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MARINA MARQUES BONOMO

AVALIAÇÃO ECOTOXICOLÓGICA DE UM NOVO POTENCIAL FORMICIDA: ANÁLISES *IN VITRO* E *IN VIVO*

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos, como requisito parcial para obtenção do título de Doutor em Ciências.

Orientador: Profª Dra. Marisa Narciso Fernandes

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Folha de Aprovação

Assinaturas dos membros da comissão examinadora que avaliou e aprovou a Defesa de Tese de Doutorado da candidata Marina Marques Bonomo, realizada em 22/06/2018:

Profa. Dra. Marisa Narciso Fernandes
UFSCar

Profa. Dra. Heloisa Sobreiro Selistre de Araujo
UFSCar

Profa. Dra. Rose Maria Carlos
UFSCar

Profa. Dra. Maria Aparecida Marin Morales
UNESP

Profa. Dra. Adrislaine da Silva Mansano Dornfeld
USP

A alegria não chega apenas no encontro do achado, mas faz parte do processo da busca. E ensinar e aprender não pode dar-se fora da procura, fora da boniteza e da alegria.

Paulo Freire

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RESUMO

Uma das principais formas de combate a pragas na agricultura e silvicultura é a utilização de iscas tóxicas, nas quais o novo composto obtido a partir da complexação do flavonoide hesperidina com o metal Magnésio (II) (Mg(hesp)) apresenta alta ação específica no controle de formigas-cortadeiras. Dessa forma, este trabalho teve como objetivo avaliar os efeitos de diferentes concentrações do complexo químico Mg(hesp) (0, 0.1, 1, 10, 100 e 1000 ng mL^{-1}), por meio da avaliação de biomarcadores citogenéticos e bioquímicos *in vitro* (cultivo celular de hepatócitos de *Danio rerio* – ZF-L) e *in vivo* (exposição do peixe Neotropical curimbatá, *Prochilodus lineatus*). A exposição a Mg(hesp) causou grandes impactos na população celular (confluência e morfologia) e na atividade de organelas específicas (mitocondrial e lisossomal), afetando também a estabilidade celular por meio de aumentos na geração de espécies reativas de oxigênio (EROs), principalmente nas maiores concentrações (100 e 1000 ng mL^{-1}). As respostas bioquímicas envolveram a ação de GSH e MT como moléculas antioxidantes e de biotransformação. A influência genética do composto pode também ser detectada nas alterações nucleares e nos eventos de instabilidade de DNA, levando a alterações no ciclo celular nos hepatócitos de *zebrafish*, porém com provável ativação de mecanismos de reparo. Danos de membranas celulares e eventos de apoptose e necrose não foram observados. Os efeitos celulares de Mg(hesp) em ZF-L representam consequências do comprometimento de diversas atividades das organelas celulares, o que possivelmente ativou mecanismos alternativos de morte celular. As avaliações *in vivo* também mostraram influências do Mg(hesp), com alterações hematológicas, provavelmente influenciando a disponibilidade de oxigênio, acompanhada por eventos de instabilidade de DNA (aumento da fragmentação e de anormalidades nucleares) em eritrócitos e hepatócitos de *P. lineatus*. Entretanto, a ausência de formações significativas de micronúcleos pode ser um indicativo de uma ação eficaz de mecanismos de reparo de DNA. As respostas bioquímicas em hepatócitos de *D. rerio* não apresentaram um padrão claro, porém foram eficazes em prevenir danos às membranas celulares, considerando a ausência de aumento na LPO. Assim, os resultados deste estudo indicam que os efeitos por estresse causados pela exposição a Mg(hesp) ocorreram principalmente após 24 h de exposição, enquanto os menores danos observados após 96 h sugerem uma potencial capacidade de recuperação dos organismos, enfatizando a importância da continuidade na investigação das influências causadas por Mg(hesp) e o promissor uso deste composto.

Palavras-chave: metal-inseticida; contaminação aquática; *Prochilodus lineatus*; zebrafish; citogenética; biomarcadores bioquímicos.

ABSTRACT

The use of toxic bait represents an important tool when dealing with important agriculture and silviculture plagues, where hesperidin flavonoid complexation with magnesium(II) generates a compound (Mg(hesp)) with high effect against cutting ants. The aim of this study was to evaluate the effects of different concentrations of Mg(hesp) (0, 0.1, 1, 10, 100 e 1000 ng mL^{-1}) using cytogenetic and biochemical biomarkers *in vitro* (zebrafish hepatocytes – ZF-L) and *in vivo* (Neotropical fish *Prochilodus lineatus*). Mg(hesp) has great impact in cell population (confluence and morphology) and in activity of cellular organelles (mitochondria and lysosome), in addition to affect cellular stability through generations of reactive oxygens species mainly in higher concentrations (100 e 1000 ng mL^{-1}). Biochemical responses include GSH and MT role as antioxidant and biotransformation molecules. Genetic influence of Mg(hesp) could also be detected in nuclear abnormalities and DNA instability, leading to alterations in cell cycle of zebrafish hepatocytes but with a probable activation of repair mechanisms. Membrane damage and apoptosis and necrosis pathways were not observed. Cellular effects of Mg(hesp) in ZF-L represent consequences of effects in several cellular compartments, which probably lead to alternative death cell pathways. *In vivo* analyses also showed Mg(hesp) influences, including hematological alterations, impacting on oxygen availability. Events of DNA instability (increasing in DNA fragments and nuclear abnormalities) in erythrocytes and hepatocytes also were observed. However, the absence of micronuclei formation is a strong indicative of an effective action of repair mechanisms. Biochemical responses in *P. lineatus* did not presented a clear pattern, but were effective in prevent membrane damages. Thus, results indicate that Mg(hesp) influences occurred mainly after 24 h of exposure, while fewer damages were observed after 96 h, suggesting a potential capacity of organism recovery, emphasizing the importance of more assessments of Mg(hesp) effect and the promising use of this compound.

Keywords: metallic-insecticide; water contamination; *Prochilodus lineatus*; zebrafish; citogenetics; biochemical biomarkers.

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LISTA DE SÍMBOLOS E ABREVIATURAS

7AAD = 7-amino-actinomicina D

AU = unidade de absorbância

CAT = catalase

CBMN = ensaio do micronúcleo com bloqueio da citocinese

CHCM / MCHC = concentração de hemoglobina corpuscular média

CytI = índice de citotoxicidade

D. rerio = *Danio rerio*

DMSO = dimetilsulfóxido

EDTA = ácido etilenodiaminotetraacético

EROS/ROS = espécies reativas de oxigênio

FBS = soro bovino fetal

FU = unidade de fluorescência

GenI = índice de genotoxicidade

GPx = glutationa peroxidase

GSH = glutationa reduzida

GST = glutationa S-transferase

H_2DCFDA = diacetato de 2′,7′-diclorodihidrofluoresceina

H_2O_2 = peróxido de hidrogênio

Hb = hemoglobina total

HCM / MCH = hemoglobina corpuscular média

Hct / Htc = hematócrito

hesp = hesperidina (5,3´-dihidroxi-4´-metoxi-flavanona-7-ramnoglucosídeo)

LDH = lactato desidrogenase

LPO = peroxidação lipídica

Mg(hesp) / $Mg(hesp)_2(phen)$ = complexo químico hesperidina-fenantrolina-Magnésio(II) ($MgC_{68}H_{74}N_2O_{30}.2H_2O$)

Mg^{2+} = íon Magnésio

MNi distribution = índice de distribuição celular de micronúcleos

MT = metalotioneína

MTT = brometo de 3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazólio

MutI = índice de mutagenicidade

NC = controle negativo

NDI = índice de divisão nuclear

O_2 = oxigênio

P. lineatus = *Prochilodus lineatus*

PBS = tampão salino fosfato

PE = ficoeritrina

PI = iodeto de propídio

POP = poluentes orgânicos persistentes

RBC = red blood cells (contagem de eritrócitos)

RCF = força centrífuga relativa

SOD = superóxido dismutase

TB = teste de exclusão do Azul de Tripan

VCM / MCV = volume corpuscular médio

ZF-L = linhagem celular de fígado de zebrafish

β-NADH = dinucleotídeo de β -nicotinamida e adenina

SUMÁRIO

APRESENTAÇÃO

O presente trabalho se insere na área de ecotoxicologia de contaminantes orgânicos, com ênfase na contaminação aquática de novos compostos. Como objeto de estudo foi escolhido um complexo químico recém-sintetizado com ação inseticida e promissora visão de mercado, sendo avaliados seus efeitos iniciais *in vitro* em uma linhagem celular de *Danio rerio* (zebrafish), a ZF-L, utilizada como modelo, bem como uma avaliação *in vivo*, utilizando uma espécie de relevância ecológica local, o peixe Neotropical *Prochilpdus lineatus*, o curimba. Este estudo envolveu a realização de análises citogenéticas, fisiológicas e bioquímicas.

Esta tese foi estruturada com uma breve introdução acerca dos assuntos envolvidos, seguida dos objetivos do trabalho e, como resultados, os três artigos originados da pesquisa. Todos os artigos são expostos em inglês e com formatação referente às principais gerenciadoras de periódicos almejadas.

O primeiro artigo trata sobre os resultados citotóxicos obtidos na análise da linhagem ZF-L, incluindo efeitos na população e densidade celular, bem como análises de danos de membrana, de organelas intracelulares e dos principais mecanismos de morte celular.

O segundo artigo aborda os efeitos genotóxicos, mutagênicos e bioquímicos do composto sobre a linhagem ZF-L, com análises de integridade de DNA (ensaio do Cometa, anormalidades nucleares e micronúcleo), influência no ciclo celular e alterações nos sistemas antioxidantes e de biotransformação, enzimáticos e não-enzimáticos.

Já o terceiro artigo expõe os resultados obtidos com a exposição de *P. lineatus* ao composto, com avaliações hematológicas, citogenéticas (em sangue e fígado) e bioquímicas (em fígado).

A tese se encerra com as considerações finais acerca dos três trabalhos desenvolvidos e as referências utilizadas para a introdução.

1 INTRODUÇÃO

Muitos insetos são considerados importantes pragas na silvicultura e agricultura brasileira por causar grande perda do cultivo (ZANETTI et al., 2003). As formigas-cortadeiras (gênero *Atta* e *Acromyrmex*) causam o desfolhamento das plantas e correspondem às principais pragas das áreas de reflorestamento brasileiro, podendo dizimar até 15% de uma safra, representando acima de 75% dos custos e tempo no controle de pragas (LOFGREN; VANDER-MEER, 1986; ZANUNCIO, 1993; URBAS et al., 2007; DECIO et al., 2013).

Dentre os métodos utilizados no controle das formigas-cortadeiras, a utilização de iscas tóxicas tem se mostrado altamente eficaz. Essas iscas são carregadas para o interior do ninho, afetando todos os indivíduos do formigueiro (DECIO et al., 2013). Sendo assim, vários estudos têm sido realizados para o combate desses insetos pragas, partindo-se da utilização de íons inorgânicos metálicos nas iscas, os quais atuam como agentes tóxicos, complexados a substâncias naturais, visto que essa combinação aumenta significativamente a taxa de consumo das iscas pelas formigas (ORVIG; ABRAMS, 1999; VALÉRIO, 2005).

No entanto, os pesticidas podem contaminar águas subterrâneas e superficiais via percolação da água no solo através de rachaduras das rochas, processos de lixiviação e escoamento superficiais, comprometendo, portanto, a qualidade da água utilizada por animais e humanos, afetando direta ou indiretamente toda a biota local (DONOSO et al., 1999; BRIGANTE et al., 2002; FILIZOLA et al., 2002; MACARY et al., 2014). Mesmo em concentrações ínfimas, os pesticidas representam riscos para algumas espécies dos corpos aquáticos com capacidade de bioacumulação, concentrando essas substâncias em até 1000 vezes (EICHELBERGER et al., 1971). Adicionalmente, os fatores climáticos das áreas afetadas também exercem grande influência no comportamento dos agroquímicos, com poucos estudos desenvolvidos acerca do efeito de agroquímicos em ambientes tropicais (DAAM; VAN DER BRINK, 2010).

A avaliação toxicológica de substâncias químicas possibilita o monitoramento e verificação precoce das possíveis consequências causadas pelos xenobióticos aos organismos expostos, onde os ensaios citotóxicos e genotóxicos são ferramentas importantes na determinação destes danos (SILVA, 2003). Para tal, o uso de espécies sensíveis e que habitam áreas de interesse e susceptíveis a impactos são

necessárias. Neste contexto, células de peixes são frequentemente utilizadas como biomarcadores. O uso da experimentação *in vitro* é amplamente utilizado na Toxicologia e Farmacologia, fornecendo dados de efeitos primários com alta reprodutividade, maior sensibilidade e rapidez, com resultados confiáveis e mais precisos (BROADHEAD et al., 2000; BERNAUER et al., 2005). Adicionalmente, avaliações *in vivo* se mantém como importante ferramenta para compreender os efeitos de compostos diversos na complexidade de um organismo. *Prochilodus lineatus*, conhecido popularmente como curimba ou curimbatá, é considerado um excelente organismo para detecção de contaminantes e toxicidade de várias substâncias presentes no ambiente aquático. Trata-se de uma espécie sensível, que é amplamente distribuída em toda região sudeste do Brasil e de grande interesse econômico (LIEBEL et al., 2011).

1.1 Pragas de cultivo e o uso de pesticidas na agricultura

A agricultura representa uma atividade econômica indispensável na produção de alimentos, de influência significativa no desenvolvimento de diversos países. O setor agroindustrial contribui para o desenvolvimento de populações que vivem no campo e em pequenas cidades. No Brasil, representa 20% das exportações, 12% do PIB e gera empregos para 22% da população ativa (FERRER et al., 2006). Dentre os cultivos existentes, o país apresenta alta representatividade no mercado de exportação de café, frutas cítricas (principalmente laranja) e açúcar, estando o complexo soja (grão, farelo e óleo) em constante crescimento de produção e renda (LANDIM, 2010; NETO, 2011).

O desenvolvimento dos cultivos e investimentos constantes na agricultura estão diretamente relacionados com a necessidade imediata de espaços físicos e ampliação dos campos de produção. Da mesma forma, uma maior produtividade está relacionada com a prática de monoculturas, o que afeta na diversidade vegetal, animal e na qualidade do solo (CARVALHO, 2000). Adicionalmente, a prática de monoculturas influencia no desenvolvimento e permanência de insetos com potencial para se tornarem pragas, considerando a abundância de alimentos específicos e escassez de predadores naturais (ZANETTI et al., 2003).

No Brasil, um dos principais insetos considerados pragas da agricultura são representantes dos gêneros *Atta* e *Acromyrmex*, conhecidos como formigas-cortadeiras. Pertencentes à família Formicidae (ordem Hymenoptera), esses insetos

são responsáveis por grandes prejuízos em cultivos e pastos, cortando os vegetais e transportando os pedações para o formigueiro, onde este material servirá para cultivo do fungo simbionte (*Leucoagaricus gongylophorus*) do qual se alimentam (HÖLLDOBLER; WILSON, 1990; FORTI, 2000). Dessa forma, as formigas-cortadeiras, com exceção de ambientes ecologicamente equilibrados, podem dizimar até 15% de uma safra, representando mais de 75% dos custos e tempo gastos no controle de pragas de culturas agrícolas, áreas de reflorestamento e pastagens no Brasil (LOFGREN; VANDER-MEER, 1986; ZANUNCIO, 1993; URBAS et al., 2007).

O incentivo no desenvolvimento, produção e uso de agrotóxicos surgiu como necessidade para acompanhar o desenvolvimento econômico e social das cidades, para que a demanda da produção agrícola fosse assegurada. Dentre os controles químicos desenvolvidos para o controle das formigas-cortadeiras, as iscas formicidas são amplamente utilizadas, por apresentar maior eficiência, possuindo uma ação tóxica retardada, alta taxa de mortalidade para o alvo após 14 dias do oferecimento, além de menores danos ambientais (VALÉRIO, 2005). Assim, a constante reformulação das propriedades químicas destas iscas é um reflexo da procura por componentes que apresentam alta eficiência, porém possuam baixa toxicidade e bioacumulação em outros animais.

1.2 Complexos metal-flavonoides como inseticidas

Dentre os princípios ativos de maior utilização em iscas tóxicas ao longo do tempo, estão inclusos inseticidas como o organoclorado dodecacloro, banido em todo território nacional devido à sua alta toxicidade ambiental e o composto sulfluramida (N-etil-perfluooctano sulfonamida), atualmente utilizado na formulação de novas iscas. Porém, de forma similar, o uso de sulfluramida também apresenta alto potencial tóxico ao ambiente e demais organismos expostos, tendo sido proibido em diversos países (OLIVEIRA, 2012; SILVA, 1993). Dessa forma, a busca por princípios ativos eficazes em sua ação inseticida, porém com baixos efeitos ambientais ainda é um desafio.

Certos metabólitos secundários produzidos por diversas plantas apresentam atividade protetora à ação de predadores. Dentre eles, os flavonoides são uma classe altamente diversificada de compostos polifenólicos, com ação antioxidante e anti-inflamatória (EMIM et al., 1994), tendo também uma potencial atividade inseticida, principalmente contra formigas-cortadeiras da espécie *Atta sexdens rubropilosa*

(ZANETTI et al., 2004). A hesperidina (5,3´-dihidroxi-4´-metoxi-flavanona-7-ramnoglucosídeo, hesp) é um dos flavonoides mais abundantes, amplamente encontrada em frutas cítricas (GROHMANN et al., 2000).

A utilização de complexos metálicos apresenta grande utilidade nas indústrias farmacêuticas, químicas e agrícolas, aumentando a biodisponibilidade dos compostos e amplificando as atividades biológicas de flavonoides anteriormente livres (DE SOUZA; DE GIOVANI, 2004). Adicionalmente, alguns íons, como Mg^{2+}, possuem fundamental importância para proteção das plantas contra patógenos e predadores (SHARVELLE, 1961). Assim, a alta capacidade de ligação e interação com importantes alvos biológicos que centros metálicos apresentam estimula o desenvolvimento de pesquisas para avaliar o uso e desenvolvimento de complexos metálicos com substâncias naturais como moléculas bioativas (ORVIG; ABRAMS, 1999).

A obtenção do composto flavonoide-metal a partir da complexação da hesperidina com Magnésio (II) gera uma molécula de formulação $MgC_{68}H_{74}N_2O_{30}.2H_2O$, a qual apresenta a fenantrolina como agente quelante (OLIVEIRA, 2012; FERNANDES et al., 2015). O complexo, referido neste trabalho como Mg(hesp), corresponde a um pó de coloração amarela com picos de absorbância na faixa de 260 a 300 nm e picos de emissão variáveis de acordo com o solvente utilizados: intensa emissão a 467 nm quando em dimetilsulfóxido, porém sem emissão produzida quando dissolvida em água ou ciclohexano. O composto apresenta alta estabilidade fotoquímica, com estabilidade química em faixas de pH de 3 a 9 e valores de solubilidade na água de 472 µg mL^{-1} (OLIVEIRA, 2012; OLIVEIRA et al., 2013).

Quanto ao modo de ação do composto, Mg(hesp) atua por inibição irreversível da atividade da enzima acetilcolinesterase, cujo papel corresponde à hidrólise específica do neurotransmissor acetilcolina nas sinapses colinérgicas (FILGUEIRA, 2008; LI et al., 2010; OLIVEIRA, 2012). Apesar de presente em todos os vertebrados e invertebrados, a enzima em questão apresenta particularidades gênicas distinta entre os dois grandes grupos. O complexo Mg(hesp) apresenta potencial ação seletiva, com 100% de inibição da atividade enzimática em extratos de *A. sexdens rubropilosa,* em contrapartida à ausência de inibição da acetilcolinesterase em extratos de peixe elétrico (*Electrophorus electricus*). Valores de ação inseticida foram obtidos com resultados similares ao princípio sulfluramida, com S_{50} de

aproximadamente 5 dias, atuando por ingestão, letais em baixas concentrações, inodoros, não repelentes, com efeito tóxico retardado e mortalidade abaixo de 15% após o primeiro dia e acima de 85% após o décimo quarto dia (OLIVEIRA, 2012). Adicionalmente, Mg(hesp) não apresentou efeitos tóxicos em ensaios de bioluminescência de *Vibrio fishceri* e em análises de citotoxicidade com a linhagem celular tumoral humana HeLa (OLIVEIRA, 2012).

1.3 Contaminação aquática por agroquímicos e o uso de bioindicadores no monitoramento ambiental

A maioria das espécies existentes nos diferentes nichos ecológicos habita ecossistemas aquáticos, os quais cobrem cerca de dois terços do planeta (JHA, 2004). Dessa forma, a utilização de pesticidas na agricultura pode levar à contaminação de águas superficiais e subterrâneas por diversos eventos, podendo levar a efeitos tóxicos para a biota local, bem como para a saúde humana quando em contato (CEREJEIRA et al., 2001).

O transporte de agroquímicos para os corpos d'água é influenciado por diversos fatores, como clima, características do solo, formas de aplicação e propriedades químicas e ambientais dos compostos utilizados. Por eventos de escoamento superficial, os agroquímicos podem atingir as águas superficiais e, por infiltração, águas subterrâneas. Adicionalmente, estes eventos podem levar à contaminação dos sedimentos, transferência para os organismos do ambiente e no próprio local, por volatilização e precipitação de chuvas (NIMMO, 1985).

A contaminação por agroquímicos nos ambientes aquáticos afeta a biota residente de diferentes maneiras, conforme a disponibilidade e capacidade de bioacumulação e adsorção desse xenobiótico e características dos próprios organismos, como tipo de alimentação e posição na cadeia trófica (VENTURA et al., 2008). Adicionalmente, os fatores climáticos das áreas afetadas também exercem grande influência no comportamento dos agroquímicos, sendo diferente em condições temperadas ou tropicais, com poucos estudos desenvolvidos acerca do efeito de agroquímicos neste último ambiente (DAAM; VAN DER BRINK, 2010). Dessa forma, o biomonitoramento utilizando espécies representantes dos ambientes alvo são de extrema importância, possibilitando a maior compreensão dos diferentes efeitos que a exposição aos agroquímicos pode causar.

Portanto, a aplicação de biomarcadores e ensaios de toxicidade com diversas espécies representa uma metodologia eficiente e comumente aplicada (BARATA et al., 2008; ECHEVARRÍA-SÁENZ et al., 2012). No contexto de contaminação de ambientes aquáticos, biomarcadores em peixes permitem detectar efeitos específicos de poluentes, considerando que a fisiologia desses vertebrados aquáticos é mais conhecida que a de invertebrados e os mesmos apresentação grande importância ecológica, econômica e social.

Métodos de cultivo *in vitro*, como culturas de linhagens celulares já estabelecidas, são extensamente utilizados em estudos toxicológicos para identificar diversos "*endpoints*", visando elucidar os efeitos genéticos da ação de diversos compostos (BARON et al., 2012). A espécie *Danio rerio* (Hamilton, 1822, Cyprinidae), também conhecida como zebrafish, peixe-zebra ou paulistinha é um importante modelo para estudos de mecanismos de respostas biológicas, possuindo 70% de similaridade com o genoma humano (SIEBEL et al., 2015). Dentre as diversas linhagens celulares padronizadas a partir de indivíduos de *D. rerio*, a linhagem ZF-L (*zebrafish* liver) foi desenvolvida a partir dos hepatócitos do animal, com alta sensibilidade a compostos, capacidade metabólica hepática e ciclo celular de 72 horas (GHOSH, 1994; FENT, 2001; CHEN et al., 2014; BCRJ, 2018).

Por outro lado, a investigação *in vivo* ainda se mantém como uma importante forma de avaliar efeitos de xenobióticos frente à complexidade sistêmica dos organismos. Considerando a importância do uso e avaliação de espécies nativas e de grande relevância ecológica e econômica, optou-se no uso de espécimes de *P. lineatus* (Valenciennes, 1847, Prochilontidae). Popularmente conhecido como curimba ou curimbatá, é uma espécie dulcícola, com alta fecundidade e hábito migratório na época reprodutiva. Presente na região Neotropical, possui alta importância econômica e sua distribuição se restringe a locais pouco impactados. Adicionalmente, por se alimentar de detritos, estando exposta às substâncias químicas que possam ter ficado retidas nos sedimentos e por ter sua biologia bem conhecida e apresentar uma alta sensibilidade a poluentes, esta espécie é considerada um bioindicador em potencial, sendo apropriada para testes de toxicidade (MARTINEZ; CÓLUS, 2002; PEREIRA-MADUENHO; MARTINEZ, 2008).

1.4 Biomarcadores citogenéticos e bioquímicos

A exposição a xenobióticos leva a diversas mudanças celulares, bioquímicas, moleculares, fisiológicas e/ou comportamentais ao nível de organismo. Dessa forma, quantificar e qualificar tais alterações, relacionando-as com o xenobiótico, compreende uma das definições para biomarcadores (PEAKALL, 1994). A identificação das interações e efeitos subletais entre os contaminantes e indivíduos possibilita a ação de práticas remediadoras e preventivas, sendo cada vez mais incluídas em planos de avaliação de contaminação ambiental (JESUS et al., 2008).

Biomarcadores celulares e moleculares, muito utilizados no campo da citogenética ambiental, correspondem a ferramentas de alerta altamente sensível e precoce, auxiliando em estratégias de biorremediação, podendo indicar a exposição aos poluentes e a magnitude dos efeitos no organismo (CAJARAVILLE et al., 2000). Em peixes, a utilização de biomarcadores permite a compreensão dos mecanismos de ação dos contaminantes e o nível de influência na biota aquática, onde parâmetros genotóxicos e citotóxicos são amplamente utilizados para a detecção rápida de efeitos adversos, principalmente aliando testes *in vitro* e *in vivo* (PORTO et al., 2005).

As células estão susceptíveis a diversas influências, sendo estas expressas em diferentes formas de dano. Rupturas na cadeia de DNA, modificações nos nucleotídeos, alterações cromossômicas, entre outros, são efeitos detectáveis por diversos ensaios toxicológicos, como o Ensaio Alcalino do Cometa (SINGH et al., 1988; PORTO et al., 2005). Os danos diretamente relacionados ao material genético de um organismo podem passar por processos de reparo próprios do aparato celular, revertendo o dano inicialmente causado. Porém, em situações mais danosas, xenobióticos podem levar a consequências em outros níveis da composição celular, como alterações na viabilidade e atividade de diversas organelas e estruturas, podendo ser detectados por diversos testes, como a avaliação da viabilidade mitocondrial pelo Ensaio do MTT (MOSMANN, 1983), integridade lisossômica por meio da técnica do Ensaio de Retenção do Vermelho Neutro (BACICH; BORENFREUND, 1990) e a integridade de membrana, pelo Teste de Exclusão de Azul de Tripan (STROBER, 1997).

Os biomarcadores fisiológicos e/ou bioquímicos fornecem informações relacionadas a alterações funcionais e metabólicas frente à exposição a um contaminante (AMORIN, 2003). Dentre os biomarcadores fisiológicos, os

componentes celulares e a composição do sangue dos peixes têm sido utilizados como uma indicação de seu bem-estar (CLAUSS et al., 2008). O hematócrito (Hct), a concentração de hemoglobina (Hb) total e a contagem de eritrócitos (red blood cells - RBC), assim como os índices hematimétricos (volume corpuscular médio – VCM; hemoglobina corpuscular média – HCM; concentração de hemoglobina corpuscular média – CHCM) são sensíveis a mudanças fisiológicas ocasionadas pelo estresse (HOAR et al., 1992; CLAUSS et al., 2008).

O estresse pode ser definido como uma resposta adaptativa que permite ao peixe enfrentar um agente estressor, de forma a manter a homeostase (BARTON, 2002). Dentre as possíveis vias metabólicas ativadas para superar situações de estresse, os biomarcadores bioquímicos podem ser citados, atuando nas vias antioxidantes ou de biotransformação.

As linhas de defesa à ação de espécies reativas de oxigênio (EROs), que são produzidas pela ação direta ou metabolização de xenobióticos, incluem, dentre outras, as enzimas superóxido dismutase – SOD, catalase – CAT, os marcadores não enzimáticos, como a glutationa reduzida – GSH e os marcadores de danos celulares (peroxidação lipídica – LPO). Por outro lado, mecanismos de biotransformação podem atuar de forma direta, neutralizando ou facilitando a metabolização e eliminação do tóxico, a citar a enzima glutationa S-transferase – GST, novamente a molécula GSH e o conteúdo de metalotioneína – MT (VAN DER OOST et al., 2003; PRIETO et al., 2006).

Dessa forma, a integração dos diferentes biomarcadores possibilita uma maior compreensão sobre os efeitos biológicos do agroquímico, seu mecanismo de ação, sugerindo relações de causa-efeito, permitindo, assim, a intervenção prévia a consequências irreversíveis.

2 OBJETIVOS

2.1 Objetivo Geral

Este trabalho tem como objetivo avaliar os efeitos da possível contaminação do ambiente aquático devido à utilização de complexos químicos (hesperidinas e Magnésio II) aplicados como inseticidas no controle de formigas cortadeiras (*Atta sexdens rubropilosa*), por meio da avaliação de biomarcadores bioquímicos e citogenéticos *in vitro* (cultivo celular de hepatócitos de *D. rerio* – ZF-L) e *in vivo* (*P. lineatus*).

2.2 Objetivos Específicos

- Avaliar, *in vitro*, os efeitos do complexo químico hesperidina-Magnésio(II) (Mg(hesp)) em hepatócitos de *D. rerio* (linhagem ZF-L), por meio de biomarcadores citotóxicos (morfologia e confluência, densidade populacional, integridade de membranas celulares, viabilidade mitocondrial e lisossomal e detecção de apoptose e necrose);

- Avaliar, *in vitro*, os efeitos do complexo químico Mg(hesp) em hepatócitos de *D. rerio* (linhagem ZF-L), por meio de biomarcadores genotóxicos, mutagênicos e bioquímicos, bem como alterações no ciclo celular;

- Avaliar, *in vivo*, os efeitos do complexo químico Mg(hesp) em *P. lineatus* (curimbatá), por meio de biomarcadores hematológicos, citotóxicos e bioquímicos.

3 RESULTADOS

3.1 Artigo 1

In vitro cytotoxic effects of a novel potential formicide in zebrafish (_Danio rerio_) hepatocyte cell line

ABSTRACT

The development of novel agrochemical compounds to reduce the use of pesticides with high ecological impact is relevant. Complexation of hesperidin flavonoid and phenatrolin with magnesium (Mg(hesp)) results in high insecticidal activity, specifically for controlling leafcutting ants, an important pest in crops of tropical regions. *In vitro* cytological biomarkers were used to assess the primary effects of Mg(hesp) on fish cells, as this insecticide can reach the aquatic environment. The cytotoxic effects of Mg(hesp) were evaluated at different concentrations (0, 0.1, 1, 10, 100 and 1000 ng mL^{-1}) in a zebrafish hepatocyte cell line (ZF-L). Mg(hesp) has great impact on cell population (confluence/morphology) after 24 h exposure to high concentrations (10 and 1000 ng mL^{-1}). Mitochondrial activity and lysosomal retention ability decreased as Mg(hesp) concentration increased. Cell membrane injury, apoptosis, and necrosis were not observed. The cellular effects of Mg(hesp) are a consequence of failure in cellular organelle activities and could include activation of alternative cell death mechanisms. However, after 96 h exposure, the toxic effects of Mg(hesp) may be mitigated, even at high concentrations, enabling cellular population recovery. These data provide important information on the initial effects of Mg(hesp) on fish cells, indicating the potential mechanisms of the cellular response and stimulating further analysis to elucidate the cellular pathways.

Keywords: metallic-insecticide; ZF-L cell line; cell death; aquatic contamination; cytological biomarkers.

RESUMO

O desenvolvimento de novos compostos agroquímicos com o intuito de reduzir o uso de pesticidas com alto impacto ecológico possui alta relevância. A complexação do flavonoide hesperidina com fenantrolina e magnésio(II) resulta em um composto (Mg(hesp)) com alta atividade inseticida, especialmente no controle de formigas-cortadeiras, uma importa praga de cultivos tropicais. Biomarcadores citológicos *in vitro* foram utilizados para avaliar os efeitos primários de Mg(hesp) em células de peixe, considerando que este inseticida pode atingir ambientes aquáticos. Diferentes concentrações de Mg(hesp) (0, 0.1, 1, 10, 100 e 1000 ng mL^{-1}) foram testadas na linhagem celular de hepatócitos de zebrafish (ZF-L). Mg(hesp) causou grandes impactos na população celular (confluência e morfologia) após 24 h exposição em altas concentrações (10 and 1000 ng mL^{-1}). A atividade mitocondrial e a capacidade de retenção lisossomal decaíram com o aumento das concentrações do composto. Danos de membranas celulares e eventos de apoptose e necrose não foram observados. Os efeitos celulares de Mg(hesp) representam consequências do comprometimento de diversas atividades das organelas celulares, o que possivelmente ativou mecanismos alternativos de morte celular. Entretanto, após 96 h de exposição, os efeitos tóxicos de Mg(hesp) foram mitigados, mesmo em altas concentrações, possibilitando uma recuperação da população celular. Os dados obtidos fornecem informações importantes acerca dos efeitos iniciais de Mg(hesp) em células de peixe, indicando os potenciais mecanismos de resposta celular, estimulando maiores estudos para elucidar as vias celulares ativadas.

Palavras-chave: metal-inseticida; linhagem ZF-L; morte celular; contaminação aquática; biomarcadores citológicos.

1 INTRODUCTION

The industry of agrochemicals for pest control is continuously investing in the development of new compounds to improve silviculture and crop practices and increase food production. However, such compounds may adversely affect the environment and reach aquatic ecosystems through atmospheric deposition, surface run-off or leaching, threating non-target organisms, including fish (Akerblom, 2004). The development of compounds with selective pesticide potential is relevant to reducing the application of those with high ecological impact.

Several secondary metabolites produced by plants present preventive action against pests; among theses, flavonoids constitute a class of polyphenolic compounds with potential insecticide activity (Wang et al., 2016). One of them, hesperidin (hesp), is present at high concentrations in citrus species but is highly hydrophobic, which limits its application. Metallic complexes with these flavonoids increase the hydrophilicity of compounds, amplify the biological activities of previously free flavonoids, and are highly applicable in the agricultural, chemical, and pharmaceutics industries (De Souza and De Giovani, 2004; Oliveira, 2012). Magnesium (II), which is an essential metal for numerous biological functions, plays an important role in enzyme activity in both plants and animals and is also vital for plant protection, acting to stabilize macromolecules and maintain of enzyme activities, including those involved in the antioxidant system (Oliveira, 2012; Guo et al., 2016).

Metal chelation of hesp to Magnesium (II) results in a complex $[Mg(phen)_2(hesp)$, referred as Mg(hesp), with considerable hydrophilicity and potentially greater redox stability than free hesperidin (Fernandes et al., 2015; Oliveira et al., 2013). This Mg(hesp) complex has high insecticide activity against the leafcutting ant *Atta sexdens rubropilosa*, an important pest specie in tropical crops, at concentrations around 140 ng mL^{-1} (0.2% by weight of the diet) with mortality rates similar to known commercial insecticides, such as sulphluramide (Fernandes et al., 2015; Oliveira, 2012, Zanetti et al, 2004). Preliminary in vitro biological assessment of this complex demonstrated the incorporation and low cytotoxic potential against tumor human cells (HeLa) (Oliveira et al., 2013); however, little is known about specific effects and action mechanisms in aquatic vertebrates.

Xenobiotic exposure may lead to biochemical, physiological and cellular changes, affecting animal health to different degrees. Quantitative measurement using biomarkers allows the identification of sub-lethal effects and interactions between

contaminants and organisms, enabling the application of mitigating and preventive actions, which are incorporated into environmental legislations at an increasing rate (Jesus and Carvalho, 2008). Moreover, *in vitro* cytotoxic biomarkers are highly sensitive tools and essential to the rapid assessment of the primary effects of xenobiotics, indicating the magnitude of exposure effects and its potential mechanisms of action (Hussain et al., 2018). Thus, the aim of this study was to evaluate the cellular responses of a novel potential pesticide on the zebrafish hepatocyte cell line - ZF-L, an excellent biological model for *in vitro* studies.

2 MATERIALS AND METHODS

2.1 Complex characterization

Hesperidin complexed with magnesium (II) (Fernandes et al., 2015) was obtained from the Inorganic and Bioinorganic Photochemistry Laboratory of Chemistry Department of Federal University of São Carlos, São Paulo, Brazil. The synthesis of $Mg(phen)_2(hesp)$, which will be referred as Mg(hesp), was performed according to Oliveira et al. (2013) and its formulation ($MgC_{68}H_{74}N_2O_{30}.2H_2O$) includes the presence of a phenanthroline molecule as a chelating agent. Mg(hesp) is a yellow powder that presents absorbance peaks at 260-300 nm and variable emission peaks according to the solvent used: emission is intense at 467 nm when Mg(hesp) is dissolved in dimethyl sulfoxide, dimethylformamide or 1-octanol and no emission is produced when dissolved in water or cyclohexane (Oliveira et al., 2013). The complex has high photochemical stability, chemical stability in the pH range from 3 to 9, and values of water solubility equal to 472 µg mL^{-1} (Oliveira et al., 2013).

2.2 Experimental conditions

Considering the absence of established limit levels for new chemicals in the environment, the Mg(hesp) concentrations used in this study were selected a range of 6 concentrations to compose an overall view of possible toxic effects of the Mg(hesp) compound: 0.0 (negative control), 0.1, 1.0, 10, 100 and 1000 ng mL^{-1}, which were obtained by serial dilution in sterile phosphate-buffered saline (PBS) using a low frequency (40 KHz) ultrasound bath (Q335D, QUIMIS®, Brazil). The mixture of diluted Mg(hesp) in the culture medium respected a limit of 1% of the total working volume. Two exposure periods (24 h and 96 h) were selected to evaluate the Mg(hesp) effects on cells. To ensure that the results reflected only the effects of Mg(hesp), the cells

were also exposed only to PBS (1% of total working volume) in all assays, in which viability was fully maintained (data not shown). Assays were performed in technical triplicates and in three independent experiments.

2.3 Hepatocyte cell culture

The zebrafish (*Danio rerio*) hepatocyte cell line - ZF-L- was obtained from the Rio de Janeiro Cell Bank, Rio de Janeiro Federal University and maintained in culture flasks (75 cm^2) at 28ºC in medium containing 40% RPMI 1640 medium (Sigma Aldrich, St Louis, USA), 50% Leibovitz's L-15 medium (Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (FBS, 1492-500 lot, Cultilab, Campinas, Brazil) and 1% antibiotic/anti-mycotic solution (penicillin/streptomycin and Fungizone, Sigma Aldrich, St Louis, USA).

Considering its cell cycle (around 72 h), cells were sub-cultured twice/three times per week, respecting the confluence limit and viability. Experiments were carried out using 15 - 25 culture passages, respecting 24 h of stabilization to ensure that the cells were adhered to the plates at the time of exposure, which was confirmed by microscope visualization. Each assay was performed in three independent experiments, each one in triplicate. Different seed concentrations were applied according to exposure time and the size of the plates to avoid cellular stress due to over population. Moreover, treatments were performed without FBS addition to assure that effects were assessed under conditions of metabolically active cells.

2.4 Cytotoxic assays

2.4.1 Confluence and morphology

The effect of Mg(hesp) concentrations on culture confluence and cell morphology were verified after 24 h of exposure. ZF-L cells were seeded in 24-wells plates at a density of $5x10^5$ cells per well. After culture stabilization, cells were exposed to various Mg(hesp) concentrations for 24 h, and each well was analyzed. Culture images were obtained at 40x magnification using an inverted light microscope (Nikon Eclipse TS100) coupled to a video camera (Moticam 1000 – 1.3MP Live Resolution).

2.4.2 Population density

Cells were seeded in 24-wells plates at a density of $5x10^5$ and $3x10^5$ cells per well for 24- and 96 h exposure periods, respectively. After cell stabilization (24 h),

Mg(hesp) treatments were performed. Following each exposure, cells were trypsinized and aliquots of cellular suspension were mixed with the same volume of 0.4% trypan blue dye (Sigma Aldrich, St Louis, USA). Thereafter, 10 µL of cell/dye mixture were applied in each chamber of counting slides (two reads per replicate) and analyzed in a TC20^{TM} Automated Cell Counter (Bio-Rad Laboratories, Inc.). Data were reported as cell density of viable cells (cells per mL).

2.4.3 Cell membrane integrity

To assess cellular membrane integrity, both the trypan blue dye exclusion test (TB) and the lactate dehydrogenase (LDH) leakage assay were performed. The TB protocol followed was as described in section 2.4.2 and cells with damaged membranes were differentiated from those with intact ones by detection of dye incorporation (Strober, 1997). The results were reported as percentage of live cells.

Lactate dehydrogenase activity in the culture medium (LDH leakage) was quantified as described by Han et al. (2011). Cells were seeded in 6-well plates at a density of 16 x 10^5 and 9 x 10^5 cells per well for 24 h and 96 h of exposure, respectively. After Mg(hesp) exposure, plates were briefly shaken to homogenize the LDH released into the medium. The medium was collected from each well, transferred to microtubes, centrifuged (12,000 x *g* for 5 min at 4 ºC) and aliquots of supernatant (40 µL) were added to a LDH substrate solution (260 µL, 0.26 mM β-NADH, 0.62 mM sodium pyruvate, 0.05 M potassium phosphate buffer). Absorbance kinetics were measured in a spectrophotometer (SpectraMax® M5, Molecular Devices, USA) at 340 nm and analyzed using the Soft Max Pro Software (version 5.4). The results were expressed as a percentage of the negative control (NC).

2.4.4 Mitochondrial reduction activity

Mitochondrial reducing activity was analyzed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay according to Mosmann (1983). Cells were seeded in 96-well plates at a density of 2.5 x 10^5 and 1 x 10^5 cells per well for 24 h and 96 h period of exposure, respectively. Following Mg(hesp) exposure, cells were incubated for 4 h in FBS free medium with 0.5 mg mL^{-1} of MTT and then washed with PBS. Thereafter, 200 µL of DMSO was added to each well for cell dissolution and liberation of formazan products from mitochondrial MTT metabolism. The absorbance was read in a spectrophotometer (MRX-TC, Dynex

Technologies, USA) at 540 nm and the results were presented in absorbance units (AU).

2.4.5 Lysosome retention ability

A colorimetric analysis was performed to assess lysosome retention ability, in which viable cells can incorporate the supravital dye neutral red (Repetto et al., 2008). Cells were seeded in 96-well plates at a density of 2.5 x 10^5 and 1 x 10^5 cells per well for 24 h and 96 h of exposure, respectively. Following Mg(hesp) exposure, cells were incubated in 40 µg mL^{-1} neutral red solution (3-amino-7-dimethylamino-2-methylphenazine hydrochloride; Sigma Aldrich, St Louis, USA) for 2 h. Thereafter, plates were washed with PBS and, then, 150 µL of distaining solution (50% ethanol 96%, 49% deionized water and 1% glacial acetic acid) were added in each well and homogenized in a plate shaker. The absorbance was read at 540 nm in a spectrophotometer (MRX-TC, Dynex Technologies) and data were presented in absorbance units (AU).

2.4.6 Apoptosis and necrosis rates

To assess apoptosis and necrosis effects, 24 wells were seeded with density of 5 x 10^5 and 3 x 10^5 cells per well for 24 h and 96 h of exposure, respectively. To ensure that quick responses to Mg(hesp) exposure were not being overlooked, an additional exposure period of 10 h (2.5 x 10^5 cells per well) was carried out. The assay was performed using an Annexin V–PE staining kit (BD Biosciences, NJ – USA). After exposure, plates were centrifuged (12,000 rpm) for 5 min at 4 ºC, washed with PBS and, 200 µL of binding buffer were added per well. Afterwards, Annexin V phospholipidbinding protein conjugated with PE fluorochrome and 7AAD vital dye were applied (2.5 µL per well) for 15 min to detect, respectively, apoptotic and necrotic cells. The cellular suspension was analyzed using a flow cytometer (BD Accuri C6; BD Biosciences, NJ, USA), counting 15,000 events differentiated in four different cell statuses (viable, necrotic, recent apoptosis and late apoptosis). Data were reported in percentage.

2.5 Statistics

Data were presented as median (first quartile – third quartile). All data were tested for the normal distribution (Shapiro-Wilk's test). Non parametric data were then

analyzed applying Kruskal-Wallace followed by Dunn's multiple comparison test as post hoc analyses ($P < 0.05$). Apoptosis/necrosis data were evaluated through contingency tables and a chi-square test ($P < 0.05$). Spearman's rank correlation coefficient was used to determine correlations within variables and reported only when significance ($P < 0.05$ and $r_s > 0.45$) was detected. All statistical analyses were performed in InfoStat (Di Rienzo et al., 2010).

3 RESULTS

Exposure to Mg(hesp) had a clear impact on cell morphology and cell population (confluence) after 24 h of exposure at high concentration. Adherent ZF-L cell morphology has an epithelial-like shape and maintained this shape at confluence in the NC (Fig. 1A, B); however, after exposure from 10 to 1000 ng mL^{-1}, many cells showed a spherical/deformed shape (Fig. 1C).

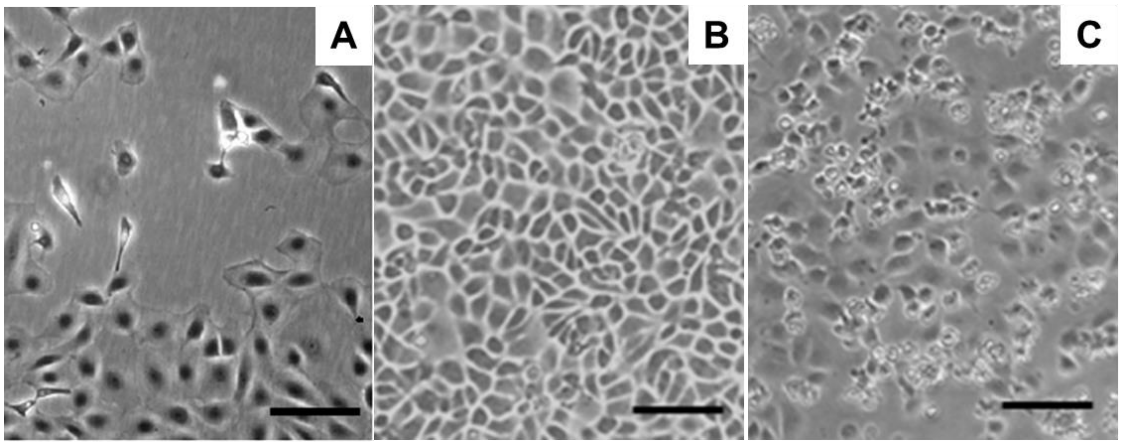

Figure 1. ZF-L cell line morphology. A. Morphology of ZF-L cell line obtained from Cell Bank (ATCC® and Rio de Janeiro Cell Bank) at low density. Note the epithelial appearance of cells; B. Cell morphology from negative control at high density; C. Cell morphology after 24 h exposure to 1000 ng mL^{-1} of Mg(hesp). Note the spherical/deformed shape of many cells. Scale bar = 100 µm.

After 24 h of exposure, the cell population (confluence) showed a progressive decrease in density as the Mg(hesp) concentration increased from 10 to 1000 ng mL^{-1} (Fig. 2). A significant decrease in ZF-L cell population density occurred after exposure to 100 and 1000 ng mL^{-1} Mg(hesp) in both periods, 24 and 96 h (Fig. 3A, B and Table S1), reaching up to 47% relative to the negative control (NC) after 24 h and 68% after a 96 h exposure (Fig. 3C).

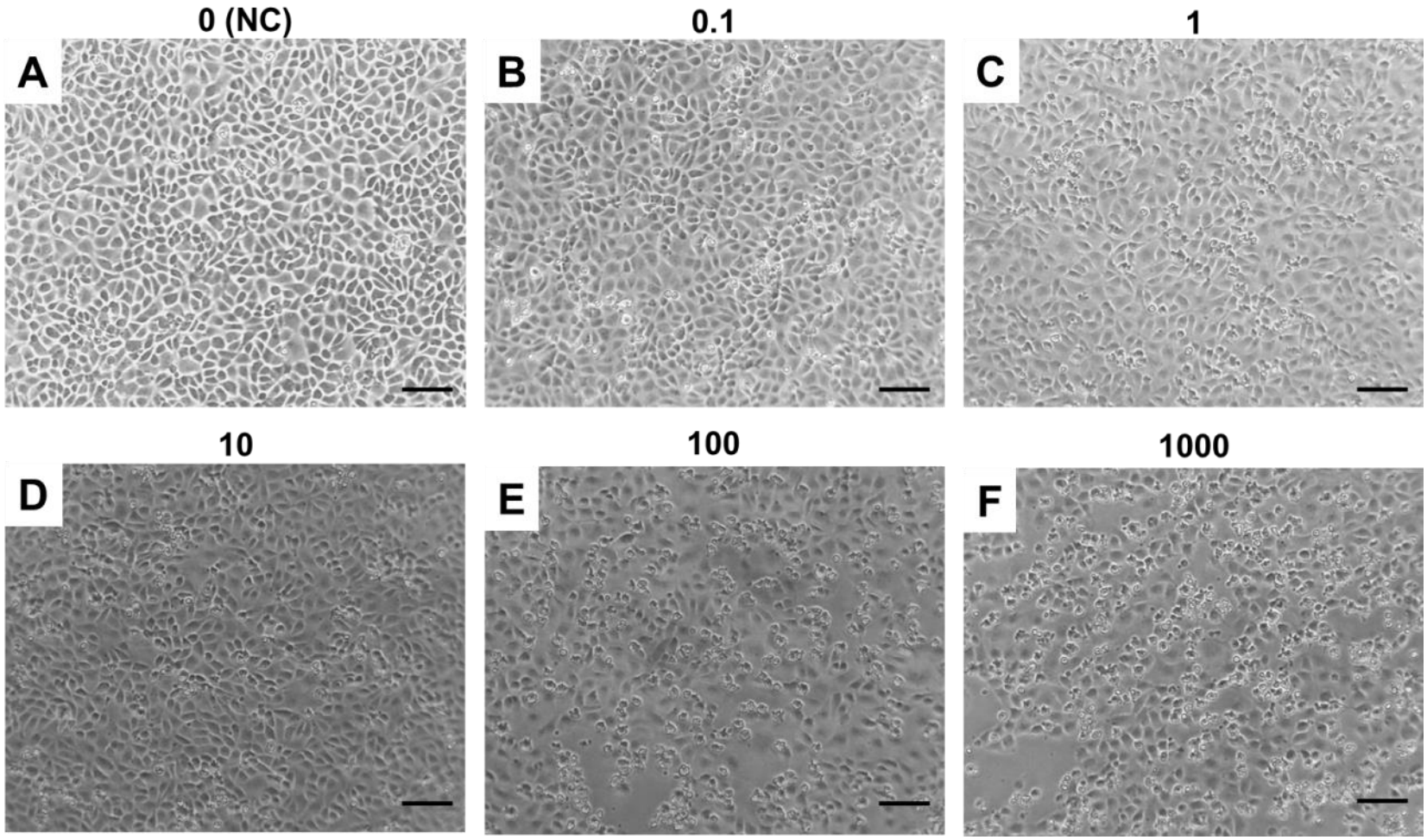

Figure 2. Confluence of ZF-L cell line after a 24 h exposure to Mg(hesp). A: Negative control (NC) [0 ng mL^{-1} Mg(hesp)]; B to F: ZF-L exposure to 0.1 to 1000 ng mL^{-1} Mg(hesp). Scale bar = 100 µm.

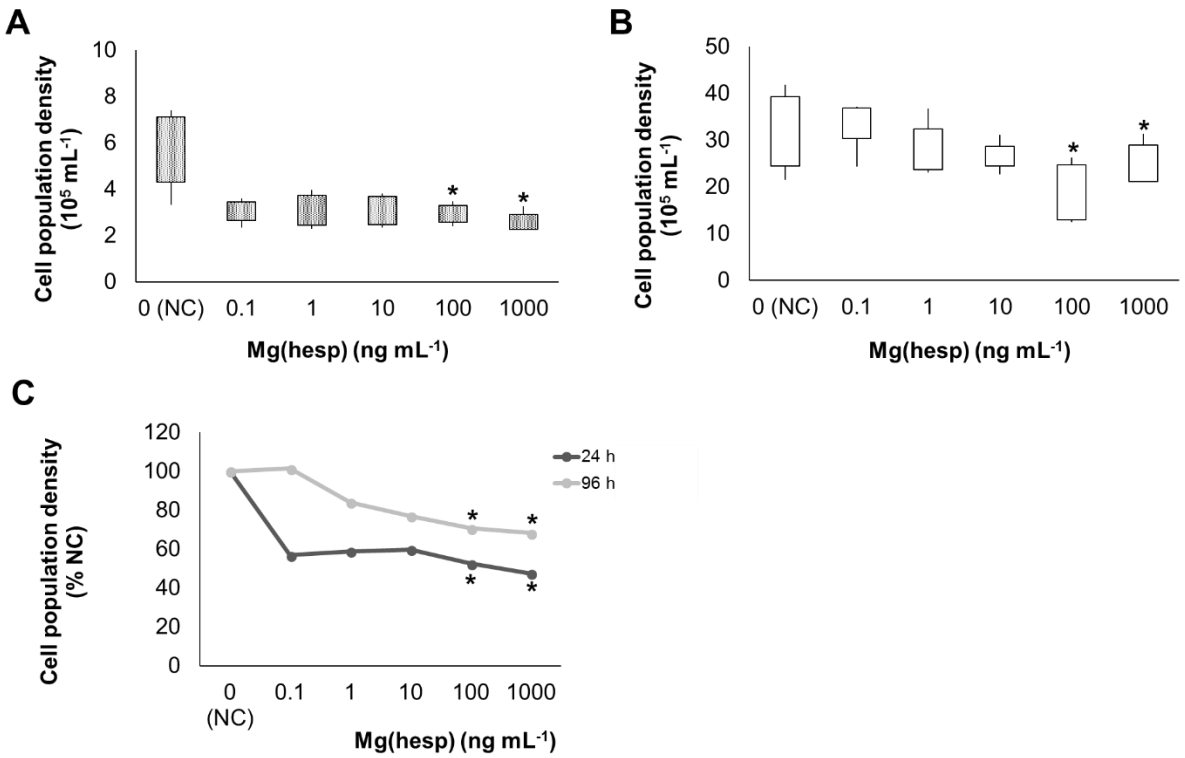

Figure 3. ZF-L cell line population density after 24 (A) and 96 (B) h of exposure to Mg(hesp). *indicates significant difference from negative control (NC), $P < 0.05$. C: Population density percentage relative to negative control (% NC) after 24 and 96 h of exposure to Mg(hesp).

The assessment of cytotoxicity in intracellular organelles showed severe reduction of mitochondrial activity (MTT assay) after 24 h of exposure to Mg(hesp) varying from 35 % at concentrations as low as 1 ng mL^{-1} to 61 % at 1000 ng mL^{-1}, the highest concentration (Fig. 4A, C and Table S1). Meanwhile, after 96 h of exposure to Mg(hesp), the reduction of mitochondrial activity (15 %) was significant only at concentrations equal to or higher than 10 ng mL^{-1} (Fig. 4B, C).

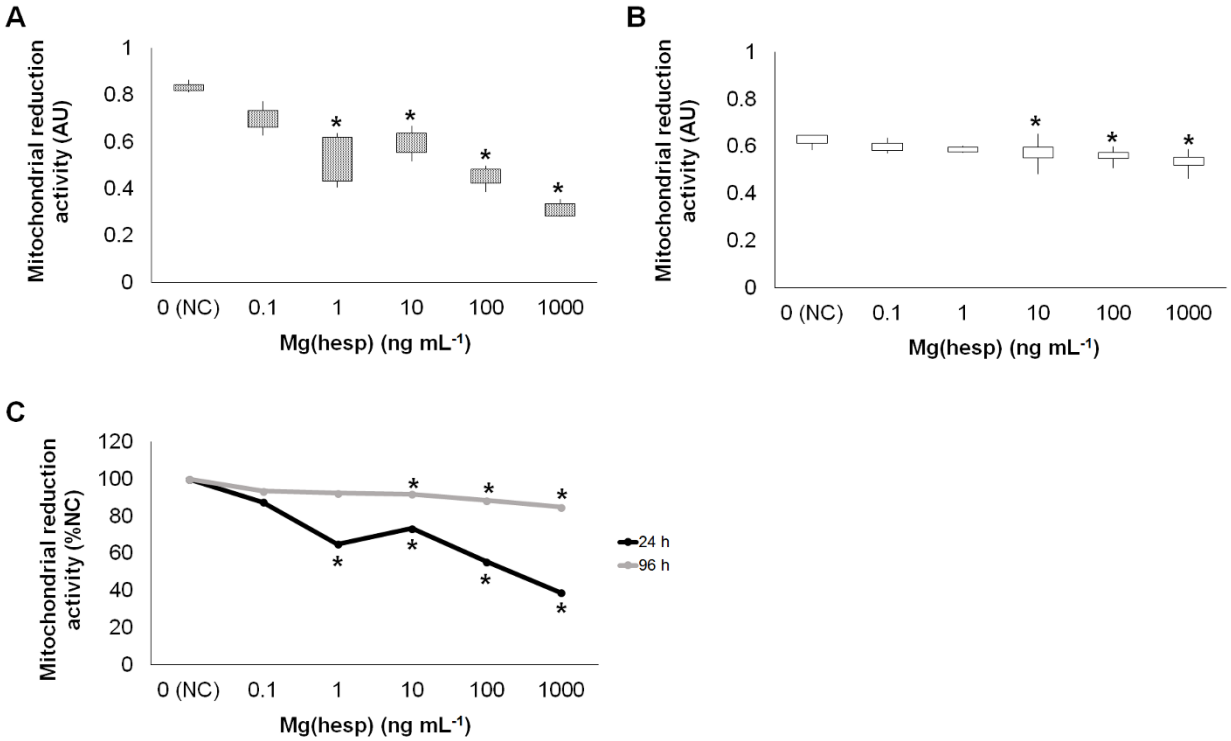

Figure 4. ZF-L cell line mitochondrial activity reduction after 24 (A) and 96 (B) h of exposure to Mg(hesp) *indicates significant difference from negative control (NC), $P < 0.05$. C. Mitochondrial activity reduction percentage relative to negative control (% NC) after 24 and 96 h of exposure to Mg(hesp).

Lysosomal integrity decreased from 12 % to 34 % of NC after 24 h of exposure to all Mg(hesp) concentrations (0.1 to 1000 ng mL^{-1}); after 96 h of exposure, the lysosomal integrity decreased by up to 18% only at Mg(hesp) concentrations of 100 and 1000 ng mL^{-1} (Fig. 5 and Table S1).

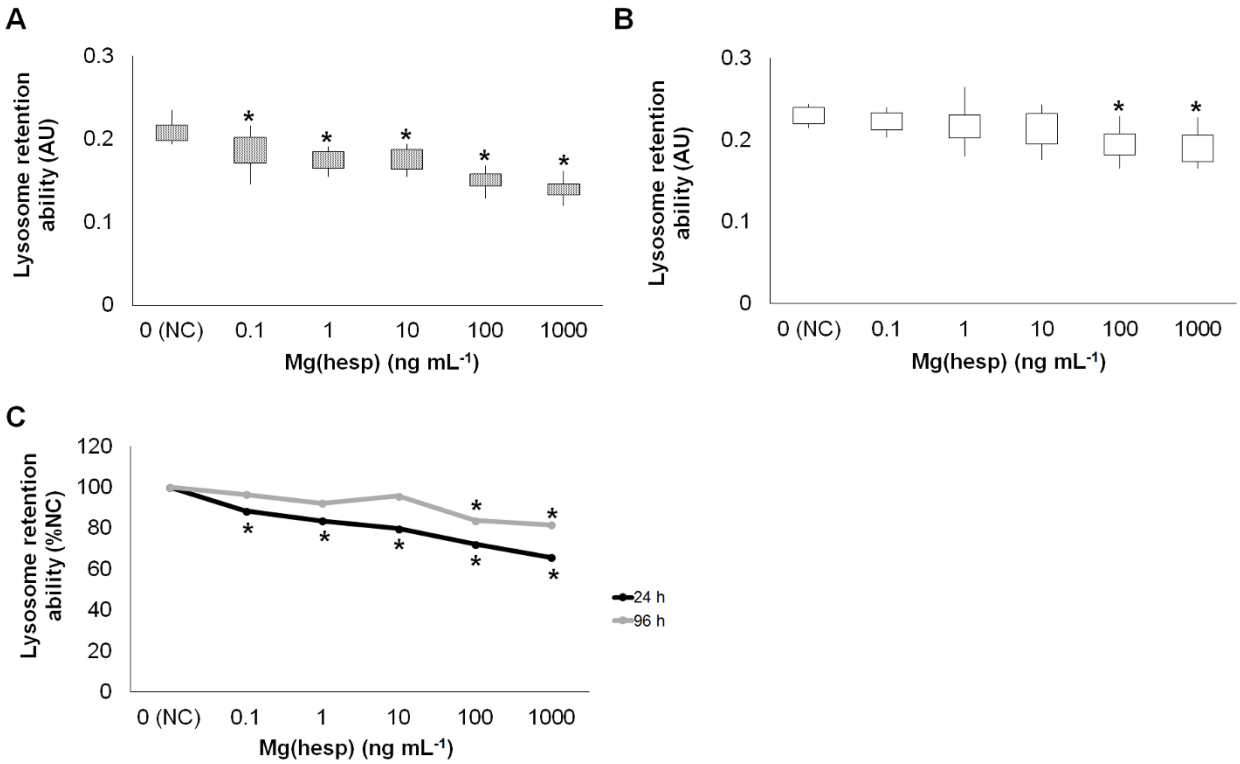

Figure 5. ZF-L cell line lysosome retention ability after 24 (A) and 96 (B) h of exposure to Mg(hesp). *indicates significant difference from negative control (NC), $P < 0.05$. C. Percentage of lysosome retention ability relative to negative control (% NC).

On the other hand, evaluation of cell membrane integrity (Fig. 6 and Table S1) showed no significant differences between treatments in both membrane integrity assays (trypan blue dye input and LDH release in extracellular medium).

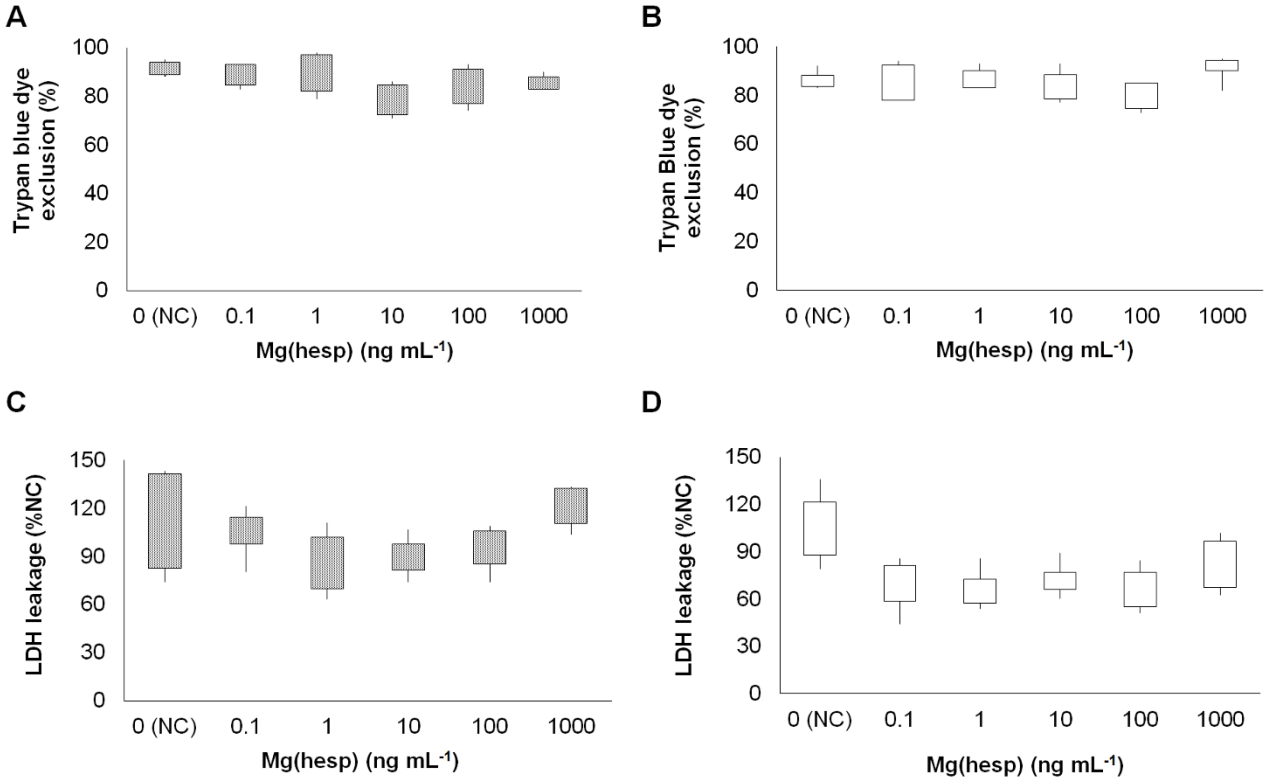

Figure 6. Membrane integrity assays performed in the ZF-L cell line after 24 and 96 h of exposure to different concentrations of Mg(hesp). A and B: Trypan blue dye exclusion test results after 24 and 96 h of exposure, respectively. C and D: Lactate dehydrogenase (LDH) leakage assay after 24 and 96 h exposure, respectively.

The activation of apoptotic or necrotic processes was not significant during any exposure period (10 h, 24 h and 96 h) and Mg(hesp) concentrations (Figures 7 and S1 and Table S2).

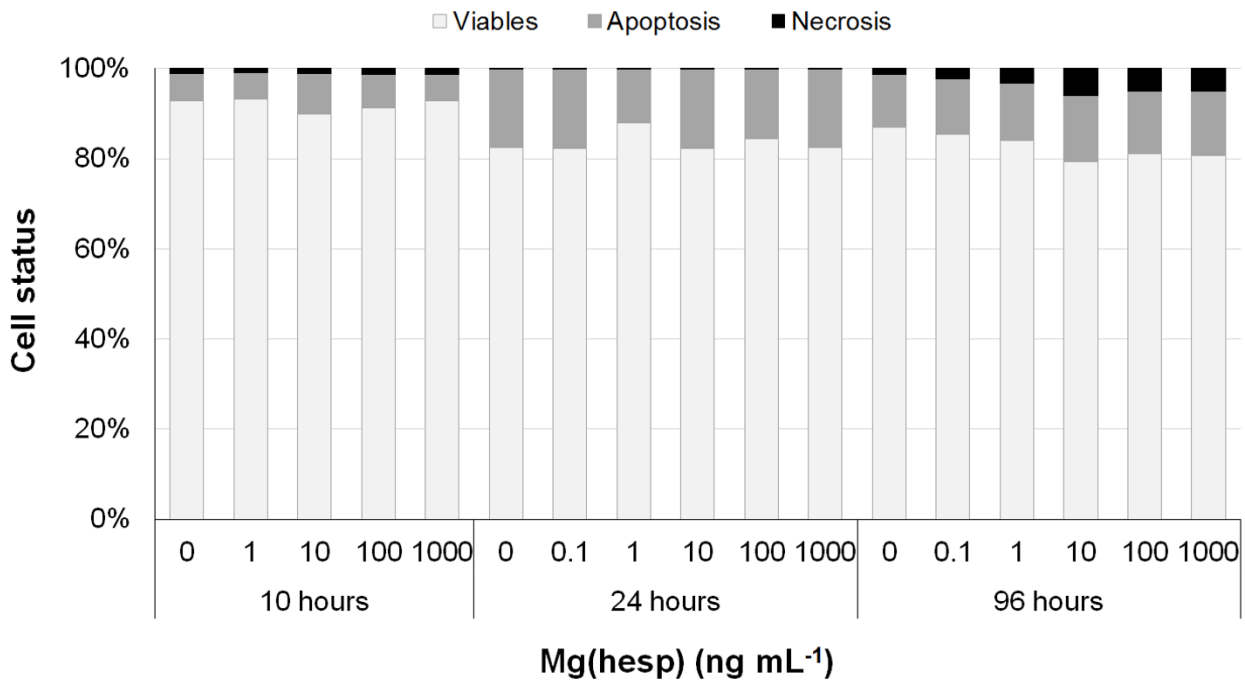

Figure 7. Live (viable), apoptotic and necrotic percentages of the ZF-L cell line after 10, 24 and 96 h of exposure to different concentrations of Mg(hesp). *indicates a significant difference from negative control (NC), $P < 0.05$.

Significant correlations were found between cytotoxic biomarkers. After 24 h exposure to Mg(hesp), cellular density correlated with the reduction in mitochondrial activity (r_s 0.54) and lysosomal retention ability (r_s 0.49), and the reduction in mitochondrial activity correlated with lysosomal retention ability (r_s 0.93). After a 96-h exposure, correlation occurred between reduction of mitochondrial activity and lysosomal retention ability (r_s 0.46).

4 DISCUSSION

This study showed a complex interaction of Mg(hesp) with ZF-L cells, with a strong influence on the cell population. The confluence and alterations in cell morphology evidenced a powerful visual tool to detect the impact on the cell population, being the first indicator of cytotoxic effects of Mg(hesp) at high concentrations (100 and 1000 ng mL^{-1}). The ZF-L cell line from the negative control presented an epithelial appearance at confluence as described by Ghosh et al. (1994); however, the spherical/deformed morphology of many ZF-L cells after exposure to high Mg(hesp) concentrations was associated with a direct effect of this compound on the cells and

not to a loss of adherence as before microscope observation the cells was washed with PBS to remove residual detached cells.

The visual observation was confirmed by the quantification of cellular density, which showed significant decrease, especially after a 24-h exposure at the two highest Mg(hesp) concentrations (100 and 1000 ng mL^{-1}), reaching up to 53% of the negative control. Considering the absence of FBS as a growth factor in the medium during Mg(hesp) exposure, such an effect can be related to a direct influence on cell viability, excluding impacts on cellular growth capacity.

There are many toxic cytological pathways when the cells are subjected to xenobiotic stressor, and the toxic effects may be a consequence of different action mechanisms on the cellular compartments. The cell death process still belongs to a research field full of new discoveries divided into passive (resulting from an overwhelming damage) and active (where the cell triggers processes to its own demise) mechanisms (Tait et al., 2014). The reduction of cell density observed at high Mg(hesp) concentrations was not related to cellular membrane stability, considering that both assays performed (trypan blue dye exclusion and LDH leakage) did not detect membrane damage. The necrosis detection assay corroborated the findings obtained in the trypan blue and LDH assays, evidencing that such a death mechanism was not the reason for the decrease in cell density. Necrosis is generally a passive proinflammatory mechanism in which cell death results from several responses, including loss of membrane integrity (Bortner and Cidlowski, 2016). Furthermore, necrosis is directly associated with increased plasma membrane permeability that can be monitored by cellular leakage of lactate dehydrogenase or uptake of trypan blue dye or other some fluorescent marker (Orreinus et al., 2011). On the other hand, apoptosis pathways involve stereotypic morphological changes, such as the formation of apoptotic bodies and phosphatidylserine outsourcing to the extracellular surface of the cell (Hasanzadeh et al., 2017), which enables the use of annexin V staining as an apoptosis detection protocol (Bortner and Cidlowski, 2016). Such alterations and cellular death pathways were not triggered after Mg(hesp) treatments.

However, any effect on some intracellular organelle or structure (e.g., activity and integrity) limits the cells' capability of forming a compensatory response to overcome toxicity. The decrease in cell density was probably related to a decrease in lysosomal integrity and mitochondrial metabolic activity at high concentration exposures. In lysosomal retention ability assessment using the neutral red uptake

assay, the amount of retained dye is usually proportional to the number of live cells; however, such retention can also be affected by alterations in lysosomal functioning (Repetto et al., 2008). Similar responses can also be considered when estimating mitochondrial activity by MTT assay, and the reduction in mitochondrial activity is indicative of failure of energetic processes, which can lead to deficits in the energy required to maintain cellular metabolic integrity. The correlation between lysosomal retention ability and mitochondrial activity assays indicates that the reductions in cell density were the consequence of such subcellular dysfunctions or alternative cell death mechanisms at high Mg(hesp) concentrations.

Alternative active pathways of cell death may involve some nonapoptotic/necrotic processes, which might occur as a result of progressive loss of mitochondrial function, ultimately leading to a bioenergetic crisis and cell death (Tait et al., 2014). In addition, some stress factors can activate autophagy processes as an alternative pathway for cell death (type II cell death) in which the massive destruction of cytosolic compounds and/or organelles can be consequences of extensive formation of autophagic vacuoles leading to a total failure of cellular functions and viability (Maiuri et al., 2007).

On the other hand, in comparing both exposure periods, 24 and 96 h, the effects of Mg(hesp) after 96 h of exposure were not so severe as those after 24 h of exposure, which could be related to the cell's adaptive capacity to overcome stressful conditions or the liver cell's ability to metabolize xenobiotics. Such comparisons as emphasized by Crawford (2009), provide information on an organism's ability to recover or resist the adverse effects of a pesticide or identify the potential for latent effects.

Many toxicology assays must be interpreted with caution, considering that detection of such significant results represents an alert to investigate specifics mechanisms that can be triggered, particularly when dealing with novel chemical compounds with promising applications, such as Mg(hesp). The data provided by this preliminary study indicate that, despite causing stress responses and cellular damage to in vitro aquatic models, Mg(hesp) at high concentrations (1000 ng mL^{-1}) can have its toxic effects mitigated after longer periods, enabling the cellular population to initiate recovery processes.

Effective pesticide results of Mg(hesp) are obtained at concentrations around 140 ng mL^{-1} in solid bites (Oliveira, 2012). Thus, potential contamination of aquatic environments as a consequence of Mg(hesp) use as a pesticide will probably be

among the lowest concentrations evaluated in this study, which did not presented significant toxic effects. Aquatic contamination depends mainly on the soil's capacity to filter, immobilize and degrade organic and inorganic compounds (Keesstra et al., 2012). Furthermore, the Mg(hesp) concentrations used in this study (from 0.1 to 1,000 ng mL^{-1}) represent rates that are above the generally accepted limits (< 1.0 ng mL^{-1}) for organic contamination in aquatic ecosystems (Brazil, 2005).

Assessing pesticide effects by using different assays is a crucial step to understanding the mechanisms of cellular responses, particularly when dealing with novel chemicals. The data collected in this study provide important information on screening of the initial effects of Mg(hesp) indicating its cytotoxic influences and the potential mechanisms of the cellular response and stimulating further analysis to elucidate cellular pathways triggered and, moreover, to integrate *in vitro* and *in vivo* responses.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

Table S1. Cytotoxic assays results performed in ZF-L cell line after 24 and 96 h of exposure to different Mg(hesp) concentrations. Values are expressed as median (first quartile – third quartile). Letters indicates significant difference inside same period of exposure ($P < 0.05$). AU – absorption units.

Exposure Treatments (ng mL^{-1})		Cytotoxic assays				
		Cell density ($x10^5$ mL^{-1})	Cell membrane integrity		Mitochondrial activity (AU)	Lysosome retention ability (AU)
			Trypan blue dye exclusion (%)	LDH leakage (% of NC)		
24h exposure	**0 (NC)**	5.270 (4.295 - 7.125) [a]	89.0 (89.0 - 94.0) [a]	100.00 (82.55 - 141.32) [a]	0.826 (0.819 - 0.845) [a]	0.212 (0.198 - 0.217) [a]
	0.1	2.990 (2.640 - 3.455) [a]	87.0 (84.5 - 93.0) [a]	108.31 (97.48 - 114.51) [a]	0.723 (0.662 - 0.734) [a]	0.186 (0.171 - 0.202) [b]
	1	3.100 (2.445 - 3.725) [a]	92.0 (82.0 - 97.0) [a]	82.80 (69.72 - 101.93) [a]	0.537 (0.431 - 0.619) [b]	0.176 (0.164 - 0.185) [b]
	10	3.150 (2.475 - 3.670) [a]	77.0 (72.5 - 84.5) [a]	86.79 (81.23 - 97.61) [a]	0.606 (0.555 - 0.635) [b]	0.168 (0.164 - 0.187) [b]
	100	2.770 (2.580 - 3.290) [b]	84 (77.0 - 91.0) [a]	92.32 (85.24 - 106.0) [a]	0.459 (0.423 - 0.482) [b]	0.152 (0.143 - 0.158) [b]
	1000	2.500 (2.255 - 2.914) [b]	85.5 (83.0 - 88.0) [a]	124.08 (110.38 - 132.42) [a]	0.321 (0.285 - 0.334) [b]	0.139 (0.133 - 0.146) [b]
96h exposure	**0 (NC)**	33.150 (24.500 - 39.325) [a]	85.5 (83.75 - 88.25) [a]	100.00 (88.05 - 121.37) [a]	0.635 (0.613 - 0.646) [a]	0.234 (0.220 - 0.240) [a]
	0.1	33.600 (30.375 - 36.875) [a]	81.5 (78 - 92.5) [a]	67.81 (58.80 - 81.41) [a]	0.595 (0.583 - 0.614) [a]	0.226 (0.212 - 0.234) [a]
	1	27.85 (23.700 - 32.425) [a]	86.5 (83.0 - 90.0) [a]	69.19 (57.46 - 72.62) [a]	0.588 (0.575 - 0.597) [a]	0.216 (0.203 - 0.231) [a]
	10	25.500 (24.425 - 28.625) [a]	83.0 (78.5 - 88.5) [a]	71.05 (66.07 - 76.91) [a]	0.584 (0.551 - 0.597) [b]	0.224 (0.195 - 0.233) [a]
	100	23.400 (12.925 - 24.725) [b]	83.0 (74.5 - 85.0) [a]	72.36 (55.02 - 76.99) [a]	0.562 (0.549 - 0.574) [b]	0.196 (0.182 - 0.208) [b]
	1000	22.600 (21.075 - 28.900) [b]	93.0 (90.25 - 94.25) [a]	83.11 (67.13 - 96.70) [a]	0.540 (0.522 - 0.553) [b]	0.191 (0.174 - 0.206) [b]

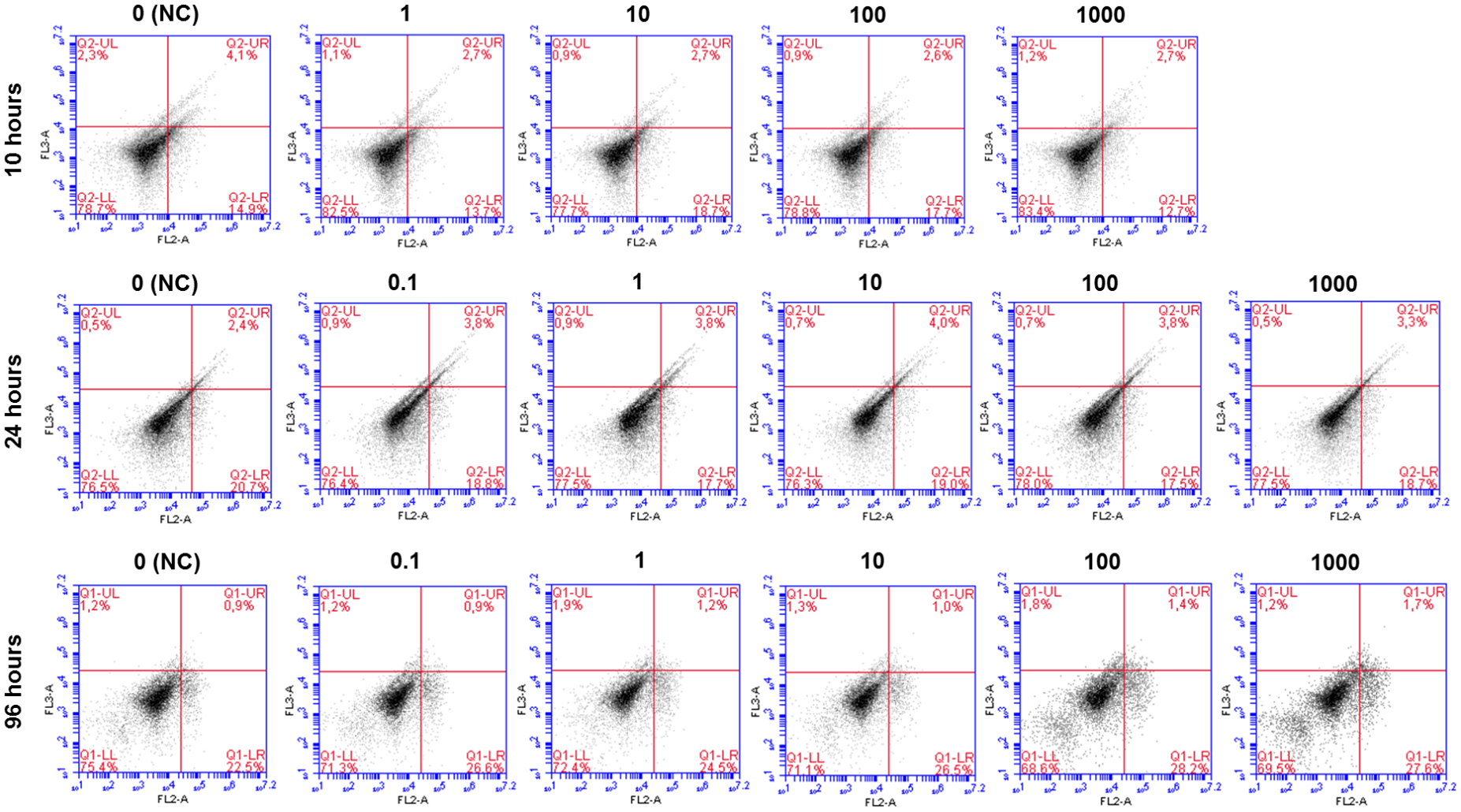

Figure S1. Apoptotic and necrotic cell death induced by different concentrations of Mg(hesp) on ZF-L cell line after 10, 24 and 96hours exposure. Viable cells are shown in Q1-LL; initial apoptosis in Q1-LR; final apoptosis in Q1-UR and necrosis in Q1-UL, measured using flow cytometry.

Table S2. Results from apoptosis and necrosis analyses performed in ZF-L cell lines after different exposure periods of Mg(hesp). Values are expressed as mean ± standard error. No significative difference was detected (P < 0.05).

Exposure Treatments (ng mL^{-1})		Apoptosis/Necrosis assay		
		Viables (%)	Apoptotic (%)	Necrotic (%)
10h exposure	**0 (NC)**	92.35 ± 0.45	6.60 ± 0.20	1.05 ± 0.05
	1	93.20 ± 0.30	5.95 ± 0.70	0.85 ± 0.45
	10	89.85 ± 1.45	8.90 ± 1.10	1.25 ± 0.30
	100	91.35 ± 0.15	7.40 ± 0.10	1.25 ± 0.25
	1000	92.90 ± 0.05	5.85 ± 0.15	1.25 ± 0.15
24h exposure	**0 (NC)**	82.35 ± 0.78	17.45 ± 0.78	0.20 ± 0.01
	0.1	82.20 ± 1.66	17.64 ± 1.59	0.16 ± 0.06
	1	83.05 ± 1.63	16.76 ± 9.74	0.19 ± 0.07
	10	82.23 ± 0.35	17.60 ± 0.46	0.17 ± 0.06
	100	84.36 ± 0.91	15.50 ± 0.96	0.14 ± 0.06
	1000	82.43 ± 1.50	17.44 ± 1.45	0.13 ± 0.06
96h exposure	**0 (NC)**	87.00 ± 1.70	11.70 ± 1.63	1.30 ± 0.02
	0.1	85.34 ± 2.57	12.40 ± 2.60	2.26 ± 0.12
	1	84.04 ± 2.77	12.61 ± 2.59	3.35 ± 0.15
	10	79.30 ± 2.04	14.65 ± 1.99	6.04 ± 0.21
	100	81.18 ± 3.32	13.83 ± 3.32	4.99 ± 0.06
	1000	80.71 ± 4.21	14.21 ± 4.06	5.08 ± 0.21

3.2 Artigo 2

Genotoxic, biochemical and cell cycle assessment in zebrafish (*Danio rerio*) hepatocyte cell line (ZF-L) of a novel metaloinsecticide

ABSTRACT

This study evaluated genotoxic and biochemical responses of ZF-L hepatocytes to a novel potential pesticide (metal-flavonoid complex Mg(hesp)), where results obtained have great importance, considering that the lack of previous information and the complexity of whole organisms triggered responses by new chemical compounds. Mg(hesp) affects cell stability by increasing reactive oxygen species (ROS) in both exposure times (24 h and 96 h). CAT activity decreased after 24 h of exposure, which could be related with pesticide influences in its expression. GSH and metallothionein seemed to be main biotransformation responses, avoiding overwhelming oxidative damages, corroborated by the absence of increases in lipid peroxidation values. ROS influence, however, had strong influences on DNA integrity, mainly after 24 h. In Comet Assay, increases in head intensity accompanied by decreases in percentage of DNA in tail along with high rates of nuclear abnormalities and cell cycle alterations (increase in Sub-G1 phase and decrease in S-phase) could be associated with chromosomal instability and triggering of DNA repair complexes. Thus, genetic stress responses due to Mg(hesp) concentrations were observed in high rates (up to 1 ng mL^{-1}), which are close to dosage of application and that are above general limits for organic contamination in aquatic ecosystems, emphasizing the promising use of such compound.

Keywords: pesticide; comet assay; micronucleus; antioxidant; biotransformation.

RESUMO

O presente estudo avaliou as repostas genotóxicas e bioquímicas em hepatócitos ZF-L frente à exposição a um novo potencial pesticida (o complexo metal-flavonóide Mg(hesp)), onde os resultados obtidos são inéditos, considerando a ausência de literatura prévia e a complexidade das respostas desencadeadas em organismos por novos compostos químicos. Mg(hesp) afetou a estabilidade celular por meio de aumentos na produção de espécies reativas de oxigênio (EROS) em ambos os períodos de exposição (24 h e 96 h). A atividade da CAT diminuiu após 24 h de exposição, o que pode estar relacionado com uma provável influência do pesticida em sua expressão. GSH e metalotioneína se mostraram as principais respostas de biotransformação, evitando danos oxidativos exacerbados, fato corroborado pela ausência de variações nos valores de peroxidação lipídica. As influências de EROS, entretanto, apresentaram fortes efeitos na integridade do DNA, principalmente após 24 h. No Ensaio do Cometa, aumentos na intensidade da cabeça do cometa foram acompanhados por reduções na porcentagem do DNA na cauda o que, em adição às altas taxas de anormalidades nucleares e alterações no ciclo celular (aumento na fase Sub-G1 e redução na fase S) podem estar associados com eventos de instabilidade cromossômica e a ativação de mecanismos de reparo de DNA. Portanto, as respostas de estresse genético devido à exposição à Mg(hesp) foram observadas em altas concentrações (até 1 ng mL^{-1}), valores próximos à dosagem de aplicação sugerida e que estão acima dos limites gerais de poluentes orgânicos em ambientes aquáticos, enfatizando o promissor uso do composto.

Palavras-chave: pesticida; ensaio do cometa; micronúcleo; antioxidante; biotransformação.

1 INTRODUCTION

Agriculture activity is essential for food production and represents one of the most important economic areas in several countries (Carvalho et al., 2000; Oliveira, 2012). In this scenario, agrochemicals have been an alternative to mitigate crop losses caused by such plagues. Despite its efficacy, the intense use of such compounds can contaminate the environment including the aquatic ecosystems and threatening non-targeting organisms (Akerblom, 2004). Therefore, the development and application of agrochemicals with lower ecological impact and selective action are under industrial focus which includes the manipulation of natural substances with potential pesticide action.

Some micronutrients such as magnesium (Mg II) and plant metabolites from flavonoids class, such as hesperidin has important roles in plant biological functions, including the defense action against plagues (Zanetti et al., 2004). Metal-flavonoid complexes magnify the flavonoids biological activities and become them more water soluble (De Souza and De Giovani, 2004; Oliveira, 2012). The complex $[Mg(hesp)_2(phen)]$, here referred as Mg(hesp), has high insecticide activity against leafcutting ant, *Atta sexdens rubropilosa*, an insect which causes extensive damages to *Pinus* sp., *Eucalyptus* sp. plantation and other culture crops. This complex application as bites (concentrations around 140 ng mL^{-1} – proportion of 0.2% by weight of the diet) reaches mortality rate similar to the common commercial insecticide, the sulphluramide (Oliveira et al., 2013; Fernandes et al., 2015). Sulphluramide is an intensively used insecticide to control ants in the South America, however, it is classified as a persistent organic pollutant (POP) and toxic for non-target animals (Schnellmann, 1990; Peden-Adams et al, 2007). At now, sulphluramide has been included in the Brazilian National Action Plan to reduce and eliminate industrial POPs (Brazil, 2015) and has been prohibited in some Brazilian states (Environmental Protection Foundation of Rio Grande do Sul State, 2018).

The evaluation of the effects of the new compounds such as Mg(hesp) complex on non-target organisms have high relevance in preserve them and the environment. Previous study of Mg(hesp) complex action on the zebrafish hepatocyte cell line (ZF-L) showed potential cytotoxical effects at high concentrations (100 and 1000 ng mL^{-1}), including decreases in cell population and impairment of cellular compartments (as reported in Chapter 1). Thus, the main goal of this study was to assess the biochemical, physiological and molecular effects of this new insecticide on ZF-L cell line to elucidate

the action mechanism of Mg(hesp) complex. Biomarkers of antioxidant cell defense system, biotransformation enzyme of phase two, oxidative stress and DNA integrity as well as potential alterations in cell cycle were determined to evaluate the magnitude and potential harmful action of such new insecticide.

2 MATERIALS AND METHODS

2.1 Complex characterization

The Mg(hesp) complex was obtained from Inorganic and Bioinorganic Photochemistry Laboratory of Chemistry Department of Federal University of São Carlos, São Paulo/Brazil. The complex present as chemical formulation ($MgC_{68}H_{74}N_2O_{30}.2H_2O$) and includes the presence of phenanthroline as chelating agent, generating a yellow powder with high photochemical stability, absorbance peaks at 260-300 nm and no emission produced when dissolved in water or cyclohexane, water solubility equal to 472 µg mL^{-1} and chemical stability in pH ranges from 3 to 9 (Oliveira et al., 2013; Fernandes et al., 2015).

2.2 Zebrafish hepatocyte cell culture

Zebrafish (*Danio rerio*) hepatocyte cell line (ZF-L) was obtained from Rio de Janeiro Cell Bank – UFRJ and maintained in culture flasks (75 cm^2) incubated in medium containing 40% RPMI 1640 medium (Sigma Aldrich, St Louis, USA), 50% Leibovitz's L-15 medium (Cultilab, Campinas, Brazil) supplemented with 10% fetal bovine serum (FBS, 1492-500 lot, Cultilab, Campinas, Brazil) and 1% antibiotic/anti-mycotic solution (penicillin/streptomycin and fungizone, Sigma Aldrich, St Louis – USA) at 28° C. Cells were sub-cultured twice/three times per week, respecting limit confluence and viability, according to its cell cycle (around 72 h).

2.3 Experimental conditions

Each experiment used culture passages of number 15-25 and the Mg(hesp) exposure was carried out after 24 h culture stabilization to ensure cells were adhered (confirmed through microscope visualization). Three independent experiments were performed including technical replicates and different seed concentrations were used according to each exposure time and plates to avoid cellular stress due to super population. All Mg(hesp) treatments were performed without addition of FBS, except for cell cycle analysis.

The ZF-L cells were exposed to six Mg(hesp) concentrations: 0 (negative control, NC), 0.1, 1.0, 10, 100 and 1000 ng mL^{-1}. The Mg(hesp) stock solution was prepared using a low frequency (40 KHz) ultrasound bath (Q335D, QUIMIS®, Brazil) and the desired concentrations in each experiment were obtained by diluting the stock solution into culture medium, respecting a limit of 1% of total work volume. In addition to Mg(hesp) concentrations evaluated, cells were also exposed only to PBS (1% of total work volume) in all assays to ensure that results reflected only Mg(hesp) effects (data not shown). The cells were exposed to Mg(hesp) for 24 h and 96 h, except for cell cycle analysis in which was respect the full cell cycle period of them (72 h).

2.4 Biochemical biomarkers

2.4.1 Reactive Oxygen Species (ROS) production

The Mg(hesp) induction of ROS generation were determined by oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma–Aldrich, USA) (Wan et al., 1993; Cavalcante et al., 2014). Cells were seeded in black 96-well flat-bottom plate at concentrations of 0.25 x 10^6 cells per well for 24 h exposure and 0.1 x 10^6 cells per well for 96 h exposure. After Mg(hesp) treatments, the cells were incubated with 10 µM H_2DCFDA for 30 min and, then, suspended in phosphate-buffered saline. Fluorescence was measured with excitation/emission at 494/518 nm using a microplate reader (SpectraMax M5, Molecular Devices, USA) and ROS production was expressed in fluorescent units (FU).

2.4.2. Antioxidants and oxidative biomarkers determination

For antioxidant system and oxidative stress analyses, 75 cm^2 flasks were seeded with 10 x 10^6 cells for 24 h exposure and 8 x 10^6 cells for 96 h exposure. After treatments with different concentration of Mg(hesp) for 24 h and 96 h, cells were detached, centrifuged (12,000 rpm for 5 min at 4 ºC), immediately frozen in liquid nitrogen and preserved at -80 °C. For analyses, the cells were disrupted using a sonic dismembrator (500 Sonic Dismembrator, Fisher Scientific, USA) for 3 min (30 sec of sonication with 30 sec intervals) in ice. Samples were then centrifuged for 10 min at 10,700x *g* at 4 °C and supernatant aliquots were preserved at -80 °C until analysis.

The total protein concentration of each sample was determined according to the Bradford method (Bradford, 1976) at 595 nm using bovine serum albumin as standard. Six biochemical biomarkers were assessed: the activities of superoxide dismutase

(SOD) were measured according to McCord et al. (1977) and catalase (CAT) as described by Beutler (1975); the activity of the biotransformation enzyme of phase two, glutathione S-transferase (GST) were assessed according to the method described by Habig et al. (1974). Reduced glutathione (GSH) and metallothionein content (MT) were quantified according to Wilhelm-Filho et al. (2005) and Viarengo et al. (1997), respectively. Lipid hydroperoxide (LPO) were quantified through FOX method according to Jiang et al. (1991). All analyses were adapted to a microplate reader (SpectraMax M5, Molecular Devices, USA).

2.5 Cytological biomarkers

2.5.1 Alkaline Comet Assay

The effect of Mg(hesp) concentrations on DNA was assessed using the alkaline version of Comet Assay as described by Singh et al. (1988) with modifications (McKelvey-Martin et al., 1993; Tice et al., 2000). Flaks of 25 cm^2 were seeded with 2.5 x 10^6 cells for 24 h exposure and 1.7 x 10^6 cells for 96 h exposure. After Mg(hesp) treatments, agarose cell slides were prepared and incubated in lysis solution (100 mM ethylenediaminetetraacetic acid – EDTA, 2.5 M sodium chloride, 10 mM Trizma base, 1% Triton X-100, 20% dimethyl sulfoxide – DMSO, pH 10.0) for at least 24 h. Electrophoresis was performed at 25 V and 300 mA for 20 min. Then, the slides were fixed with absolute ethanol and stained with GelRed™ solution (Biotium, Inc). Nucleoids images were obtained with a fluorescent microscope (Olympus BX-61 with CellSens™ software, Olympus, PA-USA) at 200x magnification using a 515-560 nm excitation filter and 590 nm barrier filter. The same exposure time was applied for all slides in order to correctly compare intensities between samples. Seven hundred nucleoids were analyzed using the OpenComet© software (Gyori et al., 2014) which provided comet parameters of head intensity (in pixels) and tail DNA content (in percentage).

2.5.2 Cytokinesis-block micronucleus cytome assay (CBMN Cyt assay)

Genotoxicity, mutagenicity, cytostasis and cytotoxicity were evaluated by cytokinesis-block micronucleus cytome assay (CBMN) as described by Fenech (2007). Cells were seeded in 6-well plate at a concentration of 1.6 x 10^6 for 24 h exposure and 0.9 x 10^6 for 96 h exposure. After Mg(hesp) treatments, cytochalasin B (at final concentration of 3 µg mL^{-1}) was added in each flask and incubated for 72 h. Cell slides

were stained with 5% Giemsa and analyzed under light microscope (Olympus BX-61 with CellSens™ software, Olympus, PA-USA) at 400x magnification.

The following indexes were calculated according to Fenech (2007): Nuclear Division Index (NDI), Cytotoxicity Index (CytI), Genotoxicity Index (GenI), Mutagenicity Index (MutI) and Micronuclei Cell Distribution Index (MNi distribution). NDI was calculated by evaluating 500 viable cells per slide to determine the frequency of 1, 2, 3, 4 or more nuclei inside same cytoplasmic boundary. NDI was calculated as $NDI = (M_1 + 2M_2 + 3M_3 + 4M_4)/N$, where M_1–M_4 represents the number of cells with 1–4 nuclei and N is the total number of viable cells scored. CytI was determined by scoring apoptotic and necrotic cells in a population of 500 cells. GenI was estimated by scoring nucleoplasmic bridges and nuclear buds in a population of 1000 viable binucleated cells. MutI was calculated by quantifying binucleated cells with presence of one or more micronuclei in a population of 1000 viable binucleated cells. MNi distribution was quantified through the ratio of total number of micronuclei per total number of binucleated cells with one or more micronuclei in order to detect potential multiple micronuclei formation in cells.

2.5.3 Cellular cycle

The effect of Mg(hesp) concentrations on cell cycle was determined by flow cytometry of propidium iodide (PI)-stained cells (Ormerod, 2008; He, 2010). For this assay, 6-well plates were seeded at a density of 7 x 10^5 cells per well. To ensure that all cells were at the same stage of cell cycle to carry the assay, a period of 72 h of starvation was performed before Mg(hesp) treatments. After exposure to different concentrations of Mg(hesp) with FBS addition for a full ZF-L cell cycle (72 h), cells were fixed with 70% ethanol for 24 h at 4 °C. The cells were, then, incubated with 0.2 mg mL^{-1} RNase in the dark for 30 min at 37 °C. Binding buffer containing PI was added and incubated for 1 h in ice. The phases of cell cycle were determined on a flow cytometer (BD Accuri C6; BD Biosciences, NJ – USA). Data were reported in percentage of cell population analyzed.

2.6 Statistics

All results were presented as median (first quartile – third quartile). Data were tested for normal distribution (Shapiro-Wilk's test). Parametric data were analyzed

applying ANOVA followed by Tukey's multiple comparison test as *post hoc* ($P < 0.05$). Non-parametric data were analyzed applying Kruskal-Wally's followed by Dunn's multiple comparison test as *post hoc* ($P < 0.05$). All statistics analyzes were performed with InfoStat (Di Rienzo et al., 2010).

3 RESULTS

3.1 Biochemical biomarkers

3.1.1 Reactive Oxygen Species production

ROS production after Mg(hesp) exposure were shown in Table 1 and Figure 1. After 24 h exposure, despite the significative decrease in ROS production in 0.1 and 1 ng mL^{-1} Mg(hesp) concentrations, it increased after 1000 ng mL^{-1} Mg(hesp) exposure. At 96 h exposure to Mg(hesp), ROS production increased significantly at 100 and 1000 ng mL^{-1} Mg(hesp) concentrations.

In order to proportionally compare the different times of exposure, Figure 1 represents the percentage values relative to negative control. The percentage increasing in ROS generation at 100 and 1000 ng mL^{-1} Mg(hesp) concentrations were higher after 96 h (increases of 25% up to 52%) exposure than after 24 h exposure (increases of 6% in 1000 ng mL^{-1}) at the same concentrations.

Table 1. ROS production (FU – fluorescence units) in ZF-L cell lines after exposure to different Mg(hesp) concentrations for 24 h and 96 h. Values are median (first quartile – third quartile). Lowercase letters indicate significant differences ($P < 0.05$) inside same period of exposure.

Exposure treatments (ng mL^{-1})	ROS production (FU)	
	24 h exposure	96 h exposure
0 (NC)	77.68 (71.10 – 83.35) [a]	68.21 (64.71 – 77.73) [a]
0.1	69.55 (63.03 – 78.41) [bc]	73.72 (62.30 – 81.68) [a]
1	68.60 (64.38 – 72.05) [c]	77.47 (75.26 – 80.32) [ab]
10	76.00 (71.26 – 79.97) [ab]	78.39 (67.99 – 95.20) [ab]
100	73.80 (69.40 – 75.73) [abc]	85.85 (81.69 – 92.08) [b]
1000	82.25 (78.95 – 88.50) [d]	103.96 (100.24 – 126.88) [c]

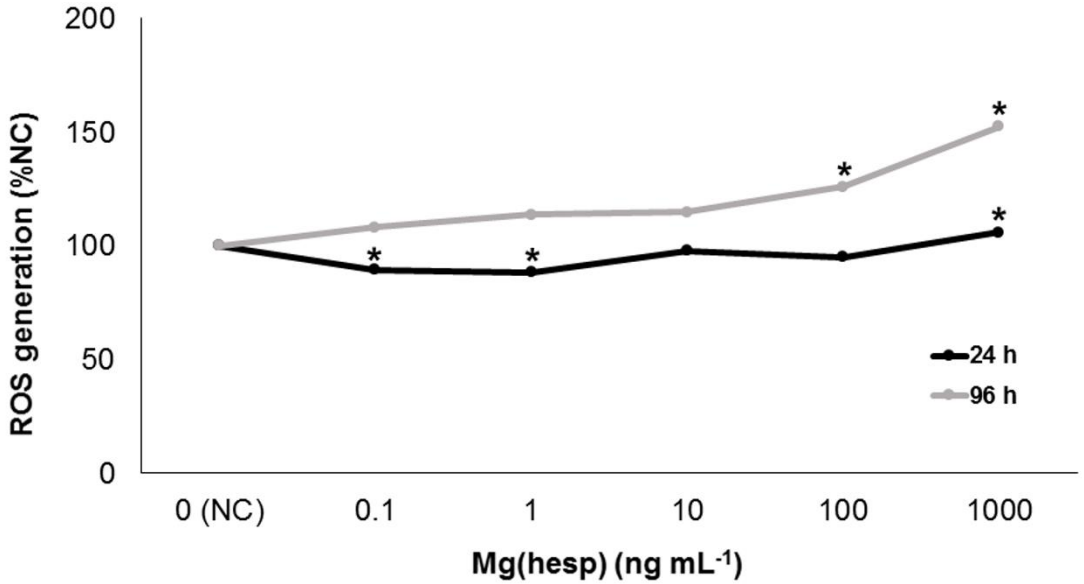

Figure 1. Response of reactive oxygen species (ROS) generation in percentage from the negative control (% NC) after 24 h (black line) and 96 h (grey line) exposure to Mg(hesp). * indicates significant difference from negative control (NC, $P < 0.05$).

3.1.2 Antioxidants and oxidative biomarkers determination

Biochemical biomarkers of antioxidant defenses, biotransformation enzyme of phase II and oxidative stress responses are shown in Table 2. Comparing the biochemical biomarkers with significant responses in different exposure times, Figure 2 represents the percentage values relative to negative control.

No alterations in GST activity was detected between ZF-L from NC and those exposed to Mg(hesp) for 24 and 96 h. The significant response of SOD activity (1000 ng mL^{-1}) after 96 h represented an increase of 46% from NC (Figure 2A), while values of CAT activity decreased up to 65% only after 24 h of exposure (Figure 2B). The variation in GSH content was up to three-fold higher than NC after exposure to 10 and 100 ng mL^{-1} Mg(hesp), while after 96 h exposure the values increased up to two-fold higher atfter exposure to 0.1 and 1000 ng mL^{-1} (Figure 2C). MT content increased up to 90% after 96 h of exposure (Figure 2D). Despite Mg(hesp) treatments may triggered some biochemical responses, effects of the compound did not included damage in cellular membrane, considering that LPO content did not differ from NC in any Mg(hesp) concentrations, in both exposure periods.

Table 2. Biochemical biomarkers (superoxide dismutase – SOD, catalase – CAT, glutathione S-transferase – GST activities, reduced glutathione – GSH, metallothionein – MT and lipid peroxidation – LPO content) in ZF-L cell lines after 24 and 96 h exposure to different Mg(hesp) concentrations. Values are expressed as median (first quartile – third quartile). Letters indicate significant differences ($P < 0.05$) in the same exposure period.

Exposure treatments (ng mL^{-1})		Biochemical biomarkers					
		SOD (SOD unit.mg $protein^{-1}$)	CAT (nmol H_2O_2.mg $protein^{-1}.min^{-1}$)	GST (nmol CDNB.mg $protein^{-1}.min^{-1}$)	GSH (µmol.mg $protein^{-1}$. mL^{-1})	MT (ng. mg $protein^{-1}$. mL^{-1})	LPO (nmol H_2O_2.mg $protein^{-1}$)
24h exporuse	**0 (NC)**	51.31 (40.17 – 63.63) [a]	16.17 (15.46 – 22.49) [a]	33.93 (23.68 – 42.80) [a]	5.64 (4.27 – 6.12) [ab]	5.37 (3.58 – 6.49) [a]	0.21 (0.20 – 0.38) [a]
	0.1	43.36 (43.18 – 45.28) [a]	7.03 (6.67 – 7.49) [b]	43.34 (35.82 – 43.48) [a]	7.13 (6.61 – 7.40) [abc]	8.62 (6.65 – 9.42) [b]	0.27 (0.19 – 0.336) [a]
	1	45.31 (44.76 – 47.33) [a]	6.97 (6.08 – 8.87) [b]	45.05 (26.14 – 49.47) [a]	4.77 (4.27 – 5.26) [a]	3.80 (3.57 – 3.96) [a]	0.17 (0.16 – 0.18) [a]
	10	43.16 (42.43 – 49.16) [a]	6.37 (6.07 – 7.75) [b]	32.56 (30.22 – 32.94) [a]	16.66 (14.49 – 18.72) [cd]	5.51 (5.41 – 5.95) [ab]	0.26 (0.22 – 0.26) [a]
	100	47.37 (44.14 – 55.49) [a]	5.67 (5.21 – 5.87) [b]	43.88 (38.82 – 47.24) [a]	34.07 (32.68 – 34.28) [d]	6.51 (6.24 – 6.70) [ab]	0.24 (0.21 – 0.34) [a]
	1000	39.50 (37.92 – 43.11) [a]	5.82 (5.70 – 6.65) [b]	34.24 (20.58 – 36.76) [a]	14.21 (11.84 – 14.71) [bcd]	5.65 (4.08 – 6.57) [ab]	0.29 (0.16 – 0.34) [a]
96h exporuse	**0 (NC)**	44.03 (43.57 – 44.75) [a]	3.69 (1.62 – 4.59) [a]	103.94 (78.59 – 125.27) [a]	12.82 (9.67 – 14.80) [a]	2.81 (2.55 – 3.46) [a]	0.11 (0.09 – 0.12) [a]
	0.1	47.23 (44.81 – 49.88) [a]	3.67 (2.89 – 3.67) [a]	149.48 (105.21 – 150.52) [a]	30.00 (25.27 – 30.47) [b]	4.42 (4.15 – 4.53) [ab]	0.12 (0.07 – 0.18) [a]
	1	44.23 (43.10 – 50.06) [a]	1.96 (1.89 – 2.02) [a]	109.68 (92.87 – 123.22) [a]	26.33 (22.55 – 29.28) [ab]	4.24 (3.61 – 4.82) [ab]	0.13 (0.13 – 0.13) [a]
	10	44.73 (39.14 – 46.69) [a]	2.93 (1.69 – 3.65) [a]	153.13 (116.63 – 164.23) [a]	21.44 (19.37 – 23.18) [ab]	3.59 (3.45 – 3.63) [ab]	0.09 (0.07 – 0.10) [a]
	100	43.07 (42.45 – 43.36) [a]	2.84 (2.57 – 3.13) [a]	103.63 (100.01 – 105.57) [a]	24.52 (17.63 – 30.38) [ab]	5.35 (4.15 – 6.31) [b]	0.12 (0.10 – 0.15) [a]
	1000	64.21 (61.94 – 65.87) [b]	2.63 (1.59 – 2.91) [a]	106.54 (90.85 – 116.72) [a]	27.92 (21.22 – 30.69) [b]	5.03 (4.40 – 5.98) [b]	0.11 (0.09 – 0.12) [a]

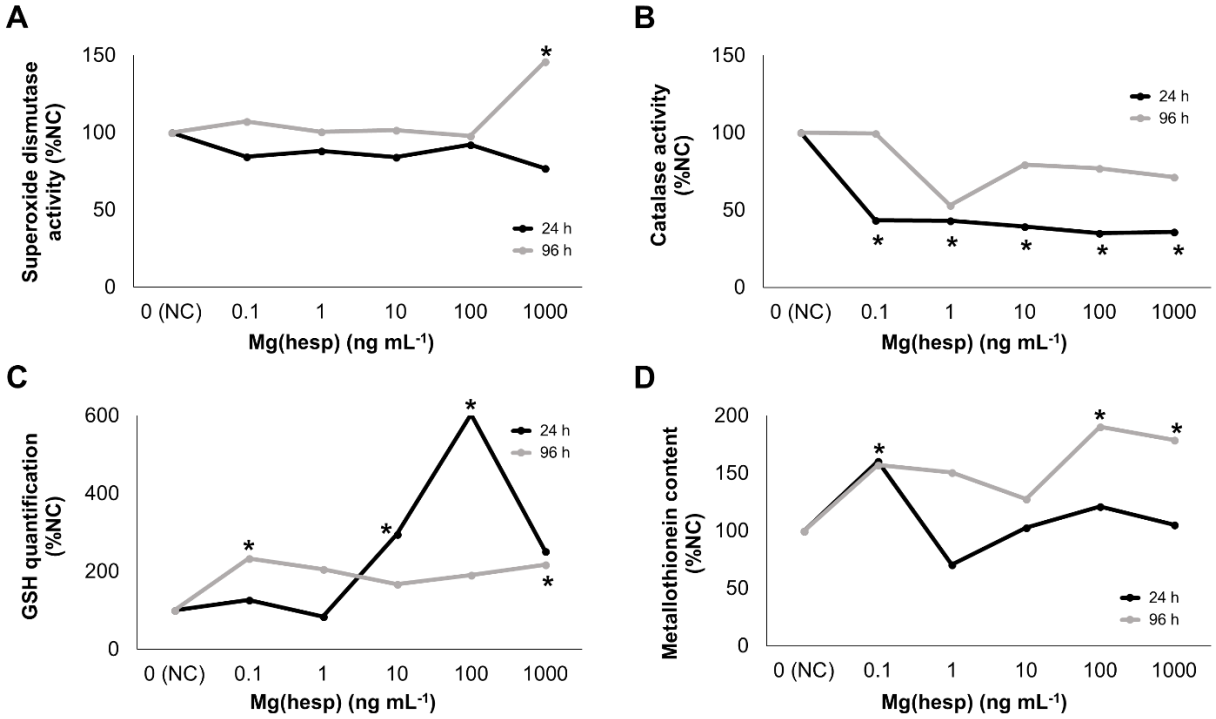

Figure 2. Response of significative biochemical biomarkers in percentage from the negative control (% NC) after 24 h (black line) and 96 h (grey line) exposure to Mg(hesp). A. Superoxide dismutase (SOD) activity response; B. Catalase (CAT) activity response; C. Reduced glutathione (GSH) quantification response; D. Metallothionein (MT) content response. * indicates significant difference from negative control (NC, $P < 0.05$).

3.2 Cytological biomarkers

3.2.1 Alkaline Comet assay

DNA integrity evaluated by Comet assay are shown in Table 3 and Figure 3. Different nucleoids morphologies are presented in Figure 4. Higher fluorescence in nucleoids (head intensity) was observed at 0.1, 100 and 1000 ng mL^{-1} Mg(hesp) concentration after 24 h exposure and in all concentrations, from 0.1 up to 1000 ng mL^{-1} Mg(hesp), after 96 h exposure. This result was accompanied by decreasing in DNA percentage in tail, which were observed in almost all concentrations (except 10 ng mL^{-1}) after 24 h and in the highest Mg(hesp) concentrations (10, 100 and 1000 ng mL^{-1}) after 96 h of exposure. Figure 3 represents the percentage relative to negative control. Variations in head intensity represented an increase of up to 11% after 24 h of exposure and up to 98% after 96 h while DNA content in tail decreased up to 47% and 60% respectively after 24 h and 96 h of exposure.

Table 3. Comet Assay analysis performed in ZF-L cell lines after 24 and 96 h exposure to different concentrations of Mg(hesp). Values are expressed as median (first quartile – third quartile). Letters indicate significant differences (P < 0.05) in the same exposure period.

Exposure treatments (ng mL^{-1})		Comet Assay	
		Head intensity (pixels)	DNA in tail (%)
24h exposure	**0 (NC)**	129.00 (112.52 – 139.15) [a]	2.89 (1.46 – 6.45) [a]
	0.1	137.59 (115.69 – 147.41) [b]	1.92 (0.89 – 4.03) [bc]
	1	123.31 (89.57 – 143.09) [a]	1.75 (1.08 – 3.12) [bd]
	10	129.76 (102.63 – 138.88) [a]	3.83 (1.74 – 10.63) [a]
	100	143.84 (134.64 – 151.27) [c]	2.18 (1.25 – 4.97) [c]
	1000	143.31 (133.82 – 152.24) [c]	1.54 (0.83 – 2.93) [d]
96h exposure	**0 (NC)**	85.95 (67.32 – 99.02) [a]	3.50 (1.64 – 7.73) [a]
	0.1	154.53 (145.09 – 166.21) [b]	3.69 (1.70 – 9.46) [a]
	1	136.34 (107.00 – 155.72) [c]	3.59 (1.75 – 14.98) [a]
	10	170.39 (156.59 – 179.67) [d]	2.04 (1.05 – 3.77) [b]
	100	160.82 (140.51 – 171.97) [b]	2.39 (1.28 – 4.57) [c]
	1000	160.06 (147.23 – 167.81) [b]	1.41 (0.75 – 3.22) [d]

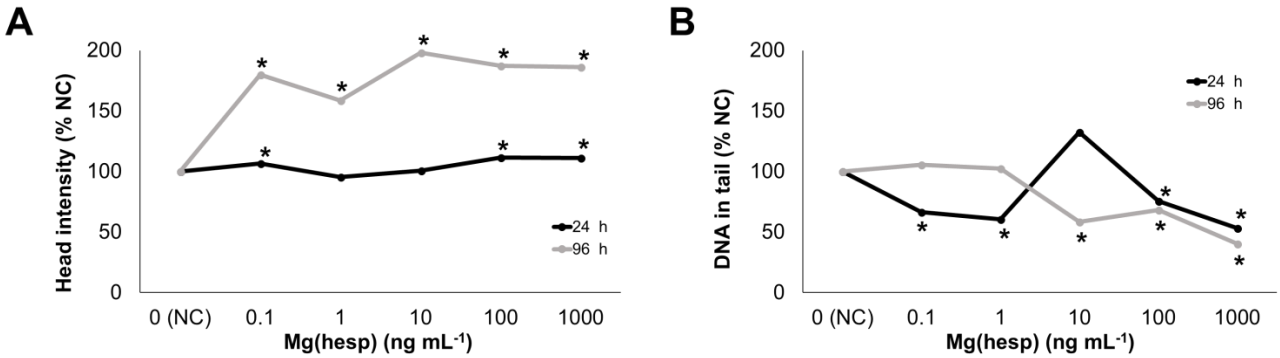

Figure 3. Percentage of head intensity (A) and DNA percentage in tail (B) in relation to negative control (% NC) after 24 h (black line) and 96 h (grey line) exposure to Mg(hesp). *indicates significant difference from NC (P < 0.05).

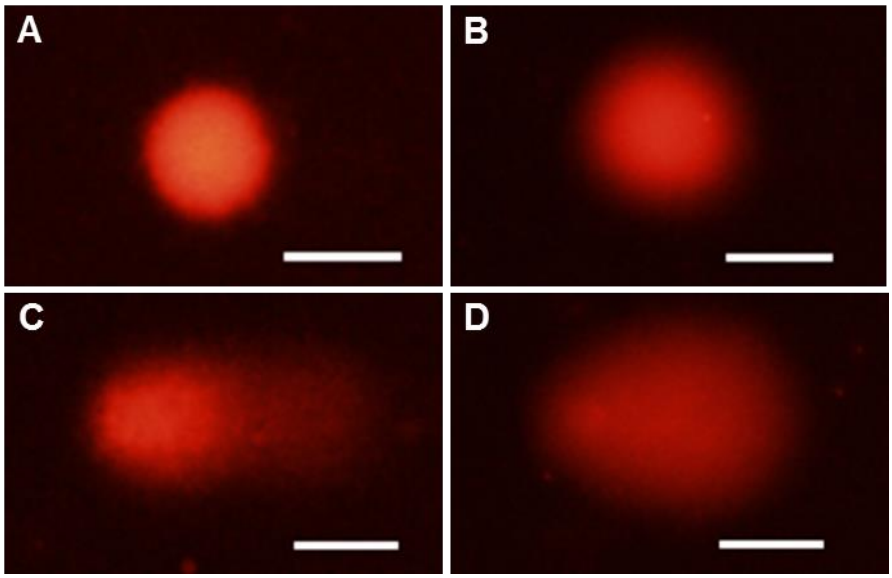

Figure 4. Nucleoids morphology after Comet Assay. A to D shows progressive decrease in head fluorescence intensity followed by increasing in DNA fragments in comet's tail. Scale bar = 50 µm.

3.2.2 Cytokinesis-block micronucleus cytome assay (CBMN Cyt assay)

The CBMN Cyt assay indexes after 24 and 96 h exposure to Mg(hesp) are shown in Table 4 and the different nuclear morphologies found in ZF-L cell line are presented in Figure 5. For NDI calculation, the mono, bi and multinucleated (three or more nuclei) cells having normal nucleus morphology and intact cytoplasm were characterized as viable (Figure 5A, B, C, D). Considering these morphological aspects, the NDI indexes calculated for each Mg(hesp) treatments after 24 and 96 h exposure ranged between 1.4 to 1.8. They were lower after 24 h exposure to 10 and 100 ng mL^{-1} Mg(hesp) and remained unchanged after 96h exposure.

The CytoI index is constituted by apoptotic (Figure 5E) and necrotic cells. Apoptosis was characterized by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear membranes (early apoptosis) or by nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane (late apoptosis). Necrosis is characterized by their pale cytoplasm, the presence of numerous vacuoles, damaged cytoplasm membrane and intact nucleus if in early stages or, when in late necrotic stage, with loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary. No significant difference

was observed in the cytotoxic index after ZF-L exposure to all Mg(hesp) concentrations compared to the NC, in both exposure periods. Necrotic cells were not observed.

GenI which consider the following nuclear abnormalities: nucleoplasmic bridges (Figure 5F), in which binucleated cells presented continuous DNA-containing structure linking the two nucleus, and nuclear buds (Figure 5G), that are similar to MNi in appearance but are connected with the nucleus via a bridge. After 24 h exposure to almost all Mg(hesp) concentrations except 1 ng mL^{-1}, GenI were significant higher than the NC and the predominant nuclear abnormalities was the nuclear buds. After 96 h exposure, no difference in GenI was detected.

For MutI, micronuclei (Figure 5H) were characterized by the same morphology and staining intensity as the nuclei but smaller (up to 1/3rd of nuclei mean diameter) than normal binucleated cells, with no link or connection with main nuclei. This index did not show significant difference from NC after all Mg(hesp) concentration at 24 and 96 h exposure. No difference was observed in MNi distribution.

Table 4. Index of nuclear division (NDI), cytotoxicity (CytoI), genotoxicity (GenI), mutagenicity (MutI) and the micronuclei distribution (MNi) of zebrafish hepatocyte cell line (ZF-L) after 24 and 96 h exposure to different Mg(hesp) concentrations. Values are the median (first quartile – third quartile). Letters indicate significant differences ($P < 0.05$) in same exposure period.

Mg(hesp) Exposure (ng mL^{-1})		CBMN assay				
		NDI	CytoI	GenI	MutI	MNi distribution
24h exposure	**0 (NC)**	1.796 (1.771 – 1.805) [a]	0.031 (0.011 – 0.034) [a]	0.020 (0.016 – 0.020) [a]	0.016 (0.013 – 0.019) [a]	1.231 (1.000 – 1.263) [a]
	0.1	1.730 (1.660 – 1.810) [ab]	0.016 (0.013 – 0.026) [a]	0.043 (0.041 – 0.056) [bc]	0.019 (0.014 – 0.020) [a]	1.143 (1.100 – 1.211) [a]
	1	1.684 (1.674 – 1.771) [ab]	0.027 (0.015 – 0.037) [a]	0.037 (0.033 – 0.041) [ab]	0.013 (0.009 – 0.020) [a]	1.000 (1.000 – 1.050) [a]
	10	1.638 (1.620 – 1.640) [b]	0.008 (0.006 – 0.020) [a]	0.061 (0.051 – 0.079) [c]	0.012 (0.011 – 0.013) [a]	1.000 (1.000 – 1.250) [a]
	100	1.681 (1.605 – 1.698) [b]	0.019 (0.016 – 0.027) [a]	0.058 (0.047 – 0.073) [bc]	0.012 (0.011 – 0.015) [a]	1.167 (1.091 – 1.200) [a]
	1000	1.732 (1.685 – 1.737) [ab]	0.038 (0.026 – 0.041) [a]	0.045 (0.043 – 0.051) [bc]	0.010 (0.010 – 0.014) [a]	1.100 (1.000 – 1.143) [a]
96h exposure	**0 (NC)**	1.566 (1.449 – 1.589) [a]	0.008 (0.002 – 0.011) [a]	0.077 (0.063 – 0.085) [a]	0.016 (0.011 – 0.020) [a]	1.182 (1.150 – 1.188) [a]
	0.1	1.566 (1.332 – 1.589) [a]	0.005 (0.002 – 0.008) [a]	0.079 (0.077 – 0.080) [a]	0.011 (0.010 – 0.016) [a]	1.188 (1.182 – 1.333) [a]
	1	1.379 (1.332 – 1.449) [a]	0.011 (0.005 – 0.011) [a]	0.065 (0.032 – 0.077) [a]	0.012 (0.010 – 0.020) [a]	1.150 (1.083 – 1.333) [a]
	10	1.379 (1.332 – 1.400) [a]	0.007 (0.005 – 0.011) [a]	0.050 (0.033 – 0.060) [a]	0.010 (0.010 – 0.012) [a]	1.100 (1.083 – 1.333) [a]
	100	1.400 (1.379 – 1.424) [a]	0.007 (0.006 – 0.011) [a]	0.050 (0.046 – 0.060) [a]	0.010 (0.005 – 0.012) [a]	1.100 (1.083 – 1.600) [a]
	1000	1.424 (1.383 – 1.437) [a]	0.012 (0.006 – 0.019) [a]	0.081 (0.070 – 0.083) [a]	0.016 (0.015 – 0.018) [a]	1.389 (1.063 – 1.400) [a]

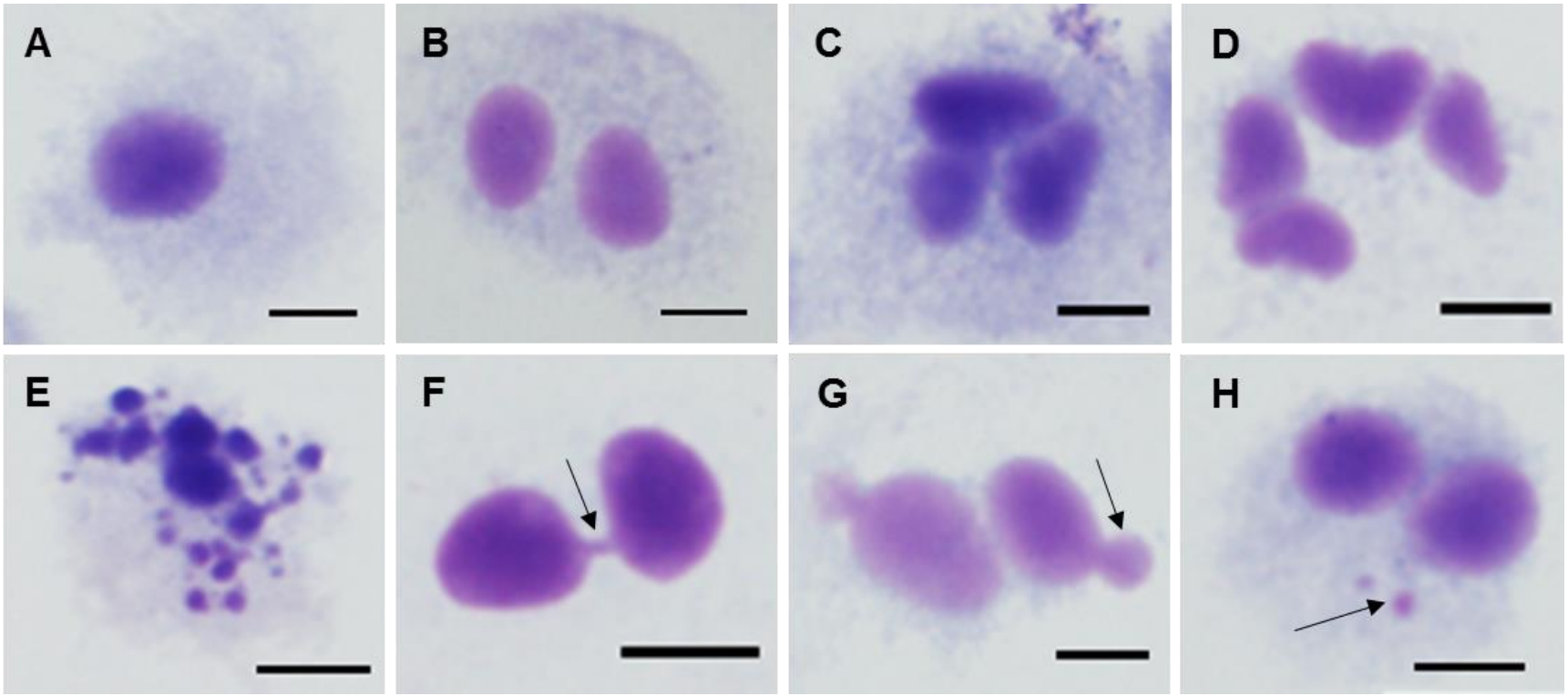

Figure 5. Morphology of the cells scored in the CBMN assay. A. Mononucleated cell; B. Binucleated cell (BN); C. Trinucleated cell; D. Multinucleated cell; E. Apoptotic cell; F. BN cell with nucleoplasmic bridge (arrow); G. BN cell containing nuclear bud (arrow); H. BN cell with micronucleus (arrow). Scale bar = 5 µm.

3.4 Cellular cycle

The percentage of cell in each cell cycle phase was presented in Figure 6A and Table S1. An increase of cell population in Sub-G1 phase was observed in 0.1 and 1000 ng mL^{-1} (Fig 6A).

PI stained cells were represented in histograms based on cell differences in DNA content between the pre-replicative phase cells (G0/G1) versus the phase that replicate DNA (S phase) versus the post-replicative plus mitotic (G2+M) (Figure 6B). Sub-G1 phase results in the presence of fragments of cells and nuclei containing small amounts of DNA and was followed by a decrease in cells in G2+M phase in 0.1 ng mL^{-1} and a decrease in S phase in 1000 ng mL^{-1} (Figure 6A, C).

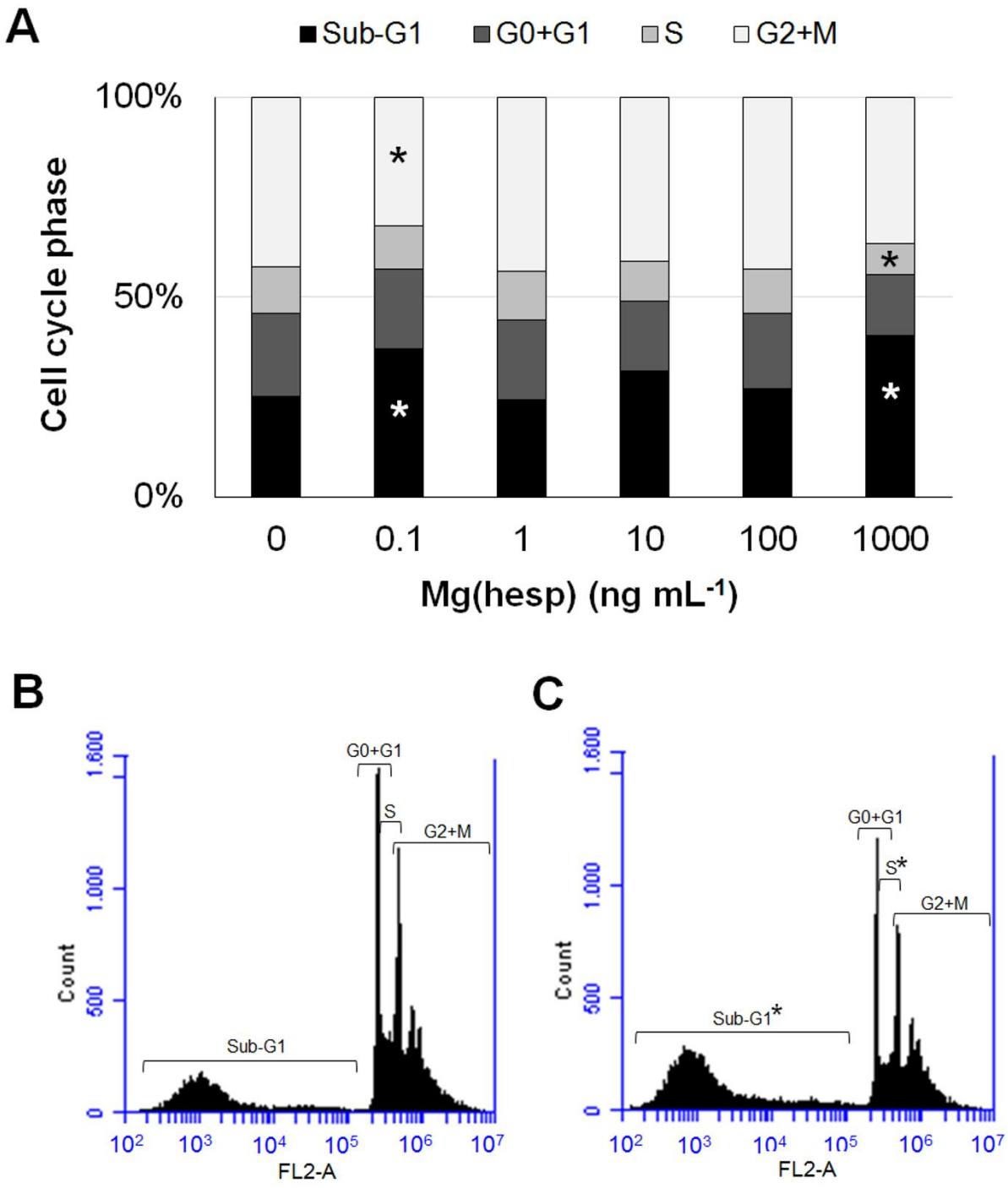

Figure 6. Mg(hesp) effect on ZF-L cell cycle (72 h). A. Percentage of PI stained cells in each cell cycle phase after exposure to different Mg(hesp) concentrations. B. Histogram showing each cell phase from negative control. C. Histogram showing each cell phase after exposure to 1000 ng mL^{-1} Mg(hesp). * indicates significative differences from negative control.

4 DISCUSSION

These results evidenced that Mg(hesp) is potentially toxic at high concentrations (1000 ng mL^{-1} for 24 h and 100 and 1000 ng mL^{-1} for 96 h) as induces ROS production. ROS generation can lead to oxidative stress, disrupting the cellular metabolism with physiological and genetic consequences, including disfunctions of cellular macromolecules like proteins, DNA, RNA, and lipids (Jabłońska-Trypuć, 2017).

After exposure to Mg(hesp), the increasing ROS induced changes in SOD activity only after 96 h, an enzyme that catalyzes dismutation of the superoxide anion to O_2 and H_2O_2. CAT is expected to react with H_2O_2 to form water and molecular oxygen protecting cells from further damage from exposure to ROS (Bresciani et al., 2015). However, CAT activity decreased after 24 h exposure. The enzyme glutathione peroxidase (GPx) play complementary roles in the elimination of H_2O_2 (Hermes-Lima, 2004; Box et al., 2007) and is also involved in the reduction of numerous organic hydroperoxides (beside H_2O_2) by glutathione (GSH) oxidation (Livingstone, 2001; Van der Oost et al., 2003). In general, GPx is activated at low H_2O_2 level while CAT is activated at high H_2O_2 levels which suggested the possible GPx response instead CAT, although GPx activity was not determined in the present study. In addition, CAT expression may be jeopardized as consequence to chemical exposure, as previously reported in zebrafish for other pesticides, such as dimethoate and alphamethrin (Ansari and Ansari, 2014a; Ansari and Ansari, 2014b).

Another biochemical pathway response against xenobiotic effects requires action of phase II biotransformation enzyme. GST mediates the conjugation of xenobiotic to reduced GSH and GSH conjugated with the xenobiotic generates a product capable to be excreted from cell (Labade et al., 2018). Although GST activity did not change, the increased GSH levels after exposure to 10 and 100 ng mL^{-1} Mg(hesp) suggested the GSH expression, as this molecule has itself an antioxidant role due to thiol groups from the cysteine amino acids which are active sites of reaction for ROS (Di Giulio and Meyer 2008). The variation of MT content suggests that this molecule may act as a scavenger of reactive oxygen intermediates as already emphasized by Zhang and Schlenk (1995). Metallothionein plays an important role in maintenance of essential metals homeostatic metabolism, protecting the organism against potential toxic effects of such compounds through sequestration and reducing mechanisms (Hamilton and Mehrle, 1986). Taken together, the antioxidant enzyme activities and the changes in

GSH as well as MT levels in response to different Mg(hesp) concentration evidenced a complex antioxidant and biotransformation defense mechanisms against this insecticide. Such pathways to avoid overwhelming oxidative damages has occurred with success, especially considering the absence of significative lipid peroxidation after Mg(hesp) treatments.

DNA damage as result of xenobiotic exposure, including ROS generation, comprehend a variety of genetic alterations such as changes in individual nucleotide bases, DNA strand breaks, gene deletions or amplification, rearrangements and the formation of adducts (Matés and Sánchez-Jiménez, 2000; Cadet et al., 2003). As alternative of such effects, cells have developed a DNA repair system, a complex mechanism to reduce levels of mutations and chromosomal aberrations, which can act by several pathways, including triggering the signal transduction events to activate the repair components and directly reversing, excising or tolerating DNA damage (Begley and Samson, 2004).

The Comet results in this study could be an indicative of relations observed between ROS generation, nuclear abnormalities and a probable formation of DNA repair complexes. For both exposure periods, an increase in head intensity was accompanied by a decrease in percentage of DNA in tail. Considering the exposure time carried out in this study (relatively long periods), DNA damages related to strand-breaks could be reverted, especially when considering the possibility of such Mg(hesp) exposure triggered DNA repair complexes. DNA repair rates from oxidative damage were previously reported in mammal cells (Collins, 2014; Coluzzi et al., 2014).

The consequences of Mg(hesp) exposure were detected as Genotoxicity index (nuclear abnormalities) mainly after 24 h. Nuclear budding, over the past decades, has been associated with chromosomal instability events in which the amplified DNA is selectively localized to specific sites at the periphery of the nucleus and is eliminated via nuclear budding (Fenech, 2007). Such alterations may represent nuclear membrane entrapment of DNA that has been left in cytoplasm after nuclear division, excess DNA that is being extruded from the nucleus, and/or triggered DNA repair complexes (Fenech, 2007). In addition, abnormal nuclear morphology induction can be a measure of chromosome instability induced by oxidative stress (Kausar et al., 2009).

Cell cycle analysis showed a peak in population in sub-G1 phase followed by a decrease in DNA synthesis capability, which could be associated with some cell death

mechanisms (Ormerod, 1998). Peaks in Sub-G1 phase may be the result of the presence of fragments of cells and nuclei containing small amounts of DNA (Ormerod, 2002), which corroborate with the effects observed in cell population (as discussed in Chapter 1). Additionally, the decrease observed in cell population during S phase after exposure to 1000 ng mL^{-1} Mg(hesp) may be a response to DNA G1/S damage checkpoint, preventing duplication phase to be proceed in order to allow cellular mechanisms to detect and try to overcome genetic alterations (Willis and Rhind, 2009). Results of this study indicates that, even causing some genetic stress responses to *in vitro* aquatic models, Mg(hesp) concentrations (up to 1000 ng L^{-1}) represents rates close to dosage of baits application (Oliveira et al., 2013; Fernandes et al., 2015) and that are above general limits (< 1.0 ng mL^{-1}) for organic contamination in aquatic ecosystems (Brazil, 2005). In addition, behavior of pollutants depends mainly on the soil capacity in filtering, immobilizing and degrade organic and inorganic compounds (Keesstra et al., 2012). Previous studies on Mg(hesp) effects in ZF-L cell-line showed a strong influence on cell population mainly after 24 h of exposure, which decreases of up to 53% of expected normal values (using control population as basis), whereas cells after 96 hours presented high recovery, which could be associated with the effectiveness of biochemical responses assessed in this study.

Understanding the new chemicals effects are always a challenge, in which crossing analysis of different mechanisms of response becomes a powerful tool. Data collected in this study provides an insight on genetic behavior of cells exposed to different concentrations of Mg(hesp), indicating that only at high Mg(hesp) concentrations, the effects on DNA can occur and repair mechanisms are triggered, with a potential recover of cell population after 96 hours. Thus, results emphasize the promising use of such compound, reinforcing the importance of continuing investigations in further Mg(hesp) effects, including *in vivo* analysis.

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SUPPLEMENTARY MATERIALS

Table S1. Results from cell cycle analysis performed in ZF-L cell lines after different exposure periods of Mg(hesp). Values are expressed as median (first quartile – third quartile). Results followed by different lowercase letters indicate significant differences ($P < 0.05$).

Exposure Treatments (ng mL^{-1})	Cell cycle phases (%)			
	Sub-G1	**G0+G1**	**S**	**G2+M**
0 (NC)	24.10 [a] (23.40 – 27.50)	19.60 [ab] (18.20 – 20.60)	11.20 [a] (10.90 – 11.90)	40.60 [ac] (36.80 – 41.70)
0.1	35.70 [b] (35.70 – 36.90)	19.20 [a] (19.00 – 20.70)	10.40 [ab] (10.10 – 10.90)	30.90 [b] (30.00 – 31.30)
1	23.10 [a] (21.00 – 25.90)	19.00 [ab] (18.30 – 19.60)	11.60 [a] (10.50 – 12.10)	41.70 [c] (40.70 – 44.40)
10	31.30 [ab] (27.50 – 31.80)	17.50 [ab] (16.00 – 18.00)	10.00 [ab] (8.70 – 10.00)	41.00 [c] (37.50 – 41.20)
100	26.00 [a] (25.00 – 27.10)	17.90 [ab] (17.70 – 18.60)	10.70 [ab] (10.40 – 11.10)	41.30 [c] (40.90 – 42.30)
1000	39.80 [b] (30.80 – 43.10)	15.00 [b] (14.50 – 18.90)	7.60 [b] (7.50 – 10.80)	36.10 [ab] (32.00 – 36.30)

3.3 Artigo 3

Physiological, biochemical and cytogenetics effects of a novel insecticide in the Neotropical freshwater fish species *Prochilodus lineatus*

ABSTRACT

This study assessed influences of a new insecticide (hesperidin complexed with magnesium(II) – Mg(hesp)) in Neotropical fish *Prochilodus lineatus* using physiological, genetic and biochemical biomarkers. Hematological evaluation showed alterations in hematocrit and erythrocytes values but with no changes occurred in hemoglobin content, a response to situations of stress due to chemical exposure that can affect oxygen content in blood, and, consequently, the ability in overcome compound effects. Comet results and erythrocyte morphology (crenated cells) and nuclear abnormalities suggested that Mg(hesp) at higher concentrations has genotoxicity effects mainly after 24 h. The percentage increases of DNA in Comet's tail and in nuclear budding formation indicate Mg(hesp) instability effects in DNA integrity. However, the absence of micronuclei formation is an indicative of successfully action of DNA repair mechanisms. High crenated erythrocytes rates can also indicate metabolic cellular alterations. In biochemical biomarkers, despite CAT activity was jeopardized in higher concentrations after 24 h, it was triggered as an antioxidant response after 96 h. Results of this study indicates that hematological and genetoxic effects of Mg(hesp) concentrations occurs generally after 24 h of exposure, while after 96 h the lower damage suggested a potential recover capability of organisms and emphasized the importance of continuous investigations of Mg(hesp) effects and its promising use.

Keywords: metallic-insecticide; aquatic contamination; hematology; comet assay; antioxidant.

RESUMO

O presente estudo avaliou as influências de um novo inseticida (hesperidina complexada com magnésio(II) – Mg(hesp)) no peixe Neotropical *Prochilodus lineatus* utilizando biomarcadores fisiológicos, genéticos e bioquímicos. A avaliação hematológica detectou alterações nos valores de hematócrito e no número de eritrócitos, porém não foram encontradas variações nos conteúdos de hemoglobina, caracterizando uma resposta frente a uma situação de estresse devido à exposição ao composto, o que pode influenciar na concentração de oxigênio no sangue, afetando severamente a capacidade do organismo em superar possíveis efeitos tóxicos. Os resultados do cometa e das análises morfológica (células crenadas) e de anormalidade nucleares dos eritrócitos sugerem que as maiores concentrações de Mg(hesp) apresentam efeitos genotóxicos, principalmente após 24 h. Aumentos na porcentagem de DNA na cauda e a formação de brotos nucleares indicam as influências do composto na instabilidade da integridade do DNA. Entretanto, a ausência de formações significativas de micronúcleos é um indicativo de uma ação eficaz de mecanismos de reparo de DNA. O aumento no número de células crenadas também pode ser considerado um indicativo de alterações metabólicas. Para os biomarcadores bioquímicos, a atividade da CAT, apesar de inibida nas maiores concentrações após 24 h, foi ativada após 96 h. Os resultados deste estudo indicam que os efeitos hematológicos e genéticos por estresse ao Mg(hesp) ocorreram principalmente após 24 h de exposição, enquanto os menores danos observados após 96 h sugerem uma potencial capacidade de recuperação dos organismos, enfatizando a importância da continuidade na investigação das influências causadas por Mg(hesp) e o promissor uso deste composto.

Palavras-chave: metal-inseticida; contaminação aquática; hematologia; teste do cometa; antioxidante.

1 INTRODUCTION

Environmental health and water quality have been the main concerns when dealing the increased agrochemical application over years, considering the development and investment in new pesticides to control crop pests follows the growing demand in food production. Aquatic contamination due to intense use of agrochemical use and consequent natural processes such as surface run-off or leaching due to careless disposal of materials, giving special importance to pesticide compounds with selective action, in order to reduce ecological impact (Akerblom, 2004; Sumon et al., 2018).

The complexation of magnesium (II) with hesperidin results in a compound ($Mg(phen)_2(hesp)$, here referred as Mg(hesp), with high efficiency to control leaf-cutter ant *Atta sexdens rubropilosa*, a tropical silviculture plague mainly for *Pinus* and *Eucalyptus* planting. Flavonoids molecules such as hesperidin belongs to a polyphenolic class having insecticide activity (Wang et al., 2016) and presents increased hydrophilicity and biological interactions when chelated with magnesium (II), an essential metal for numerous biological functions (Oliveira et al., 2013; Fernandes et al., 2015; Guo et al., 2016). Mg(hesp) induce mortality rate similar to the most known commercial insecticides, the sulphluramide (Fernandes et al., 2015; Oliveira, 2012; Zanetti et al, 2004) at concentrations around 140 μg L^{-1} (0.2% by weight of the diet) (Oliveira et al., 2013; Fernandes et al., 2015).

Pesticide contamination in aquatic environments can be evaluated by analyzing some biochemical biomarkers in different fish organs, such as blood and liver, which play important role in xenobiotics absorption, circulation, detoxification and excretion (Monteiro et al., 2005; Vieira et al., 2018). In this context, *Prochilodus lineatus* (curimbatá), a Neotropical freshwater species with ecological and economical importance, it is an excellent biological model for *in vivo* studies due to moderate sensitivity to pesticides, especially in juvenile stage (Vieira et al., 2018).

Biomarkers allows identification and characterization of mechanism actions and potential effects in different biological matrix. Biochemical biomarkers have been widely used to monitor the organism responses related to biotransformation and antioxidant system defense (Prieto et al., 2006; Hodgson et al., 2008). Physiological biomarkers provide information concerning to functional and metabolic alterations in response to xenobiotic exposure, including hematological parameters, very sensitive to changes stress-related (Clauss et al., 2008) and genetic biomarkers are applied to

investigates the consequences of xenobiotic exposure on the cellular DNA integrity, chromosomal alterations and triggering of DNA repair mechanisms (Costa, 2011). Thus, the aim of this study was to evaluate the potential biochemical, physiological and genetic effects of Mg(hesp), a novel pesticide on the Neotropical freshwater fish *P. lineatus*.

2 MATERIALS AND METHODS

2.1 Complex characterization

Hesperidin complexed with Magnesium (II) ($MgC_{68}H_{74}N_2O_{30}.2H_2O$ – Mg(hesp)), using phenanthroline as a chelating agent, was provided by Inorganic and Bioinorganic Photochemistry Laboratory of Chemistry Department of Federal University of São Carlos, São Paulo, Brazil. Mg(hesp) is a yellow powder that presents absorbance peaks at 260-300 nm, has high photochemical stability and chemical stability in the pH range from 3 to 9 and water solubility is equal to 472 mg L^{-1} (Oliveira et al., 2013).

2.2 Fishes and experimental conditions

This study was approved by the Animal Ethics Committee of the Federal University of São Carlos (Proc. N. 3475090815).

Juvenile fish specimens of *P. lineatus* (mass weight 29.55 g ± 10.82; total length 13.39 cm ± 1.43) were acquired in Santa Candida Pisciculture (Santa Cruz da Conceição, SP) and kept in the laboratory for 30 days in natural photoperiod (~12:12 hours). The aquariums were supplied with chlorine free water and constant aeration; animals were fed with commercial fish food *ad libitum* (FRI-ACQUA 40, Fri-Ribe Rações, 40% protein). After acclimation, fish were randomly divided in five groups (n=10 each group) into 120 L experimental aquariums and static exposure protocols were performed in duplicate. Feeding was suspended 24 h prior to the beginning of the experiments. The following water variables were measured before and after exposure: temperature, pH, dissolved oxygen, conductivity and Na, Ca and K concentrations.

Considering the absence of established limit levels for new chemicals in the environment, Mg(hesp) concentrations used in this study selected corresponded to a range of six concentrations to compose an overall view of possible toxic effects of the compound: 0.0 (negative control), 0.1, 1.0, 10, 100 and 1000 µg L^{-1}. The desired experimental concentrations were obtained by serial dilution into aquarium water from

a stock solution prepared using a low frequency (40 KHz) ultrasound bath (Q335D, QUIMIS®, Brazil). The mixture of diluted Mg(hesp) respected a limit of 1% of the total working volume in aquarium. Two exposure periods (24 h and 96 h) were selected to evaluate the Mg(hesp) acute effects on fishes.

After exposure, animals were anesthetized with benzocaine (0.1 g L^{-1}) and a sample of blood was collected from the caudal vein using heparinized syringe and processed for hematological parameters determination, and genotoxic and mutagenic analyses: Comet assay and nuclear abnormalities. Thereafter, fish were killed by medullar sectioning and, subsequently, the liver was immediately removed and samples were taken, frozen in liquid nitrogen and kept in -80 °C for biochemical analyses. In addition, subsamples of the liver were processed for comet assay.

2.3 Hematological biomarkers

Blood samples were immediately used for hematological analyses. Hematocrit (Htc) was determined using heparinized capillary and centrifuged at 12,000 x *g* for 3 min in a micro-hematocrit centrifuge (FANEN Centrimicro MOD.211). Readings were performed using a standardized chart. For red blood cell counts, 2 mL of formal citrate solution were added to 10 µL of blood samples and the number of erythrocytes per mm^3 of blood (RBC) was determinate using a Neubauer chamber under a light microscope (Olympus BX 51, 400x). The hemoglobin content (Hb) was estimated using the cyanmethemoglobin colorimetric method (Collier, 1944) with dilution of 10 µL of blood in 2 mL of Drabkin solution and density measurement at 540 nm (SpectraMax M5, Molecular Devices, USA). The hematimetric indexes were calculated using the hematological data: mean corpuscular volume (MCV = Htc*1000/RBC); mean corpuscular hemoglobin (MCH = RBC*1000/Hgb); and mean corpuscular hemoglobin concentration (MCHC = Hgb*1000/Htc).

2.4 Cytogenetic biomarkers

2.4.1 Alkaline Comet Assay

The genotoxicity effect of Mg(hesp) concentrations on DNA was assessed through alkaline version of Comet Assay in the blood and liver as described by Singh et al. (1988) with modifications (McKelvey-Martin et al., 1993; Tice et al., 2000). For comet assay performed in blood, 1 mL of phosphate buffer saline (PBS) was added to 5 µL of blood samples followed by mixing 10 µL of PBS diluted blood in 100 µL of low

melting point agarose (0.75% at 37 °C). For liver cells analysis was followed the protocol described by Cavalcante et al. (2008) with modifications. Liver samples were washed with PBS until visually opaque and after fine sectioning with surgical scissor, samples were transferred to microtubes and incubated for 15 min at 30 °C with 0.05% trypsin and neutralized with fetal bovine serum. At room temperature, microtubes containing liver samples were manually agitated and filtered with gaze, centrifugated for 10 min at 1000 x *g*. Thereafter, pellet was resuspended in 1 mL PBS and a 10 µL aliquot was mixed in 100 µL of low melting point agarose (0.75% at 37 °C).

Low melting agarose cellular suspensions (100 µL) were placed in precoated normal melting point agarose (1%) cell slides, covered with coverslips, kept under refrigeration (10 °C) for 20 min and then coverslips were removed. Subsequently, slides were incubated in lysis solution (100 mM ethylenediaminetetraacetic acid – EDTA, 2.5 M sodium chloride, 10 mM Trizma base, 1% Triton X-100, 20% dimethyl sulfoxide – DMSO, pH 10.0) for at least 24 hours. Electrophoresis was performed at 25 V and 300 mA for 20 min. Slides were then fixed in absolute ethanol and the analysis were performed using GelRed™ solution (Biotium, Inc). Images were obtained in cellSens™ software (Olympus, PA-USA) using an Olympus BX-61 fluorescent microscope (Olympus, PA-USA) with coupled camera at 200x magnification, and 515-560 nm excitation filter and 590 nm barrier filter. For DNA damage analysis (head intensity, tail length and tail DNA content), OpenComet© software (version 1.3) extension was performed in ImageJ (Rasband, 2016). Data were reported in percentage.

2.4.2 Erythrocyte morphology, nuclear abnormalities and micronucleus

Erythrocytes cellular alterations were evaluated, including micronucleus and nuclear abnormalities as described by Carrasco (1990), while crenated cells were classified according to Harvey (2008). An aliquot of 10 µL of blood was immediately smeared on microscope slides and allowed to air dry at room temperature. Cell slides were prepared in duplicate. After fixation in absolute methanol for 5 min slides were stained with 5% Giemsa solution. Images were obtained using an Olympus BX-61 light microscope (Olympus, PA-USA) at magnification of 400x and the analysis were performed with the cellSens™ software (Olympus, PA-USA). Mutagenicity index was calculated by quantifying erythrocytes with micronuclei in a population of five thousand normal erythrocytes per slide and genotoxicity index was determined by scoring

nuclear abnormalities/alterations classified as *bebbled*, *lobed*, vacuolated and *notched* in a population of five thousand erythrocytes per slide. Crenated cells index was also calculated in a population of five thousand erythrocytes per slide.

2.5 Biochemical biomarkers

For analysis of biochemical parameters, liver samples were homogenized in phosphate buffer 0.2 M (pH 7.8) and centrifuged at 10,000 x *g* at 4 °C for 10 min. Supernatant was used for biochemical analyses.

The total protein concentration of each sample was determined according to the Bradford method (Bradford, 1976) at 595 nm using bovine serum albumin as standard. Six biochemical biomarkers were evaluated; the activity of antioxidant enzymes, superoxide dismutase (SOD) was determined according to McCord et al. (1977) and catalase (CAT) as described by Beutler (1975); the activity of biotransformation enzyme of phase two glutathione S-transferase (GST) were assessed according to Habig et al. (1974). Reduced glutathione (GSH) and metallothionein content were determined according to Wilhelm-Filho et al. (2005) and Viarengo et al. (1997). Lipid peroxidation quantification (LPO) was done according Jiang et al. (1991). All protocols were adapted for microplate reader (SpectraMax M5, Molecular Devices, USA).

2.6 Statistics

All results were presented as median (first quartile – third quartile). Data were tested for normal distribution (Shapiro-Wilk's test). Parametric data were analyzed applying ANOVA followed by Tukey's multiple comparison as *post hoc* test ($P < 0.05$). Non-parametric data were analyzed applying Kruskal-Wally's followed by Dunn's multiple comparison test as *post hoc* ($P < 0.05$). All statistical analyses were performed using the InfoStat software (Di Rienzo et al., 2010).

3 RESULTS

3.1 Water variables

Water variables before and after Mg(hesp) exposures are given in Table 1. No significant difference was observed in any analyzed variable.

Table 1. Water variables measured before and after the exposure periods of Mg(hesp). Values in median (first quartile – third quartile). No significant difference was found among each analyzed variable. b.q.l. – below quantification limit.

WATER VARIABLES

		24 h exposure					96 h exposure				
		NC (0 µg L^{-1})	**1 µg L^{-1}**	**10 µg L^{-1}**	**100 µg L^{-1}**	**1000 µg L^{-1}**	**NC (0 µg L^{-1})**	**1 µg L^{-1}**	**10 µg L^{-1}**	**100 µg L^{-1}**	**1000 µg L^{-1}**
Temperature (°C)	**Initial**	25.6 (25.6 – 25.8)	25.4 (25.2 – 25.5)	25.2 (25.1 – 25.4)	25.6 (24.7 – 25.6)	25.6 (24.7 – 25.6)	25.2 (24.6 – 25.2)	24.7 (24.6 – 24.7)	24.7 (24.6 – 25.6)	25.6 (25.6 – 25.8)	25.4 (25.2 – 25.4)
	Final	25.6 (25.5 – 25.6)	25.6 (25.5 – 25.6)	25.1 (24.7 – 25.2)	25.6 (25.5 – 25.8)	25.6 (25.5 – 25.8)	25.6 (25.4 – 25.6)	25.2 (25.2 – 25.3)	25.5 (25.1 – 25.6)	25.2 (25.1 – 25.4)	25.2 (25.1 – 25.6)
pH	**Initial**	7.1 (7.0 – 7.1)	7.5 (7.4 – 7.5)	7.5 (7.4 – 7.6)	7.5 (7.2 – 7.5)	7.5 (7.2 – 7.5)	7.4 (7.0 – 7.5)	7.6 (7.5 – 7.6)	7.5 (7.5 – 7.6)	7.1 (7.0 – 7.1)	7.4 (7.4 – 7.5)
	Final	7.5 (7.4 – 7.5)	7.4 (7.0 – 7.4)	7.5 (7.5 – 7.6)	7.1 (7.1 – 7.5)	7.1 (7.1 – 7.5)	7.1 (7.0 – 7.5)	7.4 (7.4 – 7.5)	7.4 (7.4 – 7.5)	7.5 (7.4 – 7.6)	7.4 (7.4 – 7.5)
Dissolved oxygen (%)	**Initial**	93.5 (92.9 – 95.6)	97.9 (95.0 – 98.8)	97.9 (96.4 – 98.9)	93.5 (92.9 – 99.1)	93.5 (92.9 – 99.1)	97.6 (93.8 – 97.9)	99.1 (93.8 – 99.1)	99.1 (92.9 (99.4)	93.5 (92.9 – 95.6)	96.9 (96.4 – 97.9)
	Final	98.5 (98.0 – 98.5)	96.4 (95.6 – 97.2)	98.5 (97.9 – 99.1)	92.9 (92.2 – 97.2)	92.9 (92.2 – 97.2)	97.2 (95.6 – 97.9)	97.9 (93.8 – 98.0)	97.9 (97.2 – 98.0)	97.9 (96.4 – 98.8)	97.2 (93.8 – 97.9)
Conductivity (µS cm^{-1})	**Initial**	50.9 (50.5 – 51.0)	54.9 (53.2 – 61.7)	61.2 (54.9 – 61.7)	51.3 (50.6 – 59.6)	51.3 (50.6 – 59.6)	61.4 (51.0 – 61.7)	61.2 (59.6 – 62.1)	59.6 (50.6 – 61.2)	50.9 (50.5 – 51.0)	54.9 (51.4 – 61.7)
	Final	53.3 (51.8 – 53.8)	51.5 (51.0 – 54.0)	61.2 (59.6 – 61.7)	51.0 (50.5 – 53.2)	51.0 (50.5 – 53.2)	51.0 (50.9 – 54.9)	61.7 (54.0 – 62.1)	54.0 (51.4 – 61.2)	59.6 (54.9 – 61.7)	61.7 (54.0 – 62.1)
Na (mg L^{-1})	**Initial**	1.2 (1.2 – 1.2)	1.3 (1.3 – 1.3)	1.2 (1.2 – 1.3)	1.2 (1.2 – 1.2)	1.2 (1.2 – 1.2)	1.3 (1.2 – 1.3)	1.2 (1.2 – 1.2)	1.2 (1.2 – 1.2)	1.2 (1.2 – 1.2)	1.3 (1.2 – 1.3)
	Final	1.2 (1.2 – 1.3)	1.2 (1.2 – 1.2)	1.2 (1.2 – 1.3)	1.2 (1.2 – 1.3)	1.2 (1.2 – 1.3)	1.2 (1.2 – 1.3)	1.3 (1.2 – 1.3)	1.2 (1.2 – 1.2)	1.2 (1.2 – 1.3)	1.2 (1.2 – 1.3)
K (mg L^{-1})	**Initial**	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.7)	4.5 (4.5 – 4.7)	4.5 (4.3 – 4.5)	4.4 (4.4 – 4.5)	4.5 (4.4 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.6)
	Final	4.5 (4.5 – 4.6)	4.5 (4.5 – 4.5)	4.5 (4.4 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.4 – 4.5)	4.5 (4.5 – 4.5)	4.5 (4.5 – 4.6)
Ca (mg L^{-1})	**Initial**	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.
	Final	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.	b.q.l.

Quantification limits for Na, K and Ca equals to 0.1 mg L^{-1}.

3.2 Hematological biomarkers

Hematological data are presented in Table 2. Htc values increased significantly after 24 h of exposure in all Mg(hesp). concentrations, except in 1000 µg L^{-1}. RBC presented higher values in concentrations of 1, 100 and 1000 µg L^{-1} in both periods of exposure. Hb did not change. After 24 h of exposure, hematimetric indexes: MCV decreased after exposure to highest Mg(hesp) concentration (1000 µg L^{-1}) while MCH decreased in all concentrations analyzed; MCHC was significantly lower only after 24 h exposure to 100 µg L^{-1}.

Table 2. Hematological parameters and hematimetric indexes (mean corpuscular volume MCV, mean corpuscular hemoglobin MCH and mean corpuscular hemoglobin concentration, MCHC) after 24 and 96 h exposure to Mg(hesp) in *Prochilodus lineatus*. Values are the median (first quartile – third quartile). Letters indicate significant difference in the same period of exposure ($P < 0.05$).

HEMATOLOGICAL PARAMETERS							
Exposure treatments ($\mu g\ L^{-1}$)		**Hematocrit (Htc %)**	**Red blood cels (RBC $10^6\ mm^{-3}$)**	**Hemoglobin - Hb ($g\ dL^{-1}$)**	**Hematimetric indexes**		
					MCV (μm^3)	**MCH ($pg\ cell^{-1}$)**	**MCHC ($g\ dL^{-1}$)**
24 h exposure	**0 (NC)**	34.5 (32.0 – 37.0) [ac]	2.3 (1.6 – 2.4) [a]	10.7 (9.4 – 10.9) [a]	155.0 (140.6 – 179.6) [ab]	47.4 (41.8 – 54.0) [a]	29.4 (26.9 – 32.3) [ab]
	1	37.0 (37.0 – 40.0) [b]	2.8 (2.5 – 2.9) [b]	10.6 (9.7 – 11.4) [a]	144.3 (130.7 – 160.4) [ac]	38.1 (34.5 – 41.6) [b]	26.3 (25.0 – 28.9) [bc]
	10	37.0 (32.0 – 47.0) [ab]	2.3 (2.2 – 2.) [ac]	10.5 (8.3 – 11.4) [a]	154.6 (131.8 – 186.0) [ab]	41.2 (35.4 – 46.2) [b]	26.7 (20.5 – 31.6) [bc]
	100	41.5 (35.0 – 50.0) [b]	2.5 (2.3 – 2.6) [bc]	10.5 (9.2 – 11.2) [a]	167.0 (149.4 – 186.9) [b]	40.7 (37.2 – 42.9) [b]	24.5 (20.7 – 29.6) [c]
	1000	30.5 (24.0 – 32.0) [c]	2.5 (2.3 – 2.9) [bc]	10.1 (8.1 – 12.9) [a]	125.4 (99.6 – 146.9) [c]	42.5 (36.9 – 44.3) [b]	31.2 (26.8 – 43.0) [a]
96 h exposure	**0 (NC)**	35.0 (32.0 – 42.0) [a]	2.2 (2.0 – 2.4) [a]	10.7 (9.4 – 11.1) [a]	166.3 (139.5 – 172.4) [a]	45.9 (42.2 – 51.5) [a]	27.8 (25.0 – 31.1) [a]
	1	40.0 (32.0 – 46.0) [a]	2.6 (2.3 – 2.7) [b]	12.3 (10.0 – 13.5) [a]	159.2 (136.8 – 186.9) [a]	47.5 (41.9 – 52.3) [a]	29.8 (25.1 – 32.7) [a]
	10	39.5 (32.0 – 45.0) [a]	2.5 (1.9 – 2.7) [ab]	10.4 (9.5 – 12.6) [a]	162.5 (121.3 – 201.0) [a]	41.9 (36.5 – 46.3) [a]	27.1 (22.8 – 33.0) [a]
	100	38.5 (35.0 – 47.0) [a]	2.6 (2.4 – 2.7) [b]	11.3 (10.1 – 11.7) [a]	154.3 (134.1 – 169.9) [a]	44.1 (40.6 – 47.6) [a]	28.5 (24.0 – 32.5) [a]
	1000	35.0 (33.0 – 41.0) [a]	2.4 (2.2 – 2.7) [ab]	10.9 (10.5 – 14.7) [a]	154.4 (129.5 – 162.6) [a]	49.5 (40.5 – 57.0) [a]	32.3 (24.9 – 36.1) [a]

3.3 Cytogenetic biomarkers

3.3.1 Alkaline Comet Assay

Mg(hesp) effects in DNA integrity are presented in Table 3 and Figure 1 shows the nucleoid morphologies. Blood samples presented alterations in DNA integrity in all comet parameters in both periods of exposure to Mg(hesp). After 24 h of exposure, head intensity decreased followed by an increase in percentage of DNA content in Comet's tail in all exposure concentrations. While after 96 h, despite an initial decrease in head intensity observed only in 1 µg L^{-1} Mg(hesp), an increase occurred in 10 and 100 µg L^{-1} Mg(hesp) also followed by increases in percentage of DNA content in Comet's tail, except in 100 µg L^{-1}.

In the liver, Mg(hesp) effects after 24 h of exposure lead to an initial increase in head intensity (1 and 10 µg L^{-1}) followed by a decrease in 1000 µg L^{-1}. Percentage of DNA content in Comet's tail increased in all concentration. After 96 h head intensity values were lower in 1, 10 and 100 µg L^{-1} and a higher percentage of DNA content in Comet's tail was observed in 10 and 100 µg L^{-1}.

Table 3. Comet Assay performed in erythrocytes and hepatocytes of *Prochilodus lineatus* after different exposure periods of Mg(hesp). Values are expressed as median (first quartile – third quartile). Letters indicate significant difference (P < 0.05) between concentration in the same period of exposure.

		COMET ASSAY			
	Exposure treatments (µg L^{-1})	**Erythrocytes**		**Hepatocytes**	
		Head intensity (pixels)	DNA in tail (%)	Head intensity (pixels)	DNA in tail (%)
24h exposure	**0 (NC)**	119.8 (104.8 – 128.6) [a]	6.2 (3.9 – 11.1) [a]	81.38 (63.64 – 93.01) [ad]	4.02 (1.64 – 6.20) [a]
	1	108.8 (101.1 – 115.5) [b]	17.2 (13.3 – 25.5) [b]	116.72 (82.06 – 128.47) [b]	5.54 (2.30 – 9.50) [b]
	10	107.7 (98.2 – 115.9) [b]	9.8 (6.3 – 14.8) [c]	102.17 (74.76 – 120.17) [c]	5.12 (2.14 – 10.81) [b]
	100	93.1 (79.9 – 106.7) [c]	25.4 (17.0 – 31.9) [d]	80.49 (56.53 – 105.03) [a]	11.70 (6.11 – 21.99) [c]
	1000	99.2 (89.7 – 107.7) [d]	13.3 (9.1 – 18.0) [e]	75.69 (59.72 – 92.61) [d]	6.22 (2.26 – 9.82) [b]
96h exposure	**0 (NC)**	98.3 (81.4 – 105.7) [a]	13.2 (9.6 – 17.6) [a]	99.17 (79.08 – 121.05) [a]	10.61 (6.34 – 14.51) [a]
	1	86.6 (74.7 – 99.6) [b]	16.0 (7.5 – 25.1) [b]	91.30 (71.75 – 106.11) [b]	12.78 (5.54 – 15.28) [a]
	10	127.9 (119.9 – 135.7) [c]	18.0 (10.0 – 23.4) [b]	98.81 (94.12 – 117.76) [c]	13.05 (7.95 – 25.14) [b]
	100	111.3 (102.6 – 120.3) [d]	13.2 (9.0 – 19.4) [a]	89.62 (68.22 – 104.41) [b]	16.60 (7.95 – 25.14) [c]
	1000	96.3 (86.2 – 104.8) [a]	24.8 (18.1 – 31.3) [c]	99.37 (81.62 – 120.18) [a]	11.31 (8.50 – 17.91) [a]

Figure 1 exhibits different stages of nucleoid morphology through GelRed® fluorochrome ability of binding with nucleic acids.

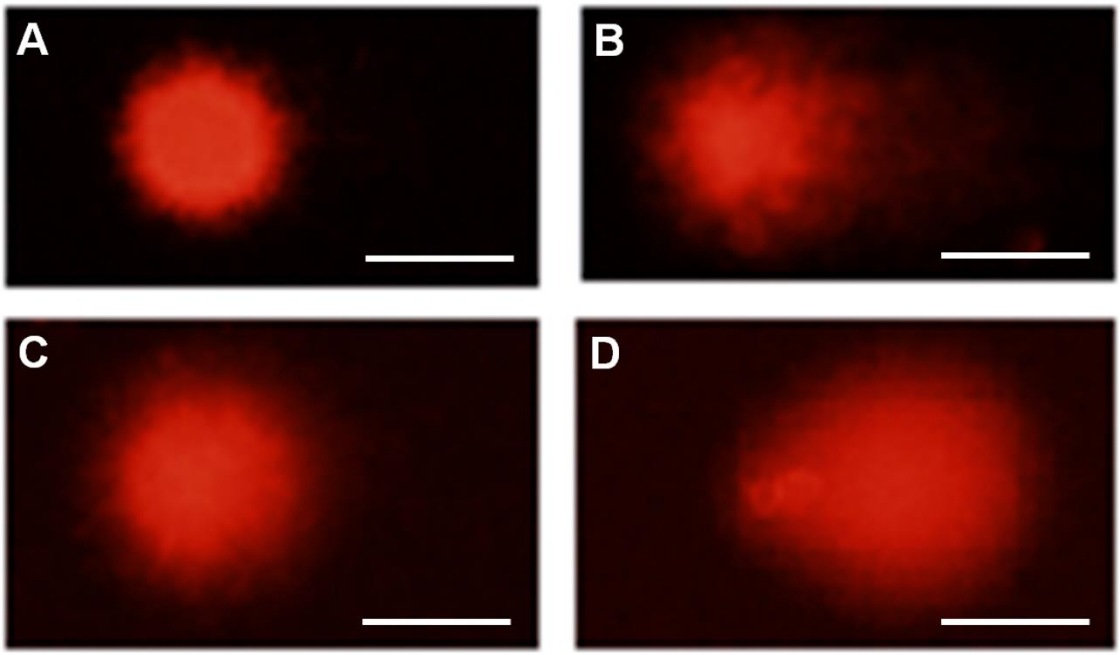

Figure 1. Nucleoid morphologies of *P. lineatus* Alkaline Comet Assay performed in liver samples. A to D shows progressive decrease in head intensity followed by increase in DNA fragments in Comet's tail. Scale bar = 50 µm.

3.3.2 Erythrocyte morphology, nuclear abnormalities and micronucleus

Changes in erythrocytes morphology (crenated cells) and the Indexes of mutagenicity and genotoxicity were show in Table 4 and Figure 2A-F shows the cell and nuclear morphologies considered in each classification.

Crenated cells, also known as echinocytes, are considered as spiculate erythrocytes in which projections are relatively spaced through cell membrane (Figure 2B). Such morphological alterations increased after 24 h exposure to the highest Mg(hesp) concentration (1000 µg L^{-1}) but, did not change after 96 h exposure to Mg(hesp).

Nuclear abnormalities (Fig. 2C, D, E, F) were scored to calculate Genotoxicity index (Table 4). *Blebbed* erythrocytes had relatively small evagination of the nuclear envelope with genetic material (Fig. 2C); *lobed* ones present nuclei with evaginations larger than the blebs (Fig. 2D); while *notched* were cells presenting a well-defined slit of uniform width extending to an appreciable depth into a nucleus (Fig. 2E); and *vacuolated* were considered when erythrocytes had a well-defined hole devoid of any

visible material (Fig. 2F). Higher levels of nuclear abnormalities, mainly *blebbled* and *notched* erythrocytes, occurred after 24 h exposure to concentrations of 100 and 1000 µg L^{-1} Mg(hesp); after 96 h exposure, no significant differences were detected.

On the other hand, the mutagenicity index did not show significant difference from controls in any Mg(hesp) concentration at both exposure period. Micronuclei (Fig. 2G) which indicate mutagenicity was characterized by smaller but morphologically identical to nuclei and with same staining intensity and no connection to mainly nuclei.

Table 4. Morphological changes and cytogenetic index calculated by scoring micronuclei generation and nuclear abnormalities in erythrocytes of *P. lineatus* after 24 and 96 h exposure to different Mg(hesp) concentrations. Values are expressed as median (first quartile – third quartile). Letters indicate significant difference ($P < 0.05$) in the same period of exposure.

MORPHOLOGICAL AND CYTOGENETIC INDEX

Exposure treatments (µg L^{-1})		Crenated cells index	Genotoxicity index	Mutagenicity index
24h exposure	**0 (NC)**	0.0000 (0.0000 – 0.0000) [a]	0.0002 (0.0000 – 0.0003) [a]	0.0017 (0.0000 – 0.0022) [a]
	1	0.0053 (0.0000 – 0.0107) [ab]	0.0131 (0.0008 – 0.0153) [ab]	0.0012 (0.0002 – 0.0016) [a]
	10	0.0046 (0.0013 – 0.0064) [ab]	0.0103 (0.0071 – 0.0109) [ab]	0.0021 (0.0013 – 0.0023) [a]
	100	0.0251 (0.0008 – 0.0378) [ab]	0.0246 (0.0154 – 0.0253) [b]	0.0011 (0.0000 – 0.0015) [a]
	1000	0.0600 (0.0000 – 0.0648) [b]	0.0290 (0.0055 – 0.0400) [b]	0.0006 (0.0000 – 0.0008) [a]
96h exposure	**0 (NC)**	0.0178 (0.0082 – 0.0207) [a]	0.0283 (0.0218 – 0.0293) [a]	0.0015 (0.0003 – 0.0017) [a]
	1	0.0193 (0.0085 – 0.0245) [a]	0.0272 (0.219 – 0.0305) [a]	0.0009 (0.0000 – 0.0010) [a]
	10	0.0168 (0.0014 – 0.0201) [a]	0.0271 (0.0244 – 0.0274) [a]	0.0006 (0.0000 – 0.0008) [a]
	100	0.0384 (0.0003 – 0.0765) [a]	0.0339 (0.0252 – 0.0426) [a]	0.0005 (0.0003 – 0.0006) [a]
	1000	0.0127 (0.0000 – 0.0167) [a]	0.0354 (0.0000 – 0.0378) [a]	0.0005 (0.0000 – 0.0001) [a]

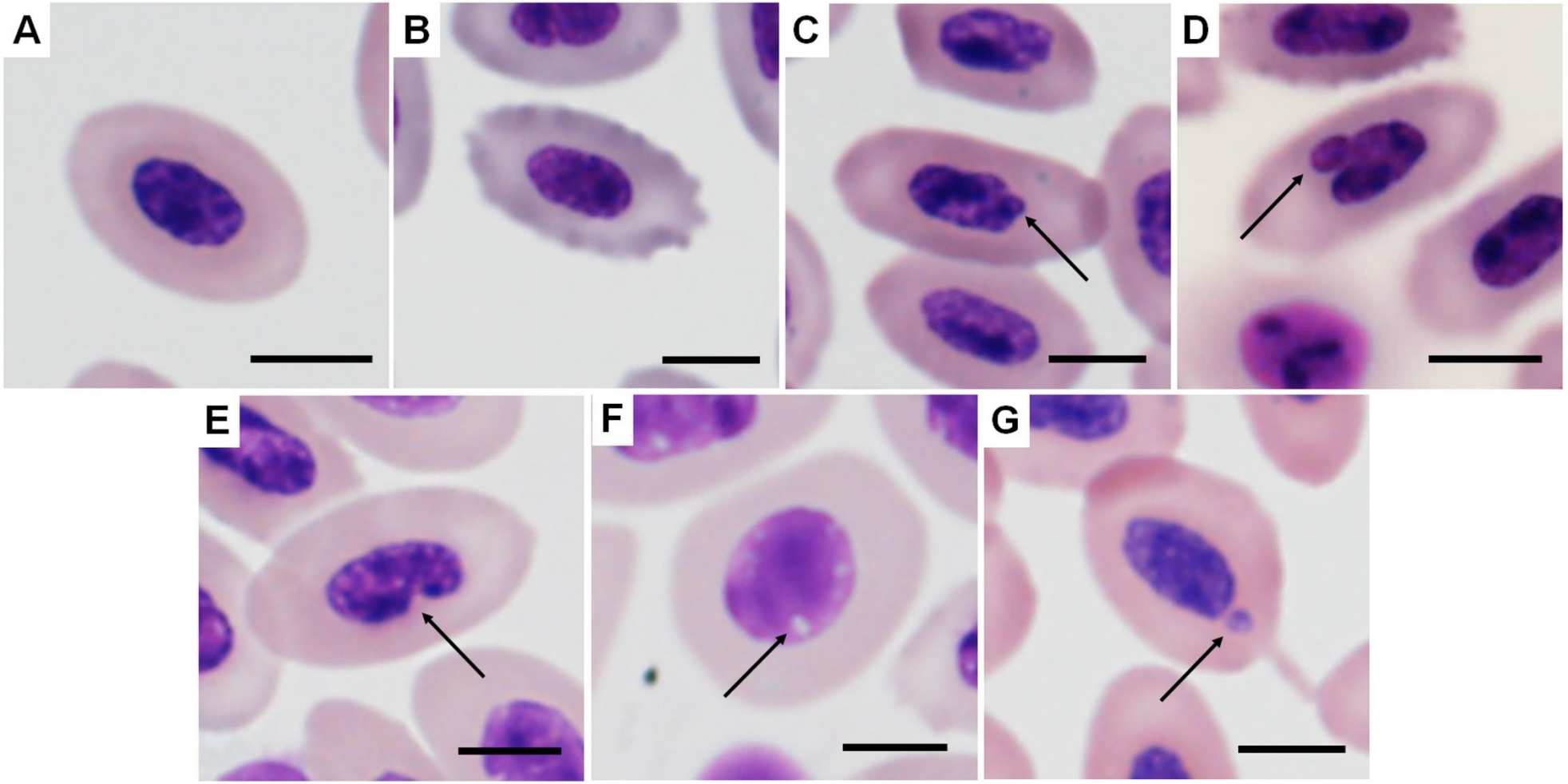

Figure 2. Morphology of erythrocytes of *P. lineatus* with scored micronucleus, nuclear abnormalities and crenated analysis. A –Erythrocyte; B – *crenated* erythrocyte; C - *blebbed* erythrocyte; D – *lobed* erythrocyte; E – *notched* erythrocyte; F – *vacuolated* erythrocyte; G – erythrocyte with micronucleus. Scale bar = 5 µm

3.4 Biochemical biomarkers

All biochemical biomarkers assessed were reported in Table 5. In order to proportionally compare the different times of exposure, Figure 3 represents the percentage values relative to negative control.

For antioxidant biomarkers, after 24 h exposure to Mg(hesp), liver analyses presented alteration of CAT activity, with decreases up to 60% at 100 and 1000 µg L^{-1}. SOD and GST did not change. Metallothionein increased around 58% after exposure to 10 µg L^{-1}. After 96 h exposure, SOD activity decreased up to 11% in 1 and 10 µg L^{-1} while CAT activity increased up to 50% at 1, 100 and 1000 µg L^{-1} Mg(hesp) concentrations. The activity of the biotransformation enzyme GST increased 45% at 10 µg L^{-1} Mg(hesp) and GSH content increased 34% in 100 µg L^{-1}. Lipid peroxidation remain unchanged after 24 h and 96h exposure to Mg(hesp).

Table 5. Biochemical biomarkers in *Prochilodus lineatus* liver after 24 and 96 h exposure to Mg(hesp). Values are expressed as median (first quartile – third quartile). Letters indicate significant difference (P < 0.05) inside same period of exposure.

		BIOCHEMICAL BIOMARKERS					
Exposure treatments ($\mu g\ L^{-1}$)		**Superoxide dismutase (U SOD mg protein^{-1})**	**Catalase (nmol H_2O_2 mg protein^{-1} min^{-1})**	**Glutathione S-transferase (mmol mg protein^{-1} min^{-1})**	**Reduced glutathione ($\mu mol\ mL^{-1}$ mg protein^{-1})**	**Metallothionein (ng mL^{-1} mg protein^{-1})**	**Lipid peroxidation (pmol H_2O_2.mg protein^{-1})**
24 h exposure	**0 (NC)**	28.75 (27.64 – 29.50) [a]	44.57 (41.73 – 49.49) [a]	20.07 (18.73 – 21.40) [a]	0.88 (0.72 – 1.34) [a]	0.40 (0.30 – 0.50) [a]	3.00 (2.00 – 6.00) [a]
	1	28.17 (28.03 – 28.57) [a]	48.22 (45.83 – 49.46) [a]	20.25 (17.83 – 21.97) [a]	0.95 (0.67 – 1.30) [a]	0.40 (0.30 – 0.50) [a]	3.00 (2.00 – 3.00) [a]
	10	24.12 (23.98 – 27.35) [a]	54.07 (47.13 – 64.64) [a]	18.67 (15.18 – 22.66) [a]	1.24 (0.71 – 1.57) [a]	0.60 (0.60 – 0.80) [b]	2.00 (2.00 – 3.00) [a]
	100	27.12 (26.97 – 27.14) [a]	18.55 (18.55 – 26.97) [b]	18.03 (12.57 – 25.35) [a]	0.74 (0.68 – 2.16) [a]	0.30 (0.20 – 0.40) [a]	3.00 (3.00 – 3.00) [a]
	1000	29.28 (26.38 0 31.72) [a]	27.49 (26.82 – 28.17) [b]	14.92 (13.63 – 18.63) [a]	1.08 (0.76 – 1.32) [a]	0.40 (0.30 – 0.50) [a]	3.00 (2.00 – 4.00) [a]
96 h exposure	**0 (NC)**	29.89 (29.19 – 31.08) [a]	30.77 (28.30 – 31.09) [a]	11.31 (10.56 – 12.73) [a]	0.62 (0.47 – 0.70) [a]	0.50 (0.40 – 0.60) [a]	6.00 (5.00 – 6.00) [a]
	1	26.51 (24.92 – 26.96) [b]	46.35 (44.93 – 47.48) [b]	8.72 (6.24 – 8.82) [a]	0.67 (0.55 – 0.68) [a]	0.30 (0.20 – 0.60) [a]	6.00 (5.00 – 6.00) [a]
	10	26.93 (24.27 – 32.29) [b]	31.67 (27.94 – 32.29) [ac]	16.42 (11.83 – 19.62) [b]	0.62 (0.46 – 0.68) [a]	0.50 (0.40 – 0.50) [a]	5.00 (5.00 – 6.00) [a]
	100	30.21 (29.63 – 30.55) [a]	38.92 (34.00 – 40.15) [bc]	11.27 (10.43 – 12.76) [a]	0.83 (0.68 – 1.03) [b]	0.60 (0.50 – 0.60) [a]	5.00 (5.00 – 6.00) [a]
	1000	27.64 (27.11 – 29.81) [ab]	38.87 (36.29 – 42.02) [bc]	11.84 (10.50 – 12.73) [a]	0.66 (0.61 – 0.73) [ab]	0.50 (0.50 – 0.50) [a]	5.00 (5.00 – 5.00) [a]

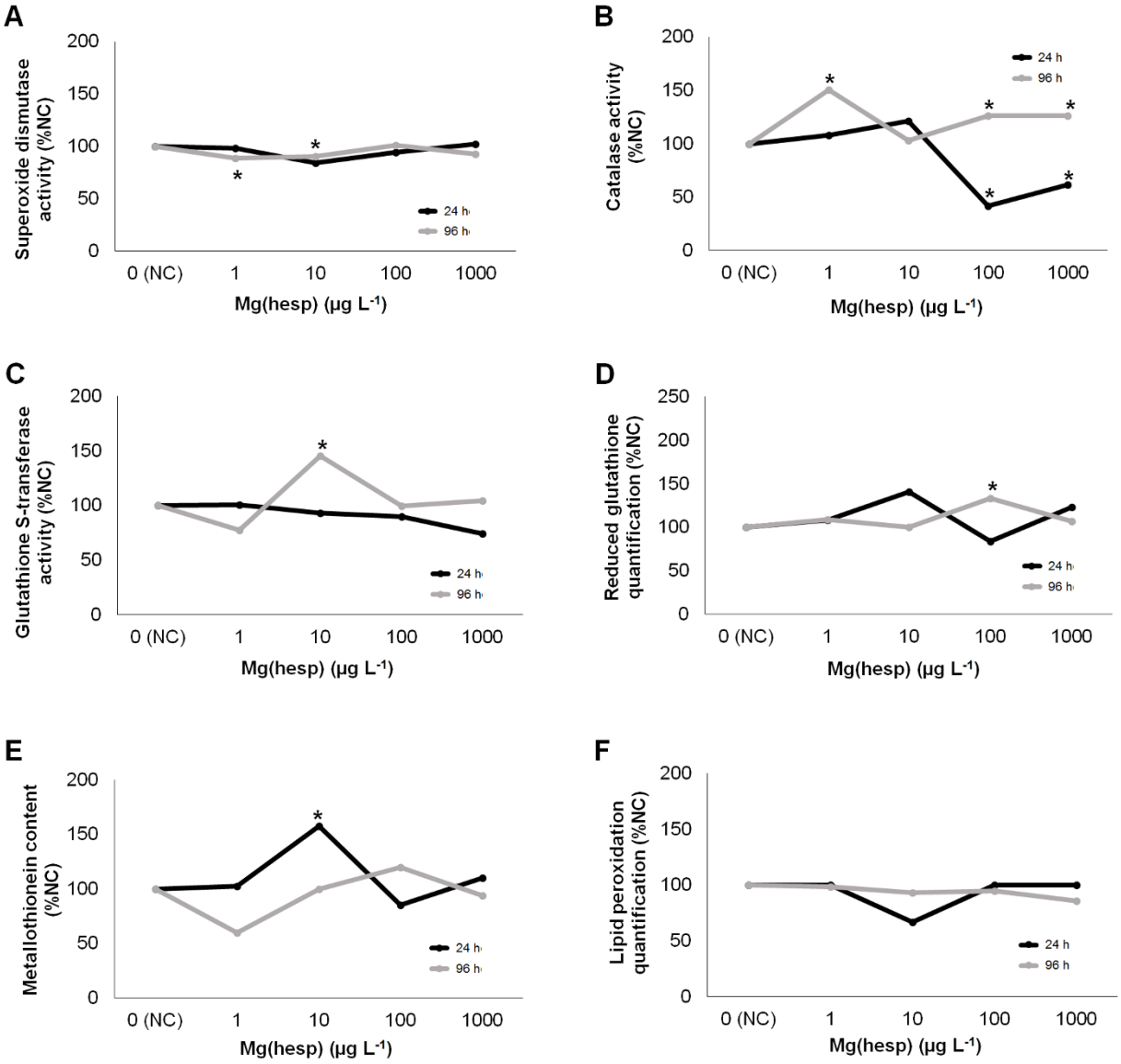

Figure 3. Percentage of control (NC) after 24 h and 96 h of exposure to Mg(hesp). A – Superoxide dismutase (SOD) activity; B – Catalase (CAT) activity; C – Glutathione S-transferase (GST) activity; D – Reduced glutathione (GSH) content; E – Metallothionein content; F – Lipid peroxidation (LPO) content.

4 DISCUSSION

Investigating biological effects of new biochemicals represents a crucial step in crop improvement with environmental consciousness. When dealing with *in vivo* analysis, the complexity of triggered responses can be clarified through assessment of several biomarkers.

Mg(hesp) effects in *P. lineatus* observed in hematological analyses showed it as a sensitive biomarker especially when analyzing aquatic contamination of pesticides and other chemicals (Pereira et al., 2013; Ramesh et al., 2018). Alterations in hematocrit values could be associated with increases in number or in volume of erythrocytes. Considering that increases in Htc values were accompanied by higher values of RBC mainly after 24 h of exposure, these results could be a response to stress condition such as the exposure to chemicals and pesticides, in which the higher number of RBC could be a direct consequence of splenic contraction stimulation, leading to a release of high quantities of erythrocytes in bloodstream (Heath, 1995). Such alterations were also detected with similar concentrations of other important pesticides, the clomazone (Pereira et al., 2013). Those changes observed in erythrocytes quantification was not followed by proportional increases in hemoglobin content, which consequently affect hematimetric parameters analyzed. MCV, MCH and MCHC significantly decreased after 24 h of exposure. Lower values of MCV and MCH also can be correlated with increases in number of immature erythrocytes in blood (Ramesh et al., 2018).

The comet assay results confirm the Mg(hesp) at high concentrations affect DNA integrity. Both, erythrocytes and hepatocytes analyzed, the increased percentage of DNA in Comet's tail in almost all concentrations of Mg(hesp) indicate instability effects in DNA integrity, which could be related with decreases observed in head intensity in all concentrations of erythrocytes analysis after 24 h of exposure and in hepatocytes analysis after 96 h (after 1, 10 and 100 µg L^{-1}). DNA instability impairs cellular metabolism and viability, affecting whole organisms and compromising its ability of trying to overcome stress conditions. However, considering the high sensitivity of Comet's assay in detect even low levels of DNA damage, such genotoxic effect could be reversed through some biomolecular pathways, including DNA repair systems, a complex genetic apparatus to reduce levels of chromosomal impairs and mutations (Begley and Samson, 2004).

The genotoxic effects initially observed by Comet assay corroborated with the genotoxicity index calculated in assessment of erythrocytes nuclear abnormalities. The formation of nuclear budding classified as *bebbled* or *lobed* has been associated with chromosomal instability events, including DNA excess that is being extruded from the nucleus and/or triggered DNA repair complexes (Fenech, 2007). Genetic impairs due to chemical exposure can lead to a variety of alterations, such as changes of individual nucleotide bases, DNA strand breaks, deletions, gene amplification and rearrangements influences (Matés and Sánchez-Jiménez, 2000; Cadet et al., 2003). However, the absence of micronuclei formation in all concentrations and times of exposure can be an indicative of successfully action of DNA repair mechanisms, considering that micronuclei may originate from clastogenic and/or aneugenic responses as an endpoint of DNA impair (Fenech, 2007).

The erythrocytes morphological and nuclear data corroborated with the evidences that the exposure to Mg(hesp) at higher concentrations affects the erythrocytes. Mature erythrocytes have smooth and elliptical nucleus shape disposed in a clear delimited cytoplasm. Despite sometimes may be artifacts resulting from improper sample collection and slide preparation, crenated erythrocytes also can be indicative of metabolic alterations, including ATP depletion (Harvey, 2008).

Mainly biochemicals pathways were not activated after 24 h exposure to Mg(hesp). The decreasing in the CAT activity at higher Mg(hesp) concentrations (100 and 1000 µg L^{-1}) may be the consequence of very high levels of H_2O_2 or other reactive oxygen species (ROS) as previously reported for Mg(hesp) in zebrafish hepatocytes cells (as discussed in Chapter 2) as nitric oxide (Brown, 1995; Purwar et al., 2011), and also for other pesticides, such as dimethoate and alphamethrin (Ansari and Ansari, 2014a; Ansari and Ansari, 2014b).

On the other hand, after 96 h exposure to Mg(hesp) exposure, CAT activity presented an opposite behavior than the observed after 24 h exposure. Such enzymatic antioxidant pathway was triggered to protect cells against further damage to its components in liver mainly in higher concentrations (100 and 1000 µg L^{-1}). Additionally, phase II biotransformation pathway against xenobiotic effects were detected only punctually, presenting higher values of GST and GSH in 10 and 100 µg L^{-1} concentrations, respectively. GST enzyme acts conjugating chemical compounds to GSH, generating a product capable of being excreted from cell, avoiding potential cellular damage and stress (Labade et al., 2018). Although the antioxidant system

showed different responses depending on Mg(hesp) exposure concentration, the final results of the activation of this system after 24 and 96 h can be considered the absence of increases in LPO in fish liver, which avoided membrane disruption.

Analyzing biological influences of new chemicals represents a challenge, especially considering scarce literature and reports of triggered response pathways. Results of this study indicates that hematological and genetic effects of Mg(hesp) concentrations occurs generally after 24 h of exposure, while after 96 h the fewer significant damage results represents a potential recover ability of organisms. The data evidence the needs of continuous investigations of Mg(hesp) effects and the promising use of such compound to control leaf ants.

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4 CONSIDERAÇÕES FINAIS

O presente estudo mostra a complexa interação entre Mg(hesp) e hepatócitos de *D. rerio* (células ZF-L) e de *P. lineatus* (curimbatá). A avaliação dos efeitos de novos compostos em ambientes aquáticos é particularmente desafiadora, considerando a ausência de literatura disponível e a complexidade das respostas desencadeadas nas diferentes matrizes bióticas. Nesse contexto, técnicas *in vitro* são essenciais para detectar efeitos primários, considerando suas diversas vantagens (SCHIRMER, 2006).

Os resultados *in vitro* obtidos detectaram uma forte influência de Mg(hesp) na população celular. As alterações visuais e análises de confluência em ZF-L se mostraram uma excelente ferramenta de detecção dos impactos causados na população celular, sendo o primeiro indicador dos efeitos citotóxicos de Mg(hesp) em altas concentrações. A redução da densidade celular observada não está relacionada com danos na estabilidade das membranas celulares, corroborado pela ausência de eventos de necrose, eliminando este mecanismo de morte celular como razão da queda da densidade celular. Entretanto, quaisquer efeitos em estruturas e componentes intracelulares limitam a capacidade celular de formar uma resposta compensatória para superar possíveis toxicidades. A citotoxicidade *in vitro* observada provavelmente está relacionada com o comprometimento de diversas atividades intracelulares, incluindo o decaimento da atividade metabólica mitocondrial e a perda da integridade lisossomal, que possivelmente desencadearem vias alternativas de morte celular em altas concentrações de Mg(hesp).

Adicionalmente, foram detectados aumentos na quantificação de EROs após exposição a altas concentrações de Mg(hesp), uma possível consequência em eventos de exposição a pesticidas, onde aumentos no estresse oxidativo podem ser danosos para as células, considerando sua capacidade em afetar o metabolismo celular (JABŁOŃSKA-TRYPUĆ, 2017). Os mecanismos de defesa ativados incluíram principalmente respostas via GSH, atuando como importante molécula biotransformadora e antioxidante, bem como a ação de MT como molécula essencial para a manutenção da homeostase metabólica, principalmente frente a efeitos de estresses oxidativos.

As anormalidades nucleares e respostas obtidas no ensaio do cometa detectadas principalmente após 24 h de exposição podem estar associadas a eventos

de instabilidade cromossômica, onde, após um evento de estresse oxidativo, o DNA amplificado é seletivamente direcionado à periferia nuclear e eliminado via brotamento nuclear (FENECH, 2007), estimulando a formação de complexos de reparo de DNA. Corroborando tais achados, as análises de ciclo celular mostraram um pico populacional na fase sub-G1, fato associado a fragmentação do DNA e mecanismos de morte celular (ORMEROD, 1998).

Quanto às análises *in vivo* realizadas em *P. lineatus*, anormalidades nucleares também foram detectadas na avaliação de eritrócitos, bem como danos de DNA pelo ensaio do cometa, confirmando efeitos de instabilidade cromossômica por exposição a Mg(hesp) possivelmente relacionada à geração de EROs e formação de mecanismos de reparo. As respostas bioquímicas detectadas nos hepatócitos nas análises *in vitro* não apresentaram um padrão claro em relação aos períodos de exposição e concentrações, porém, junto com as vias de reparo ativadas, foram eficazes em prevenir danos às membranas celulares, considerando a ausência de aumento na LPO.

Os resultados *in vitro* e *in vivo* obtidos neste estudo indicam que, apesar de causar respostas genéticas de estresse, os efeitos citotóxicos de Mg(hesp) após 96 h não foram tão severos, o que pode estar relacionado com a capacidade celular de se aclimatar e superar condições de estresse, principalmente se considerarmos o papel hepático em metabolizar xenobióticos. Adicionalmente, devemos considerar que as concentrações de Mg(hesp) avaliadas estão muito acima dos limites gerais de contaminantes orgânicos em ecossistemas aquáticos (BRASIL, 2005). Portanto, o composto Mg(hesp) apresenta promissor possibilidade de uso comercial, especialmente na concentração de 100 mg L^{-1}, considerando seus baixos efeitos subletais, ainda sendo necessário maiores estudos sobre efeitos e interações com diferentes matrizes biológicas.

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