 72 hpf) as well as the role of reactive oxygen species (ROS) in this process by pre-treatment with a classical antioxidant (*N*-acetylcysteine, NAC). Markers of reactive oxygen species production (ROS), glutathione (GSH) levels and glutathione S-transferase (GST) activity were measured along with genotoxicity (comet assay), cell death (Acridine Orange) and behavioral parameters (spontaneous movement, touch stimulation and swimming response), in order to determine potential mechanisms of embryotoxicity. According to results, MZ was able to induce morphological abnormalities such as body axis distortion, DNA damage, cell death, increased ROS generation and changes in behavioral endpoints during zebrafish development. All these toxic effects were inhibited by the pre-treatment with NAC indicating a key role of redox unbalance during MZ-induced embryotoxicity. At least in our knowledge, this is the first report on the deleterious effect of MZ to the normal embryogenesis of zebrafish. In addition, the importance of ROS generation during this pathophysiological condition was highlighted.

1. Introduction

Aquatic organisms, during the embryonic stage, have unequaled physiological characteristics such as intense and controlled cell proliferation, differentiation and apoptosis and all these physiological activities are closely related to the cellular redox homeostasis (Cole and Ross, 2001; Dennery, 2007; Hahn et al., 2015). Due to the redox status being highly dynamic, this system may be affected by physiological and/or environmental conditions. In this context, in aquatic ecosystems, organisms are in constant vulnerability because of eventual exposures to environmental contaminants, in special agrochemicals (Lushchak, 2016).

Mancozeb (MZ), a manganese and zinc-containing ethylene-bis-dithiocarbamate (EBCD) is one of the most fungicides used on a global scale (Fitsanakis et al., 2002; Runkle et al., 2017). According to international fungicide market estimates in 2014, MZ production had a 20% increase and is expected to continue to grow until 2020 (Fungicides Market, 2015). Due to the low cost and its high efficacy, this fungicide is used in several crops, like soybean, which is the most important agricultural product in Brazil (Pillar and Langue, 2015; ANVISA, 2016). According to the Brazilian Agriculture Research Agency – EMBRAPA, multiple applications up to 2 $kg \cdot ha^{-1}$ of MZ are recommended for soybean crops (Godoy et al., 2015), therefore, increasing environmental risks of this economical activity.

MZ has been claimed to present low acute toxicity to aquatic organisms, and a short persistence in the environment (Xu, 2000), however, the indiscriminate use of this chemical could deliver risks to non-target organisms, through natural processes such as surface runoff,

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drift, and leaching leading to the contamination of water resources (Rose and Carter, 2003). One of the means by which MZ can exert toxicity is associated with its pro-oxidant activity (Rath et al., 2011), by both degradation products, like ethylenethiourea (ETU) and manganese (Mn) (Costa-Silva et al., 2018; Zizza et al., 2017).

In this line, previous studies have shown a link between MZ pro-oxidant effects and toxicity (Negga et al., 2011; Srivastava et al., 2012). In addition, alterations in biomarkers of oxidative stress in adult fish exposed to this compound have been demonstrated (Atamaniuk et al., 2013; Kubrak et al., 2012; Zizza et al., 2017), pointing to oxidative stress as one of the main mechanism involved in deleterious effects induced by MZ in organisms. Specifically, oxidative stress induced by MZ in fish has been linked to Mn-derived reactive oxygen species (ROS) formation mechanisms (Costa-Silva et al., 2018; Dolci et al., 2014; Gabriel et al., 2013; Vieira et al., 2012). However, the relationship between redox alterations caused by MZ and its toxicity during fish embryo development remains poorly explored.

Alternative experimental models have been commonly used to understand the mechanism associated with the embryotoxicity induced by contaminants, among them, zebrafish (*Danio rerio*) in the embryo-larval stage is highlighted (McCollum et al., 2011). Zebrafish embryos have advantages such as sensitivity to environmental contaminants absorbed quickly by immersion (Dai et al., 2014). In addition, the embryo transparency and rapid development enable to observe morphological endpoints as quickly as the first 24 h post-fertilization (hpf) (Garcia et al., 2016). Such characteristics lead zebrafish to be a promising experimental model for the evaluation of the effects of exposure to different environmental contaminants toward the embryonic development (Bambino and Chu, 2017; Truong et al., 2011).

In the present study, we used zebrafish to assess the potential embryotoxic effects induced by MZ as well as the role of reactive oxygen species (ROS) in this process by pre-treatment with a classical antioxidant (*N*-acetylcysteine, NAC).

2. Materials and methods

2.1. Chemicals

N-acetylcysteine, Mancozeb (97.5%), 2,7-dichlorofluorescein diacetate (DCFH$_2$-DA), *N*,*N*′,*N*′-Tetramethylacridine-3,6-diamine (Acridine Orange) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

2.2. Zebrafish maintenance and reproduction

Adult *Danio rerio* (wild-type) were kept in a dedicated Zebtec® system under appropriate water conditions (pH 7.2, 400 μS conductivity, 28 °C temperature), with a 14 h/10 h photoperiod and fed on commercial flocked fish food supplemented with *Artemia* sp., as established elsewhere (Westerfield, 2000). The experimental protocols used in this work were approved by local Ethics committee (CEUA – Unipampa: protocol 003-2016). The zebrafish embryos used in the present work were obtained from the reproduction of adult specimens and maintained in system water in a B.O.D. (Biochemical Oxygen Demand) incubator at 28 °C.

2.3. Treatments

Briefly, Fig. 1 shows the experimental timeline of embryonic exposure and subsequent experiments.

2.3.1. Exposure of zebrafish embryos to MZ

Toxicological assays were based on the OECD GUIDELINES FOR THE TESTING OF CHEMICALS 236 - Fish Embryo Acute Toxicity (FET) Test, 2013 (OECD, 2013), with some modifications. A number of 50 zebrafish embryos at 5 h post-fertilization (hpf) were exposed to different concentrations of MZ (1.88; 2.81; 3.75 μM) in Petri dishes in a final volume of 30 mL. MZ concentrations were based on a previous study (Tilton et al., 2006). Embryos were kept in B.O.D. throughout the treatment period.

2.3.2. Pre-treatment of zebrafish embryos with NAC

In order to determine the involvement of ROS during the MZ-induced toxicity to zebrafish embryos, a pre-treatment with classical antioxidant *N*-acetylcysteine (NAC) was performed at different concentrations (100, 200, 300, 500, 750 μM), based on a previous study (Krishnan, 2013). In brief, 50 zebrafish embryos at 4 h post-fertilization (hpf) were exposed for 1 h to NAC and subsequently exposed to MZ in a final volume of 30 mL in Petri dishes. Dilutions of the reagents were prepared in system water and embryos were kept in BOD throughout the treatment period.

2.4. Mortality, hatching rate, and morphology

Following the treatments, embryos mortality was evaluated at 24 hpf time point. Embryos viability was analyzed by observation of eggs coagulation and absence of the heartbeat in a stereomicroscope. Morphology of the dechorionated embryos was analyzed at 48 hpf. Parameters such as body length and body axis distortion were evaluated. Hatching rate was assessed at 72 hpf. All parameters described here were assessed according to previously published protocols (Westerfield, 2000). Images were obtained in a Carl Zeiss Stemi 2000-C stereomicroscope coupled to a Moticam 2000 digital camera, zoom 1.25× and were analyzed using Image J. software.

2.5. Heart rate

The heart rate of embryos was measured at 48 hpf time point. Following treatments with MZ and/or NAC, 15 embryos of each group were acclimatized for 2 min in Petri dishes containing system water. The heart beats were counted with the aid of a Carl Zeiss Stemi 2000-C stereomicroscope coupled to a Moticam 2000 digital camera, zoom 1.25×, for 1 min, according to Xia et al. (2017).

2.6. Spontaneous movement

Spontaneous movement of zebrafish embryos was assessed in a stereomicroscope Carl Zeiss Stemi 2000-C coupled to a Moticam 2000 digital camera (1.6× zoom) by counting the number of total body axis movements performed by embryos inside their chorions (Xia et al., 2017). Following treatments, 15 embryos from each group were randomly selected for the test (a total number of 75 embryos per group). The counting was conducted at 28 hpf. Embryos were allowed to adapt for 2 min in Petri dishes containing system water and their spontaneous movement was counted for 1 min.

2.7. Touch stimulation and swimming response

Dechorionated embryos at 48 hpf were submitted to the behavioral touch response test (Saint-Amant and Drapeau, 1998). At this stage of development, the embryos remain inert in the environment, responding only to stimuli, such as touch. Briefly, one embryo at a time was gently placed in the center of a Petri dish containing 20 mL of system water and acclimatized for 2 min and then it was touched with aid of an entomologic forceps (stimulus), and then the number of stimuli needed for the first displacement. It was also determined whether the individual is capable of performing a standard escape (swim response). These behaviors were assessed by two blind operators (30 embryos per group).

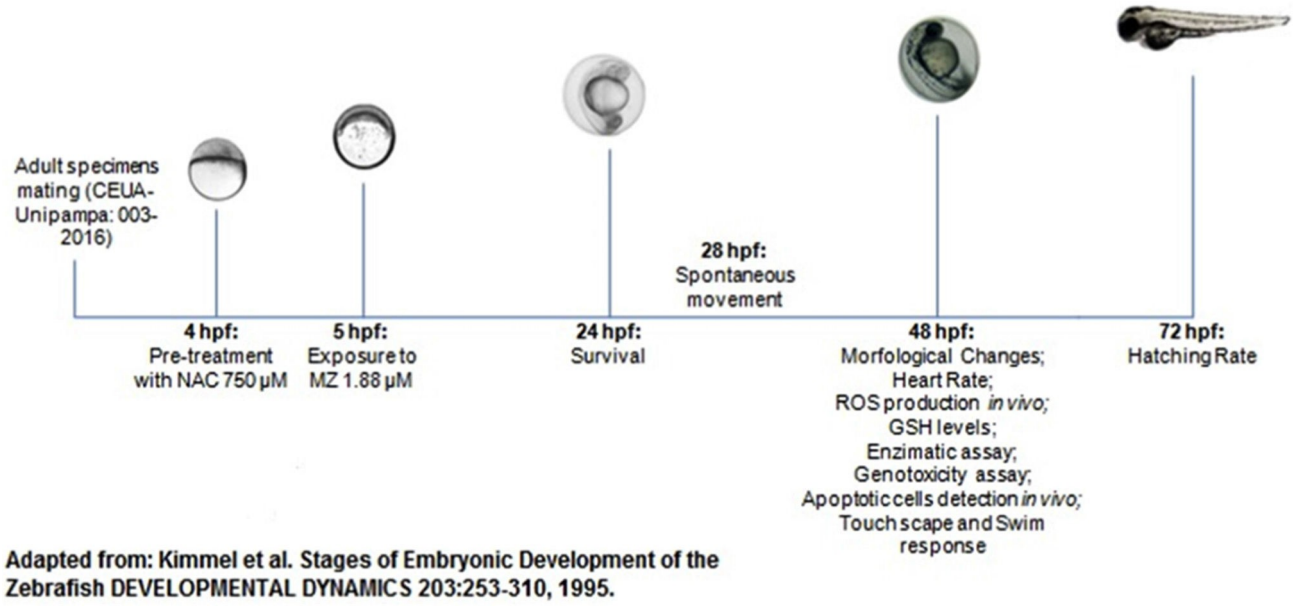

Fig. 1. Timeline of embryonic exposure and subsequent experiments.

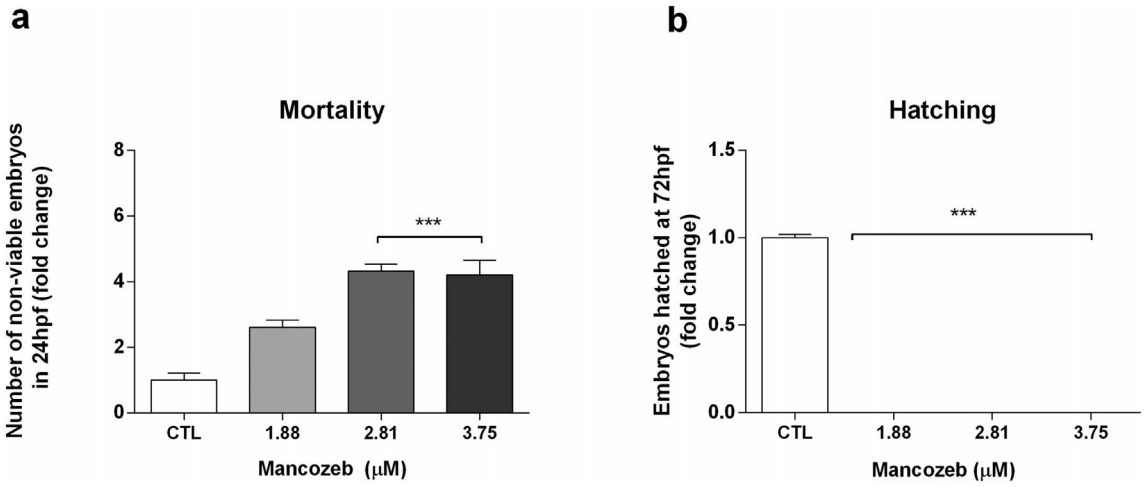

Fig. 2. Evaluation of viability and hatching of embryos treated with different concentrations of MZ. (a) The number of non-viable embryos exposed to different concentrations of fungicide MZ after 24 hpf; (b) the number of embryos hatched after 72 hpf of exposure to MZ. Data are expressed as mean ± SEM (fold change) from 200 embryos for each group and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test ***$p < 0.001$; **$p < 0.01$.

2.8. Determination of ROS generation in vivo

For the assessment of reactive oxygen species (ROS), procedures previously established were used (Anichtchik et al., 2008) with minor modifications. Briefly, embryos at 48 hpf were dechorionated and placed into 96-well plate (5 embryos per well) at a final volume 250 µL of system water. Then embryos were incubated with the fluorescent dye 2,7-dichlorofluorescein diacetate ($DCFH_2$-DA), at the final concentration of 1 µg/mL for 1 h in dark and the fluorescence intensity was measured on a microplate reader (PerkinElmer Enspire 2300) with excitation and emission spectra of 495 nm and 529 nm respectively. Changes in $DCFH_2$-DA fluorescence were also assessed in whole embryos. Embryos were mounted on microscope slides, fixed with 1.5% methylcellulose and imaged using fluorescence microscopy (Olympus 1x71) at 40× magnification, with an exposure of 500 ms, in the green light (Olympus U-RFL-T UV light). Representative images were acquired with aid of dedicated software (Q- Capture).

2.9. Determination of thiols levels

For this assay 10 embryos were used per concentration (n = 4) with 48 hpf, homogenized in 375 µL of Na + EDTA Phosphate Buffer (100 mM + 5 mM, pH 8.0) in the powerlyzer for 30 s at 2000 vibrations. 125 µL of 25% Phosphoric Acid was added to the samples and centrifuged in ultra-centrifuge for 30 min at 4 °C and 100,000*g*. To determine the thiols levels, 10 µL of the supernatant was withdrawn, 180 µL of sodium phosphate buffer, 10 µL of O-phthalaldehyde (OPT) (1 mg/mL) was added in a 96-well plate, incubated for 15 min in the dark and reading in fluorimeter at the excitation/emission wavelengths, 350/420 nm, respectively, in according to Hissin and Hilf (1976).

2.10. Preparation of the samples for enzymatic assays

For the biochemical measurements, 30 whole embryos (n = 90) at 48 hpf homogenized in 200 µL of 20 mM HEPES buffer pH 7.0 on the powerlyzer for 30 s at 2000 rpm. Subsequently, the samples were centrifuged for 30 min at 4 °C and 20,000*g* and the supernatant stored at

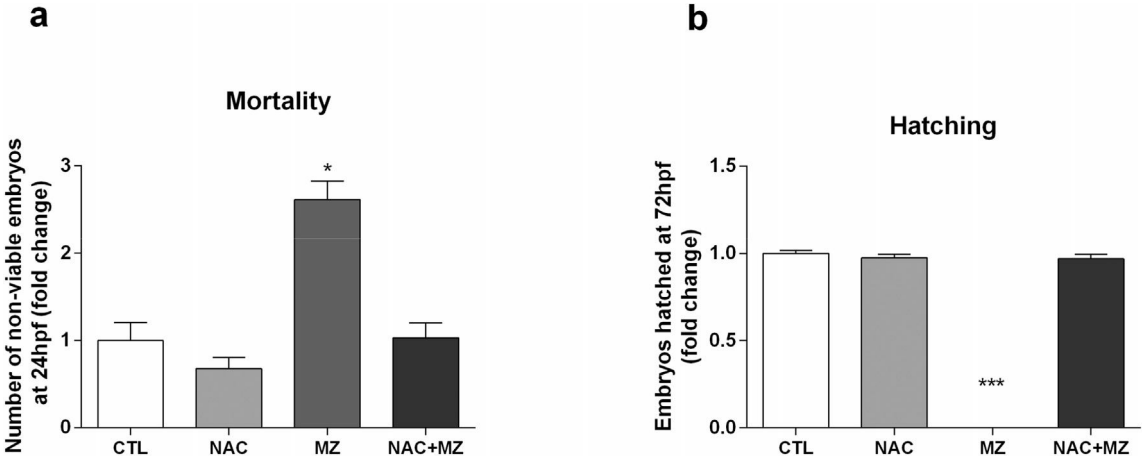

Fig. 3. Evaluation of viability and hatching of embryos treated with MZ and pre-treated with NAC. (a) The number of non-viable embryos exposed to 1.88 μM of MZ and pre-treated with NAC 750 μM after 24 hpf; (b) the number of embryos hatched after 72 hpf of exposure to MZ and NAC + MZ. Data are expressed as mean ± SEM (fold change) from 200 embryos for each group and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test ***$p < 0.001$; *$p < 0.05$.

−20 °C for further analysis.

2.10.1. Glutathione S-transferase (GST) assay

The GST activity was done on homogenate supernatant as described by Habig and Jakoby (1981), using 1-chloro-2.4-dinitrobenzene (CDNB) as a substrate primer, which is suitable for the broadest range of GST isozymes. In addition, the medium also contains phosphate buffer (0.0025 M EDTA, 0.25 M K_2HPO4, 0.25 M KH_2PO4, pH 7.0) and GSH 100 mM. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is a linear increase in the absorbance at 340 nm, it may be read for 2 min at 30 °C. One unit of GST activity is defined as the amount of enzyme producing 1.0 μmol of GS-DNB conjugate/min under the conditions of the assay ($\varepsilon = 9.60\ M^{-1}\ cm^{-1}$).

2.10.2. Glutathione peroxidase (Gpx) assay

GPx activity was determined spectrophotometrically at 340 nm by NADPH consumption for 2 min, at 25 °C, at pH 7.0 (Wendel, 1981). The homogenate supernatant was added to medium containing phosphate buffer (0,0025 M EDTA, 0.25 M K_2HPO4, 0.25 M KH_2PO4, pH 7.0), 100 mM GSH, 100 mM NADPH, 5 U/mL GR. So, the reaction was initiated by adding the tBOOH a final concentration of 10 mM. One unit of GPx will consume 1.0 μmol of $NADP^+$ from NADPH per minute ($\varepsilon = 6.22\ M^{-1}\ cm^{-1}$).

2.11. Genotoxicity

2.11.1. Comet assay

Genotoxicity will be assessed by breaks in the double helix of deoxyribonucleic acid through the comet test according to Kosmehl et al. (2008) with some modifications. To this end, slides containing 150 μL of 1% agarose (diluted in PBS) were prepared and placed at 40 °C for drying. Then, 20 embryos per group were homogenized manually with a pestle for 5 min with 1 ml of Dulbecco's Modified Eagle Medium (DMEN) plug, centrifuged for 10 min at 3000 rpm, for further removal of the supernatant and resuspension of the pellet in 1% PBS buffer. To complete the slide preparation, 10 μL of sample were placed in 90 μL of 0.75% low melting agarose, and added to the coverslip, to spread the contents. The slides were placed at 4 °C for 15 min and then added in a lysis solution containing 100 mM EDTA, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO (pH 13.0) in the dark at 4 °C overnight. After this time the slides were immersed in a neutralizing solution containing 400 mM Tris for 30 min. For the unwinding of the DNA, the slides were immersed for 30 min in a horizontal electrophoresis tank containing an alkaline buffer (12 g/L NaOH and 0.37 g/L EDTA) at 25 V and 300 mA. After the electrophoresis run, the slides were washed in distilled water, fixed with 70% alcohol for 5 min, and placed in the refrigerator for 1 h. Then the slides were stained with Sybr Green for 5 min. All slides were analyzed by fluorescence microscopy (Olympus 1x71) at 100× magnification, with an exposure of 1044.7 ms, in the green light (Olympus U-RFL-T UV light) and an image analysis system (Q- Capture). The obtained images were analyzed by ImageJ/Open Comet image analysis software.

2.12. Determination of cell death

2.12.1. Acridine Orange in vivo

For this assay were used embryos with 48 hpf dechorionated. Acridine Orange fluorescent dye was used for the detection of cells in the process of apoptosis. Five embryos were placed in a 2 mL microtube containing system water and the reagent at the final concentration of 5 μg/mL and then incubated in the dark for 30 min (Tilton et al., 2008). After, which the embryos were washed three times in system water, then fixed in slides for microscopy with 1.5% methylcellulose and images obtained by fluorescence microscopy (Olympus 1x71) at 40× magnification, with an exposure of 500 ms, in the green light (Olympus U-RFL-T UV light) and an image analysis system (Q- Capture). The images obtained were analyzed by ImageJ software; the total tail area was measured.

2.13. Total protein quantification

Protein content was determined using bovine serum albumin (BSA) as standard, in according to Bradford (1976).

2.14. Statistical analysis

Normality tests (Kolmogorov Smirnov) and homogeneity (Bartlett's test) was applied. The results were expressed as mean ± standard error (S.E.M.), for the nonparametric data the analysis was done through the Kruskal-Wallis test and Dunn's post-test. The parametric data were analyzed by the One-Way (ANOVA) and Tukey post-test, considering the significant results when $p \leq 0.05$.

1. Results

1.1. MZ exposure increases mortality and impairs embryos hatching

Embryos subjected to MZ presented altered development and compromised mortality and hatching rates at all concentrations tested (1.88; 2.81; 3.75 μM) (Fig. 2a and b, respectively) ($p < 0.001$).

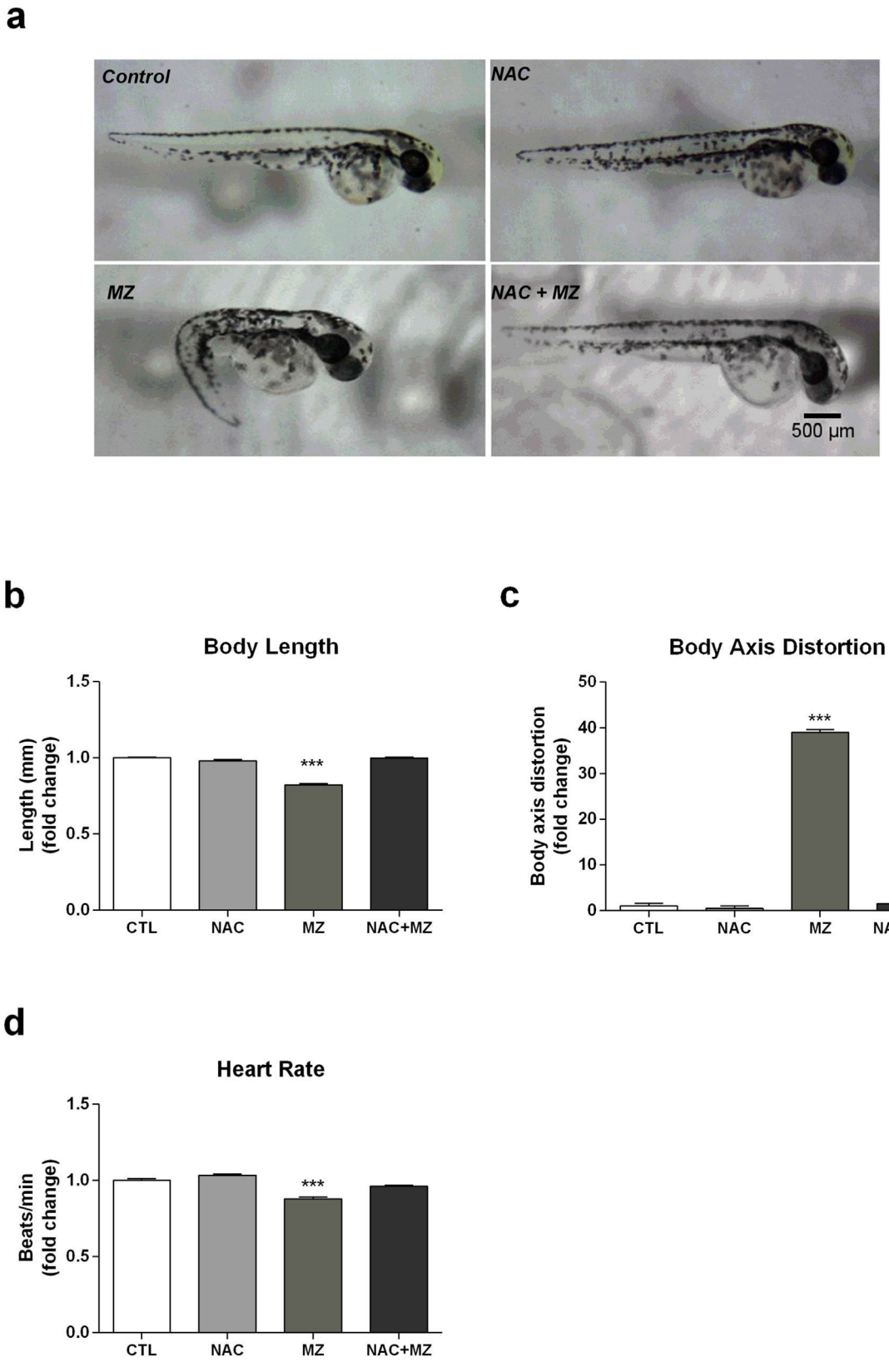

Fig. 4. Changes in embryos development and heartbeat evaluation. (a) Representative morphological alterations in embryos exposed to MZ and NAC + MZ at 48 hpf. Scale bar: 500 µm; (b) mean of body length; (c) body axis distortion; (d) heartbeat rate. All data are expressed as mean ± SEM (fold change) from 30 embryos analyzed individually for each group and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test ***$p < 0.001$.

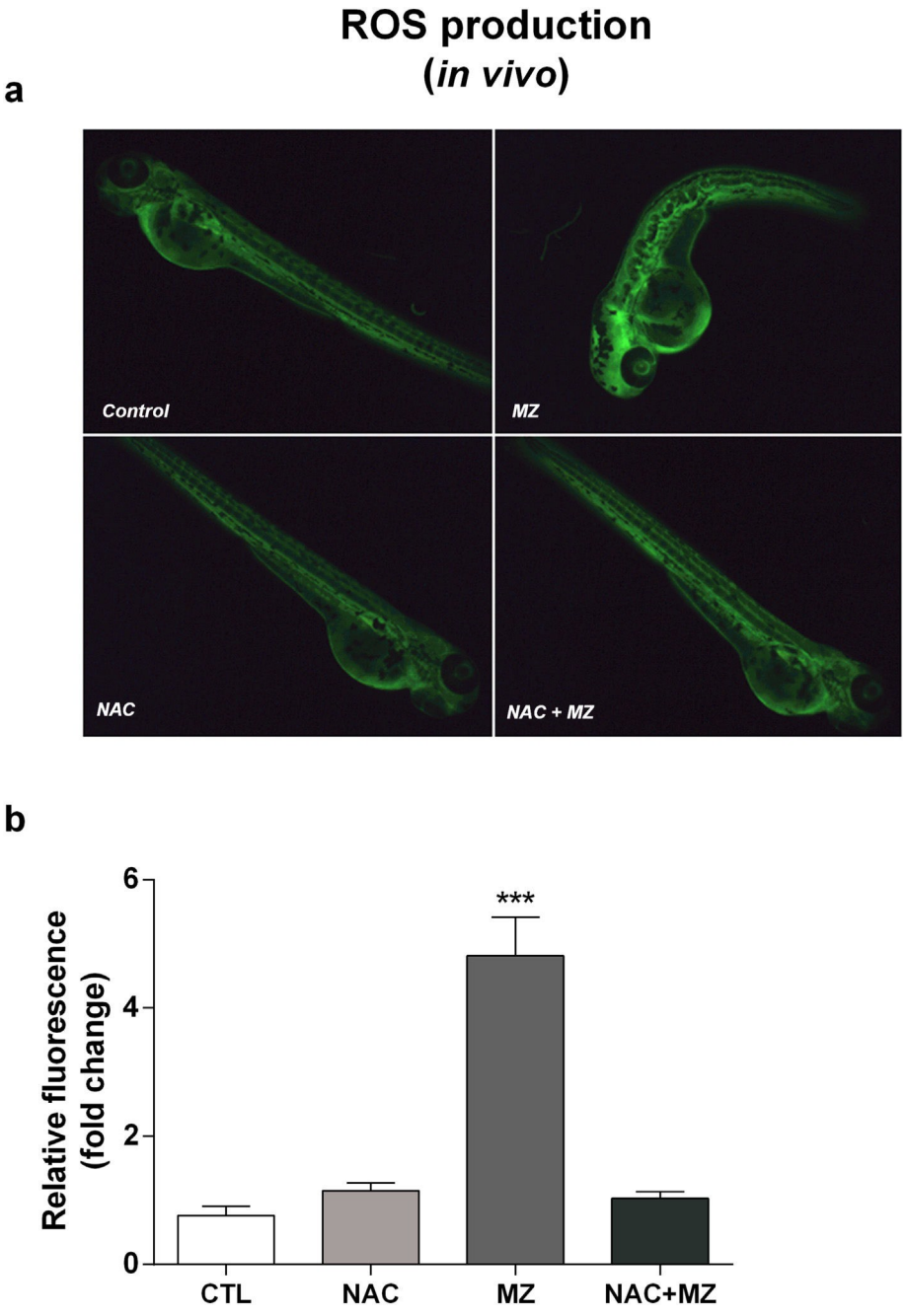

Fig. 5. Changes in ROS levels. (a) Representative *in vivo* DCF fluorescence image; (b) quantification of DCF relative fluorescence. Data are expressed as mean ± SEM (fold change) from 30 embryos analyzed individually for each group and analyzed by one-way ANOVA followed by Tukey's post-hoc ***$p < 0.001$.

Regarding the pre-treatment with NAC, different concentrations were tested in pilot experiments, based on previous literature (Krishnan, 2013). The concentration of NAC 750 μM was able to significantly block the deleterious effects of MZ. As observed, NAC 750 μM completely blocked MZ (1.88 μM) induced mortality ($p < 0.05$) and hatching alterations ($p < 0.001$) (Fig. 3a and b). Therefore, the concentrations of MZ 1.88 μM and NAC 750 μM were chosen for subsequent experiments.

1.2. *NAC prevents MZ-induced morphological and physiological abnormalities*

Exposure of zebrafish embryos to MZ 1.88 μM resulted in significant ($p < 0.001$) morphological and physiological changes, such as body axis distortion (38-fold), reduction of body length (0.18-fold), along with a decreased heart rate (0.13-fold) as compared to the control group. However, the pre-treatment with NAC was able to block all changes induced by MZ1.88 μM; as by reducing the number of embryos with body axis distortion, increasing body length (0.18-fold) and attenuating (0.8-fold) the effect on embryos heartbeat (Fig. 4).

1.3. *Oxidative parameters altered by MZ are attenuated by NAC*

MZ 1.88 μM exposure promoted a significant ($p < 0.001$) increase of ROS generation (4.05-fold) comparing to control; on the other hand, NAC pre-treatment completely blocked this condition, by reducing MZ-induced ROS to control levels, (Fig. 5a and b). In addition, MZ reduced ($p < 0.05$) the thiols levels (0.39-fold) as compared to control. Nevertheless, NAC pre-treatment prevented the decrease of thiols levels caused by MZ (Fig. 6a). NAC alone had no effect on thiols levels at the time point analyzed (48 hpf). The activities of GST and GPX did not

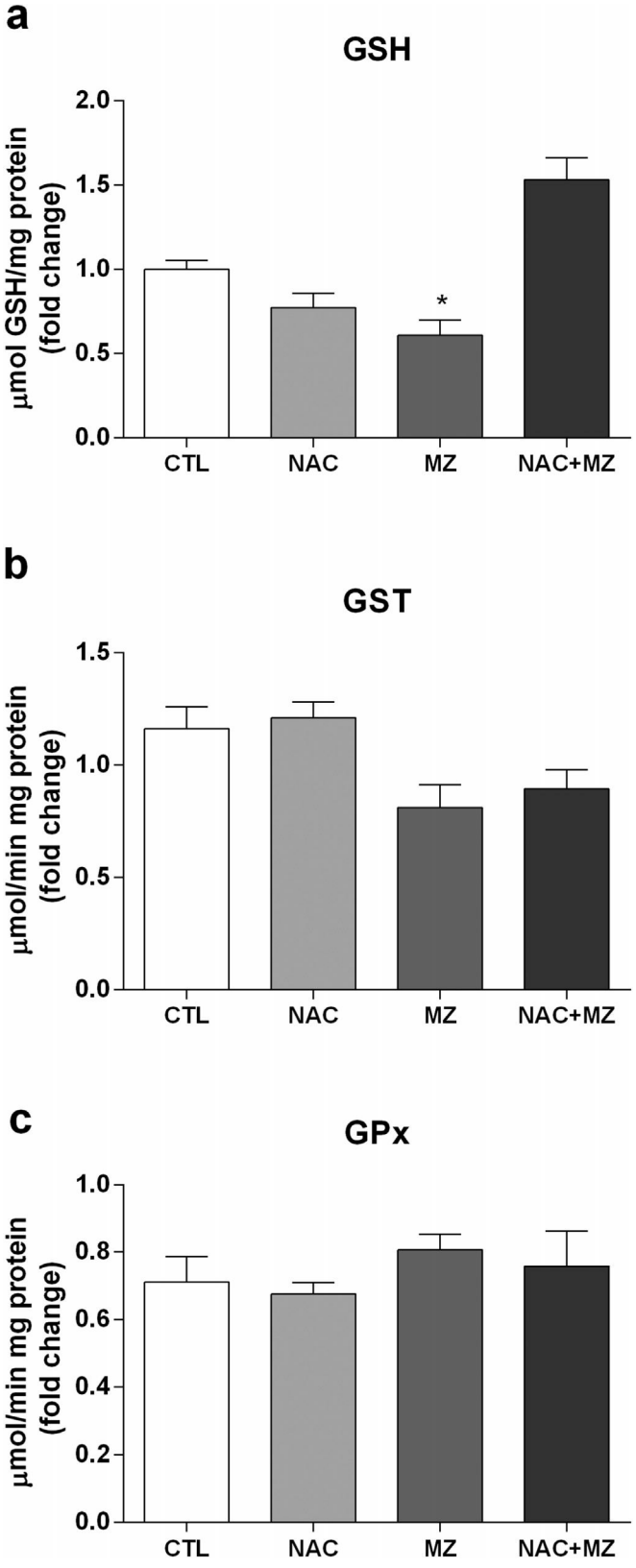

Fig. 6. Evaluation of thiols levels and enzymatic biomarkers. (a) Levels of thiols in embryos at 48 hpf. The result was expressed by µmolSH/mg protein from 10 embryos for each group in three independent experiments. Data expressed as mean ± SEM (fold change) and analyzed by one-way ANOVA followed by Tukey's post-hoc (*$p < 0.05$ compared to control); (b) GST activity at 48 hpf embryos, expressed by µmol/min mg protein from 30 embryos for each group in at least three independent experiments. Data expressed as mean ± SEM (fold change) and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test; (c) GPx activity at 48 hpf embryos, expressed by µmol/min mg protein from 30 embryos individually for each group in at least three independent tests. Data expressed as mean ± SEM (fold change) and analyzed by one-way ANOVA followed by Tukey's post-hoc.

change significantly in any groups analyzed when comparing to control. (Fig. 6b and c).

1.4. NAC prevents DNA fragmentation and cell death induced by MZ

DNA damage and apoptosis induced by MZ exposure was evaluated through the Comet assay and Acridine Orange (AO) test, respectively. A significant ($p < 0.001$) increase in comet length (0.30-fold) and tail length (0.24-fold), comparing to control, was observed in embryos exposed to MZ 1.88 µM (Fig. 7). Corroborating that, there was a significant increase ($p < 0.05$) (0.80-fold) in apoptotic cells stained with AO (Fig. 8). NAC pre-treatment completely blocked DNA fragmentation assessed as comet length and tail length parameters (Fig. 7a, b and c) and the number of apoptotic cells (Fig. 8a and b).

1.5. NAC prevents MZ-induced impairments on behavioral parameters

Embryos exposed to MZ1.88 µM presented a significant ($p < 0.001$) increase (1.08-fold) on the spontaneous movement, determined at 28 hpf; however, NAC pre-treatment restored the number of bends to control levels (Fig. 9a). The response to a mechanical stimulus (touch) was used as a measure of sensory-motor integration at 48 hpf. MZ exposed embryos responded to a tail touch with wave-like contractions of the body axis but were incapable of swimming ($p < 0.001$; $p < 0.01$, respectively), however, this impairment on motor behavior was significantly diminished (0.57-fold) and the swimming capacity was re-establish to the control levels in embryos pre-treated with NAC (Fig. 9b and c).

2. Discussion

Agrochemicals are used to improve crops productivity by controlling agricultural pests and consequently avoiding economic losses. However, excessive use and decomposition of agrochemicals into toxic by-products has adverse consequences to the environment and non-target organisms (Srivastava and Singh, 2013; Tilton et al., 2006). In this context, the fungicide MZ has raised concerns regarding its harmful effects, in special for aquatic organisms (Harris et al., 2000; Shenoy et al., 2009; Srivastava and Singh, 2014). In this study, we investigated the potential embryotoxic effects of MZ in zebrafish and the relationship between redox unbalance and developmental impairments resulted from exposure to this fungicide.

Embryogenesis is a multifactorial process and is closely related to bioenergetics, cellular signaling and redox homeostasis (Cole and Ross, 2001; Félix et al., 2016). ROS can act as signaling molecules at the cross-link of proliferation, differentiation, and apoptosis (Sauer et al., 2001). For instance, organisms in the embryonic phase are more susceptible to oxidative stress which makes them particularly susceptible to redox active pollutants (Pašková et al., 2011). Nevertheless, an overproduction of ROS can disrupt embryogenesis, thus causing morphological changes and affect the survival of exposed organisms (Dennery, 2007; McCollum et al., 2011).

We have shown here that the notochord axis distortion was a major

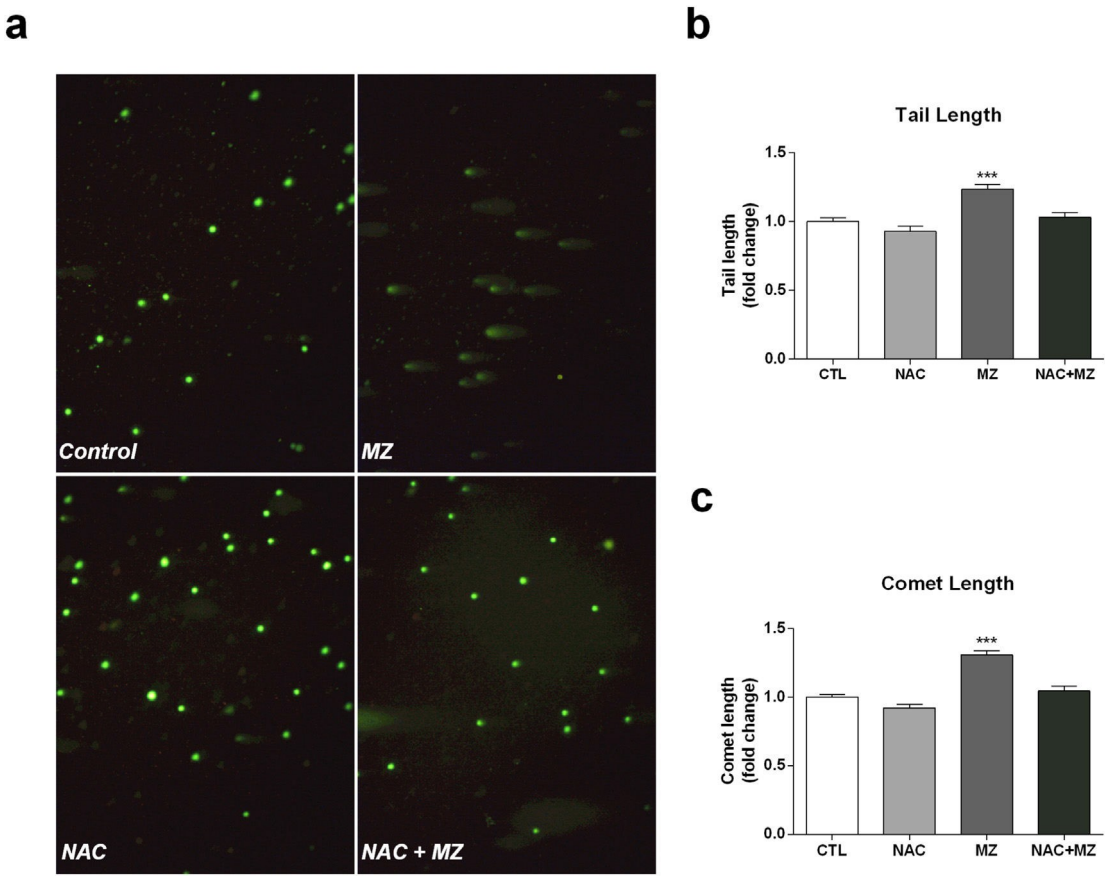

Fig. 7. Determination of genotoxicity. (a) Representative images of single cell gel electrophoresis (comet assay) of embryos at 48 hpf (control, NAC, MZ, NAC + MZ); (b) tail length; (c) comet length. Data were expressed in microns as the average (fold change) of 100 different cells and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test ***$p < 0.001$.

morphological abnormality in embryos under MZ exposure, thus, this result corroborates other works demonstrating that compounds belonging to the dithiocarbamates (DTCs) promoted morphological alterations during the development of zebrafish embryos (Haendel et al., 2004; Tilton et al., 2006; van Boxtel et al., 2010). In addition, MZ impaired the survival and hatching of exposed embryos, as well as delayed body length development and also affected physiological cardiac parameters, observed by decreases of heartbeat rates. According to Hallare et al. (2005), embryos hatching, a process that occurs 72 hpf, is related to body movements in order to break the chorion. The inability to break the egg envelope may be associated with the loss of muscle capacity due to abnormal body formation (Yamagami, 1988). In contrast, NAC was able to prevent morphological alterations, impairments on survival and hatching, and blocked the cardiac effect induced by MZ exposure. Along these lines, the hypothesis of the involvement of oxidative stress in embryotoxicity induced by MZ was reinforced and the antioxidant potential of NAC was emphasized.

In parallel to increasing ROS generation, DTCs, such as MZ, have increased potential for sulfhydryl (SH) groups oxidation, especially cellular GSH pools, thus, altering the redox state of cells (Burkitt et al., 1998; Tilton et al., 2008). On the other hand, alterations in GSH levels are frequently taken as a biomarker of redox unbalance and this can in turn promote the accumulation of ROS (Franco and Cidlowski, 2012). Exposure to MZ reduced thiols levels as compared to control and caused a disturbance in ROS steady-state levels. The pre-treatment with NAC was able to maintain thiols levels, which we assume had a positive effect on GSH pools, a major low molecular thiol which is indispensable for regulation of cell proliferation and transcription in the embryonic stage of vertebrates (Sant et al., 2017; Timme-Laragy et al., 2013), as well as to attenuate the ROS production. Previous studies have shown that NAC could increase the biosynthesis and recycling of GSH (Elbini Dhouib et al., 2016; Peña-Llopis et al., 2003), so, maintaining redox homeostasis, which plays an important role in the elucidation of MZ-induced embryonic impairments.

Our results also demonstrated that treatment with MZ induced genotoxicity determined through the biomarkers of cell death and DNA damage. DTCs have a multifactorial mode of action, one of them is the depletion of GSH, which could be linked to the activation of cell death pathways (Franco and Cidlowski, 2012). Another alternative mechanism of toxicity induced by MZ in zebrafish embryos could be closely related to manganese, the main inorganic metal compound of this fungicide. Literature has shown that this metal could promote the activation of key proteins involved in apoptosis signaling (Altenhofen et al., 2017; Bakthavatsalam et al., 2014). In addition, the major metabolite of EBDCs, ethylenethiourea (ETU), have been demonstrated to exert deleterious effects in other organisms (Belpoggi et al., 2006; Srivastava and Singh, 2013). However, MZ toxicity mechanism may be a complex association of binding between both degradation metabolite and inorganic portion of the compound (Domico et al., 2007; Li et al., 2013).

Locomotor behaviors carry out an important role in activities during zebrafish development, such as hatching, scape behavior, feeding, social, and defensive activities (Colwill and Creton, 2011). The transparency of the zebrafish embryo permits a real-time visualization of organogenesis *in vivo*. In addition, endpoints assessed during embryonic and larval stages of development usually offer an interesting platform to elucidate neurobehavioral tests (Bailey et al., 2013). The embryonic behaviors appear in a sequence of time-point and include an early, transient period of spontaneous movement, followed by responses to adverse stimuli, and capacity swimming (Brustein et al., 2003).

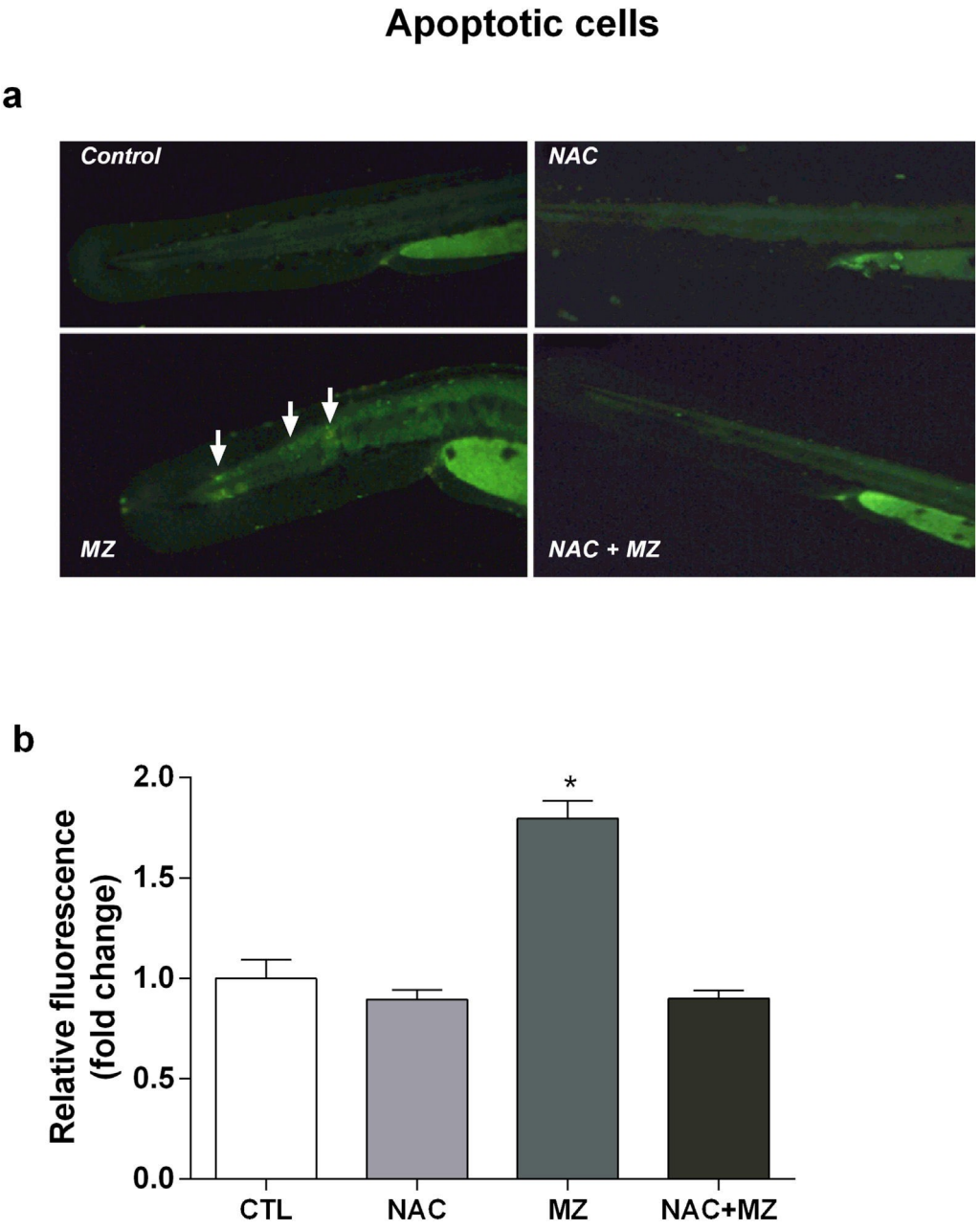

Fig. 8. Determination of cell death. (a) Apoptotic cells were determined in the tail of embryos at 48 hpf indicated by the white arrows; (b) quantification of Acridine Orange relative fluorescence. Data are expressed as mean ± SEM (fold change) from 30 embryos analyzed individually for each group and analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test *p < 0.05.

Spontaneous movement is the first motion realized by the embryo around 27 hpf and is originated from the spinal cord triggered by the development of motor neurons innervating muscles (Saint-Amant and Drapeau, 1998). This behavior has been used as a toxicological tool, to assessing toxic mechanisms of xenobiotics (Xia et al., 2017).

Our results revealed for the first time that, there was an increase in the number of spontaneous movements performed by the embryos exposed to MZ. A possible mechanism for this sensorimotor dysfunction could be related to dopamine-mediated neurotransmission, which is vulnerable to Mancozeb (Domico et al., 2007). This hypothesis can be reinforced by previous studies demonstrate a positive correlation with EBDCs, which preferentially targets dopaminergic neurons (Domico et al., 2007). Other hypothesis related MZ-induced toxicity may compromise the embryonic sensory response system to photoreceptor stimuli, causing possible hyperactivity in the exposed embryos, which may also be linked to neurotoxicity induced by the metal composition of MZ (Centonze et al., 2001; Krishna et al., 2014; Ternes et al., 2014; Tran et al., 2002). Furthermore, continued exposure to MZ caused a decrease of responsiveness to adverse stimuli. According to our results, this effect could be related to cell death in the tail region, data also showed in this study. As we know, from the possible involvement of oxidative stress in the MZ-induced toxicity, we were able to demonstrate, through the results obtained with the pre-treatment with NAC, the capacity to reduce the abnormal swimming of embryos. Thus, measurements of behavioral dysfunction are a sensitive early marker of fungicide toxicity that could be explored to examine further mechanisms of neural damage and possible therapeutic interventions by antioxidants.

In conclusion, it has become clear in the present study that MZ is a potent pro-oxidant agent capable to induce embryotoxicity in zebrafish

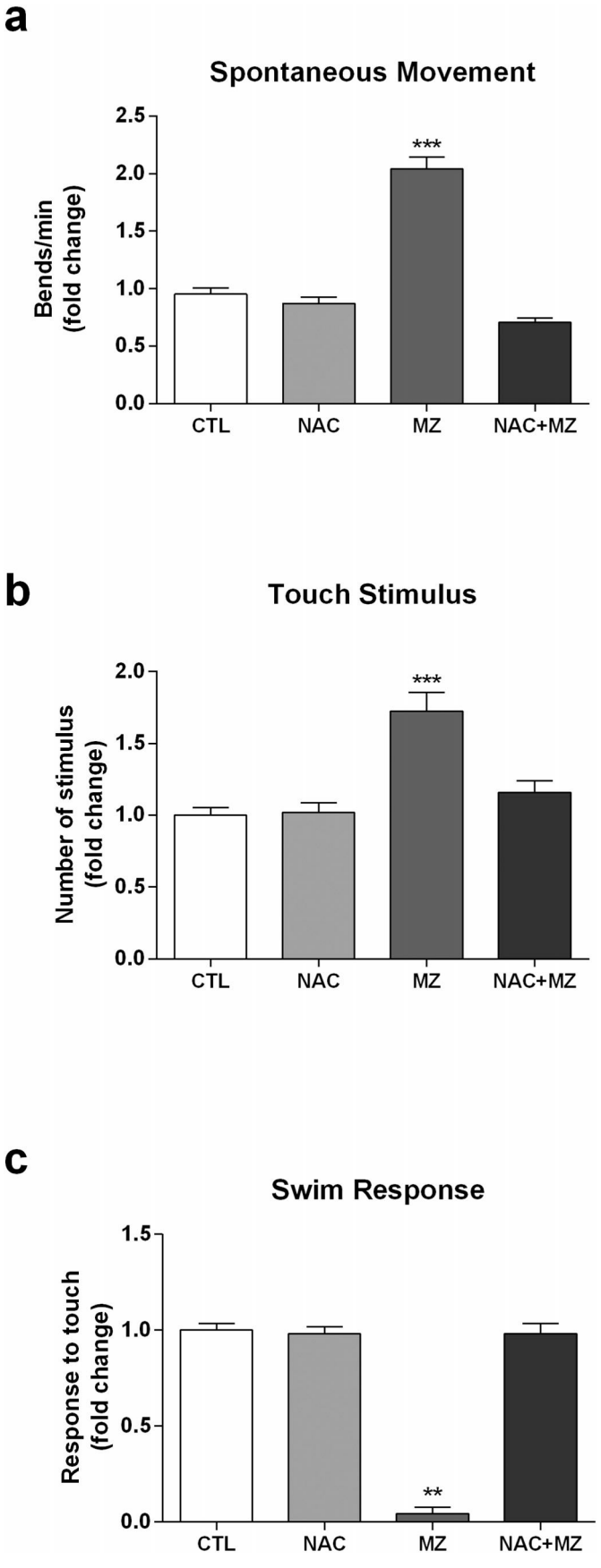

Fig. 9. Analyses of spontaneous movement and sensory-motor capacity. (a) Spontaneous movement of embryos at 28 hpf, expressed by bends/min; (b) touch stimulus, expressed as the number of stimuli necessary to first swim; (c) swim response, expressed as normal swim pattern capacity to escape to the stimuli. For all these data, 50 embryos were used individually for each group and results are expressed as mean ± SEM (fold change), statistically analyzed by Kruskal-Wallis and Dunn's Multiple Comparison Test ***$p < 0.001$; **$p < 0.01$.

via enhanced ROS generation. Considering the fact that the antioxidant NAC was able to exert a protective effect on the parameters evaluated, the hypothesis on the involvement of oxidative stress as one major mechanism related to MZ embryotoxicity is reinforced, emphasizing the key role of redox balance in the embryonic development of aquatic organisms.

Conflict of interest

None.

Transparency document

The Transparency document associated with article can be found, in online version.

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5 CONSIDERAÇÕES FINAIS E CONCLUSÕES

No capítulo I desta tese, foi possível verificar um aumento significativo nos níveis de manganês no cérebro de carpas, dessa forma, sugerindo a possível contribuição desse metal no distúrbio redox induzido pela exposição ao Mz em peixes.

Foi também verificado que a exposição a concentrações sub-letais do fungicida não induziu danos oxidativos, apesar dos níveis de espécies reativas estarem significativamente elevados. Embora, seja sugerido o efeito pró-oxidante do composto Mz, os mecanismos bioquímicos e moleculares envolvidos neste processo ainda não foram elucidados. Neste capítulo da tese, mostramos pela primeira vez a ativação do fator de transcrição Nrf2 no cérebro de carpas, no qual, é considerado elemento-chave na detoxificação de EROS, indicando este fenômeno como um importante mecanismo de adaptação frente aos efeitos neurotóxicos induzidos pelo fungicida em peixes. Tal fato pode ser reforçado pelo observado aumento da atividade enzimática de GST, GPx e GR, que são enzimas antioxidantes fundamentais nos processos de detoxificação de xenobióticos e na defesa celular contra danos oxidativos.

No capítulo II dessa tese, foi proposto um modelo para o estudo da embriotoxicidade e a possível relação dessa toxicidade ao efeito pró-oxidante exercido pelo fungicida Mz, pois ainda são escassos estudos sobre o comprometimento do desenvolvimento embrionário de peixes frente à exposição ao Mz.

Foi possível observar que a exposição de embriões de peixe-zebra ao Mz resultou em uma série de alterações em parâmetros morfológicos, fisiológicos, comportamentais e bioquímicos que são considerados chave no processo do desenvolvimento embrionário dessa espécie, indicando o grande potencial embriotóxico deste fungicida.

Como hipótese central deste trabalho, questionamos sobre o possível envolvimento do equilíbrio redox durante a embriotoxicidade induzida por Mz em peixe-zebra. Para colocar em prova esta hipótese, realizamos uma série de experimentos nos quais embriões de peixe-zebra foram pré-tratados com NAC, um poderoso antioxidante, e em seguida os embriões foram expostos ao fungicida. Como resultado, observamos que a pré-exposição à NAC bloqueou todos os parâmetros morfológicos, fisiológicos, comportamentais e bioquímicos alterados pela exposição ao Mz, confirmando assim o importante papel do estresse oxidativo na embriotoxicidade induzida pelo fungicida em organismos não-alvo, bem como o importante papel do equilíbrio redox no desenvolvimento embrionário normal de peixe-zebra.

Por fim, os dois capítulos se complementam na elucidação de mecanismos de toxicidade dentro dos modelos propostos, dessa forma, possibilitando reforçar a hipótese central do desenvolvimento dessa tese, sobre o efeito pró-oxidante do fungicida Mz, tanto na neurotoxicidade (capítulo I), assim como, na embriotoxidade (capítulo II).

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