UNIVERSIDADE FEDERAL DO PARANÁ

JOÃO LUIZ COELHO RIBAS

EFEITOS TOXICOLÓGICOS DE ANTINFLAMATÓRIOS NÃO ESTEROIDAIS EM PEIXES DE ÁGUA DOCE

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JOÃO LUIZ COELHO RIBAS

EFEITOS TOXICOLÓGICOS DE ANTINFLAMATÓRIOS NÃO ESTEROIDAIS EM PEIXES DE ÁGUA DOCE

Tese apresentada ao Programa de Pós-Graduação em Farmacologia, Setor Ciências Biológicas, Universidade Federal do Paraná.
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PARECER

A Comissão Examinadora da Tese de Doutorado intitulada "EFEITOS TOXICOLÓGICOS DE ANTINFLAMATÓRIOS NÃO ESTEROIDAIS EM PEIXES DE ÁGUA DOCE", de autoria do pós-graduando **JOÃO LUIZ COELHO RIBAS,** sob orientação da Prof.ª Dr.ª Helena Cristina da Silva de Assis e banca composta por: Prof.ª Dr.ª Helena Cristina da Silva de Assis (Presidente – Farmacologia – UFPR), Prof.ª Dr.ª Adriana Frohlich Mercadante (Patologia Básica – UFPR), Prof.ª Dr.ª Glória Emilia Petto de Souza (Física e Química – USP), Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR) e Prof.ª Dr.ª Solange Cristina Garcia (Análises – UFRGS), reuniu-se e de acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, o pós-graduando foi APROVADO. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas pela sua orientadora. Em Curitiba, 26 de agosto de 2014.

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Às três mulheres da minha vida...

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“Só porque você dança bem, não significa
que vai ser convidado para o baile.”

Michael Leboeuf

RESUMO

Fármacos antinflamatórios não-esteroidais (AINEs) são amplamente empregados na medicina humana e veterinária e apresentam potencial de contaminar água e sedimentos através de entradas de estações de tratamento de esgoto. No presente estudo, os efeitos de alguns AINEs foram analisados em peixes nativos (*Hoplias malabaricus* e *Rhamdia quelen*). A toxicidade do paracetamol, do diclofenaco e do ibuprofeno foi avaliada em cultura primária da linhagem macrofágica de rim anterior de *H. malabaricus*. Seus efeitos na viabilidade celular, produção de óxido nítrico (NO) induzida por lipopolissacarídeo (LPS) e genotoxicidade foram analisados. Na cultura celular primária, para padronização, a análise por citometria $CD11b^+$ mostrou 71,5 % de células progenitoras, 19,5 % de macrófagos e 9,0 % de monócitos. A produção de óxido nítrico induzida por LPS por essas células foi bloqueada após tratamento com dexametasona e L-NMMA. Após 24h de exposição das células ao diclofenaco (0,2-200 ng/mL), paracetamol (0,025-250 ng/mL) e ibuprofeno (10-1000 ng/mL), houve redução na produção basal de NO e inibição da produção de NO induzida por LPS em todas as concentrações testadas. A genotoxicidade ocorreu na maior concentração de diclofenaco, nas concentrações intermediárias de paracetamol e também com ibuprofeno. A toxicidade trófica do diclofenaco em *H. malabaricus* foi avaliada, sendo os peixes alimentados duas vezes por semana com *Astyanax sp*. previamente submetido à inoculação intraperitoneal (IP) com diclofenaco (0; 0,2; 2,0 ou 20,0 µg/Kg), totalizando 12 doses. A metade dos peixes recebeu carragenina IP a 1 mg/Kg e depois de 4 horas, os mesmos foram anestesiados e eutanasiados para estimativa da migração celular. Nos outros peixes (sem carragenina), os parâmetros hematológicos, a produção de NO basal e após estimulação por LPS em rim anterior, o índice hepatossomático (HSI) e a análise hepática das atividades de superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa S-transferase (GST), etoxiresorufina-O-deetilase (EROD) e catalase (CAT) foram determinadas. A glutationa reduzida (GSH) e a lipoperoxidação (LPO) foram também avaliados. Houve aumento na contagem eritrocitária e no hematócrito na menor dose de diclofenaco. A hemoglobina diminuiu na maior dose. A contagem de trombócitos aumentou em todos os grupos expostos ao diclofenaco e a contagem de leucócitos sanguíneos totais diminuiu seguindo a redução de neutrófilos. Os monócitos reduziram na maior dose. O número de macrófagos peritoneais residentes não diferiu entre os grupos, mas a migração celular reduziu após a administração de carragenina, com uma significante diminuição na migração dos polimorfonucleares. A síntese basal de NO das culturas celulares de rim anterior dos animais tratados com diclofenaco foi significantemente menor nas células dos grupos de 2 e 20 µg/Kg. A produção de NO estimulada por LPS decresceu em todos os grupos tratados. No fígado, o diclofenaco causou estresse oxidativo com aumento de LPO e de atividade da GPx. Em contraste, a atividade da GST reduziu. Os efeitos do diclofenaco em componentes do sistema imune também foram avaliados após exposição hídrica de *R. quelen* ao diclofenaco a 0,2, 2,0 e 20,0 µg/L durante 14 dias. Os peixes foram anestesiados, o sangue retirado e após eutanasia o rim anterior foi coletado. As proteínas do plasma e rim anterior envolvidas na produção de NO, migração celular e ativação do sistema complemento foram analisadas por cromatografia líquida acoplada à espectrometria de massas do tipo *tandem*. No plasma foi observada a0 inibição da expressão de receptor toll like 2 (Tlr2), fosfolipase Cγ (Plcγ), quinase quinase quinase 3 (Mekk), 1-fosfatidilinositol 3-quinase (Pi3k), proteína ativadora-1 (Ap-1), fator nuclear de polipeptídeo kappa light (Nf-kb) e proteína NO sintase induzível (iNOS). No rim anterior, a expressão de Tlr2, Plcγ, Mekk,

Pi3k, Ap1 e Nf-kb também foi significantemente inibida. Várias proteínas envolvidas na migração celular foram detectadas no plasma. Nos peixes machos, a expressão da proteína receptora de quimiocina 4 (Cxcr4), Integrina α1 (Itα1), Radixina (Rdx) e Metalopeptidase de matriz (Mmp)-17 foi inibida. Nos peixes fêmeas, a expressão de Cxcr4, Itga1, Rdx, Mmp17 e Mmp1 reduziu. No presente estudo, houve modificação na expressão da proteína componente do complemento 3 (C3), do fator B do complemento (Cfb) e da serina peptidase 1 associada à manana (Masp1), bem como de C1q e do componente do complemento 7 (C7). Adicionalmente, MHC1 no plasma diminuiu significativamente. Em síntese, os NSAIDs estudados influenciaram a produção de NO e causaram danos ao DNA nas células monocíticas oriundas de *H. malabaricus*. Os peixes apresentaram modificações hematológicas e bioquímicas quando submetidos à exposição ao diclofenaco. A inibição da expressão de muitas proteínas envolvidas na síntese de NO, migração celular e ativação do sistema complemento foi observada nos peixes estudados, o que pode comprometer os mecanismos de defesa imune inata destes animais.

Palavras-chave: Fármacos. Cultura celular. Imunotoxicidade. Genotoxicidade. Macrófago. Estresse oxidativo. Migração celular. Parâmetros hematológicos.

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are largely employed in human and veterinary medicine and have the potential to contaminate water and sediments via inputs from sewage treatment plants. Their impacts on humans and ecosystems are emerging issues in environmental health. In the present study, the effects of some NSAIDs were analyzed in native fish species (*Hoplias malabaricus* and *Rhamdia quelen*). The toxicity of acetaminophen, diclofenac and ibuprofen was evaluated on primary culture of monocytic lineage of anterior kidney from *H. malabaricus*. Their effects in cell viability, lipopolysaccharide (LPS)-induced nitric oxid (NO) production and genotoxicity were analyzed. In primary culture cell, cytometry analysis CD11b^{+} cells showed 71.5 % of stem cells, 19.5 % of macrophages and 9.0 % of monocytes. Cell viability was lower in the Ficoll compared to Percoll separation. LPS-induced NO production by these cells was blocked after treatment with dexamethasone and L-NMMA. After 24 h of cell exposure to diclofenac (0.2-200 ng/mL), acetaminophen (0.025-250 ng/mL) and ibuprofen (10-1000 ng/mL), there was a reduction in basal NO production and an inhibition of LPS-induced NO production at all tested concentrations. Genotoxicity occurred at the highest concentration of diclofenac, at the intermediary concentrations of acetaminophen and also with ibuprofen. The toxicity of diclofenac was also evaluated in *H. malabaricus* after trophic exposure, where fish were fed twice every week with *Astyanax sp*. previously submitted to intraperitoneal inoculation (IP) with diclofenac (0; 0.2; 2.0 or 20.0 µg/Kg), totaling 12 doses. In sequence, half of fish received 1 mg/Kg of carrageenan IP and after 4 hours, they were anesthetized and euthanized for cell migration estimation. In the other fish (without carrageenan), the hematological parameters, NO basal production and after LPS-stimulate in head kidney, hepatosomatic index (HSI) and liver biochemical analysis, such as activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), ethoxyresorufin-O-deethylase (EROD) and catalase (CAT) were measured. Reduced glutathione (GSH) and lipoperoxidation (LPO) were also determined. The results of trophic exposure of *H. malabaricus* showed increases in red blood cells count and in the hematocrit at the low dose of diclofenac. In contrast, the hemoglobin reduced at the highest dose. Thrombocyte count increased in all groups exposed to diclofenac and the total blood leukocyte counts decreased following the neutrophil's reduction. Monocytes decreased at the highest dose. The number of resident peritoneal cells did not differ among the groups, but the cell migration reduced after carrageenan administration, with a significant decrease in the migration of polymorphonuclear cells. The basal NO synthesis of anterior kidney cell cultures from diclofenac-treated animals was significantly lower in the cells from the groups 2 and 20 µg/Kg. The LPS-stimulated NO production reduced in all of the diclofenac-treated groups. Diclofenac also reduced HSI at the 0.2 µg/Kg. In liver, diclofenac caused oxidative stress with increased LPO and GPx activity. In contrast, GST activity decreased. The effects of diclofenac in components of the immune system were also evaluated after hydric exposure of *R. quelen* to diclofenac at 0.2, 2.0 and 20.0 µg/L during 14 days. After the exposure, fish were anesthetized and blood was taken from caudal vein. After this, fish were euthanized and the anterior kidney was collected. Plasma and kidney proteins involved in NO production, cell migration and complement system activation were analyzed using liquid chromatography tandem mass spectrometry in a shotgun proteomic approach. Results obtained after hydric exposure of *R. quelen* to diclofenac for plasma samples showed significant inhibition in the expression of toll like receptor 2 (Tlr2), phospholipase Cγ (Plcγ), kinase kinase

kinase 3 (Mekk), 1-phosphatidylinositol 3-kinase (Pi3k), activator protein-1 (Ap-1), nuclear factor of Kappa light polypeptide (Nf-kb), and the NO synthase inducible protein (iNOS). In the head kidney, the expression of Tlr2, Plcγ, Mekk, PI3K, Ap1 and Nf-kb was also significantly inhibited. Various proteins involved in cell migration were detected in the plasma. In male fish, the expression of Chemokine receptor 4 protein (Cxcr4), Integrin α1 (Itα1), Radixin (Rdx) and Matrix Metallopeptidase (Mmp)-17 was inhibited. In female fish, the expression of Cxcr4, Itga1, Rdx, Mmp17 and Mmp1 decreased. In the present study, the expression of complement component 3 protein (C3), complement factor B (Cfb) and mannan-binding lectin serine peptidase 1 (Masp1) changed as well as C1q and complement component 7 (C7). Additionally, MHC1 in plasma significantly decreased. In summary, the studied NSAIDs influenced NO production and caused DNA damage in monocytic cells from *H. malabaricus*. Fish presented hematological and biochemical changes when submitted to diclofenac exposure. The expression inhibition of many proteins involved in NO synthesis, cell migration and activation of the complement system was observed in the studied fish, which may compromise innate immune defense mechanisms of these animals.

Keywords: Pharmaceuticals. Cell culture. Immunotoxicity. Genotoxicity. Macrophage. Oxidative stress. Cell migration. Hematological parameters.

APRESENTAÇÃO

Esta tese se inicia com uma introdução, seguida de revisão bibliográfica e objetivos. A seguir, a mesma está apresentada na forma de três artigos científicos:

1. Effects of anti-inflammatory drugs in primary kidney cell culture of a freshwater fish
2. Effects of trophic exposure to diclofenac in freshwater fish
3. Immunosuppressive effects of diclofenac in a freshwater fish: a proteomic approach

Na sequência, os tópicos DISCUSSÃO GERAL E CONCLUSÕES E PERPECTIVAS apresentam comentários gerais a respeito dos resultados obtidos nos três artigos bem como as conclusões gerais da tese.

As listas de figuras, siglas, símbolos e abreviaturas e as referências referem-se ao conteúdo apresentado na introdução, revisão bibliográfica e discussão geral da tese, além dos artigos científicos.

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LISTA DE ABREVIATURAS, SÍMBOLOS E SIGLAS

AIEs - antinflamatórios esteroidais
AINEs - antinflamatórios não esteroidais
Akt - serine/threonine-protein kinase
Ap1 - activator protein-1
C1q - complement component 1
C3 - complement component 3 protein
C7 - complement component 7
Cfb - complement factor b
Cg - carrageenan
COX - ciclo-oxigenase
Cxcr4 - chemokine receptor 4 protein
Cxcrs - chemokines through receptors
FSC - forward scatter
ICAM - intercellular cell adhesion molecule
iNOS - inducible nitric oxide synthase
Itga1 - integrin alpha 1
JAM - junctional adhesion molecule
LC-MS/MS - liquid chromatography tandem mass spectrometry
Lfa1 - leukocyte function-associated antigen-1
L-NMMA - n^g-methyl-l-arginine
LPS - lipopolysaccharide
m/z - relaçãomassa/carga
Mac - membrane attack complex
Masp1 - mannan-binding lectin serine peptidase 1
Mbl - mannose-binding lectin
Mekk - kinase kinase kinase 3
Mmp1 - matrix metallopeptidase 1
Mmp17 - matrix metallopeptidase 17
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nf-kb - nuclear factor of kappa light polypeptide
NO - nitric oxide

NSAIDs	-	non-steroidal anti-inflammatory drugs
PBS	-	phosphate-buffered saline
PECAM	-	platelet endothelial cell adhesion molecule
Pi3k	-	enzyme 1-phosphatidylinositol 3-kinase
PLC gamma	-	phospholipase c gamma
Q-TOF	-	accurate-mass quadrupole time-of-flight
Rdx	-	radixin
SSC	-	side scatter
TCEP	-	tris(2-carboxyethyl)phosphine
TEAB	-	triethylammonium bicarbonate buffer
Tlr	-	toll like receptor
Tlr2	-	toll like receptor 2
TNF-α	-	fator de necrose tumoral α
VCAM	-	vascular cell adhesion molecule

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1. INTRODUÇÃO

Produtos químicos são depositados no ambiente devido a atividades humanas, resultando em deterioração do ambiente e consequentemente da biodiversidade (MACEDO-SOUZA *et al.*, 2009).

Os produtos químicos farmacêuticos são projetados para ter um modo específico de ação, e muitos deles para ter alguma persistência no organismo. Estas características os tornam um potencial alvo de pesquisas, especialmente por esses medicamentos poderem alcançar o ambiente por diversas vias, levando os animais e os seres humanos a uma exposição direta. Uma visão global e integrada do ciclo dos produtos farmacêuticos inclui não apenas as finalidades de drogas, mas também as suas consequências involuntárias para o meio ambiente e para o homem (FENT; WESTON; CAMINADA, 2006; MORGAN, 2001; RUHOY; DAUGHTON, 2008).

A ocorrência de produtos farmacêuticos no ambiente aquático é hoje em dia um problema bem estabelecido (GINEBREDA *et al.*, 2010), tornando-se uma questão de caráter científico e de preocupação pública, em busca de regulamentação para que as gerações futuras não sofram os males pela presença de produtos farmacêuticos no ambiente.Toneladas de produtos farmacêuticos das mais diferentes classes são depositadas no ambiente, após a sua utilização e excreção através das águas residuais e sistemas de tratamento de esgoto. Esses compostos são encontrados no ambiente aquático em concentrações que podem variar de ng/L a µg/L (CARBALLA; OMIL; LEMA, 2005; CHRISTEN *et al.*, 2010; GROS; PETROVIC; BARCELÓ, 2007; HEBERER; REDDERSEN; MECHLINSKI, 2002; JOSS *et al.*, 2005; KOLPIN *et al.*, 2004; OWEN *et al.,* 2009; SANTOS; APARICIO; ALONSO, 2007; SUÁREZ *et al.*, 2008; VIENO; TUHKANEN; KRONBERG, 2007).

A questão da toxicidade de produtos farmacêuticos e seus metabólitos no meio ambiente é algo que vem ganhando atenção, especialmente nos últimos anos, com real destaque sobre fármacos encontrados em esgotos ou efluentes de estações de tratamento de esgoto (KÜMMERER, 2009a,b), pois todos os produtos farmacêuticos liberados para o ambiente são considerados poluentes, uma vez que exercem efeitos biológicos (KHALAF, 2009).

Com a evolução das técnicas analíticas está sendo possível a identificação de níveis extremamente baixos de fármacos e seus metabólitos no ambiente aquático. Essas detecções remetem a estudos que tentam explicar qual o risco dessas águas à saúde e segurança do homem exposto a esses poluentes (BERCU *et al.*, 2008; GINEBREDA *et al.*, 2010; JONES; LESTER; VOULVOULIS, 2005; KOLPIN *et al.*, 2002). Contudo, a análise da ocorrência e toxicidade de todos os resíduos farmacêuticos é impraticável (KOSTICH; LAZORCHAK, 2008).

Este trabalho propôs estudar possíveis efeitos tóxicos de antinflamatórios encontrados no ambiente aquático e que poderia acarretar alterações bioquímicas, hematológicas e imunológicas. Sendo assim, nós propomos que, os antinflamatórios não esteroidais encontrados no ambiente aquático possam causar alterações na fisiologia normal no peixe, especialmente nas defesas antioxidantes e imunológicas. Especificamente o diclofenaco pode alterar tanto as funções bioquímica e hematológica, quanto a função imunológica. Dessa maneira propomos que a exposição tanto trófica quanto hídrica em espécies distintas de peixes possa apresentar tais resultados.

2. REVISÃO BIBLIOGRÁFICA

A ocorrência de fármacos de uso humano e veterinário vem sendo detectada em águas superficiais, sedimentos e esgotos domésticos no mundo todo. Embora muitos destes fármacos tenham sido submetidos a estudos toxicológicos especialmente utilizando peixes como modelo, informações sobre o seu destino no ambiente e seus riscos e efeitos tóxicos aos organismos aquáticos não são totalmente elucidadas ou compreendidas (AUKIDY; VERLICCHI; VOULVOULIS, 2014; CARMONA; ANDREU; PICÓ, 2014; CORCOLL *et al.*, 2014; FENT; WESTON; CAMINADA, 2006; GALUS *et al.,* 2013; ONESIOS; BOUWER, 2012; ORIAS; PERRODIN, 2013; PAL *et al.*, 2014; SANTOS *et al.*, 2013; STUMPF *et al.*, 1999; TANOUE *et al.*, 2014; YING *et al.* ,2013).

Quimicamente, os compostos ativos contidos em um medicamento são frequentemente formados por moléculas complexas com propriedades especiais, com diferentes funcionalidades e propriedades físico-químicas e biológicas. A maioria desses compostos tem características polares (KÜMMERER, 2009a), e são

frequentemente chamados de “micropoluentes” (COMEAU *et al.*, 2008; KÜMMERER, 2009b; MORLEY, 2009; SCHLÜTER *et al*., 2007).

Além das substâncias ativas, as formulações farmacêuticas podem incorporar adjuvantes, a exemplo de pigmentos e corantes, e também podem acarretar, em menor grau, impactos sobre o meio ambiente (KOCH *et al.*, 2005).

As características dos produtos farmacêuticos podem ser ácidas, alcalinas ou anfotéricas. Em termos ambientais, as moléculas podem assumir condições catiônicas, aniônicas, anfóteras ou de neutralidade, assumindo assim comportamentos ambientais complexos, o que pode gerar efeitos ambientais diferenciados (KÜMMERER, 2009a).

Poucos dados disponíveis existem sobre a real utilização mundial de produtos farmacêuticos, inclusive sobre o seu consumo e aplicação, que podem variar consideravelmente de um país para outro (GOOSSENS *et al*., 2005, 2007; SCHUSTER; HÄDRICH; KÜMMERER, 2008). Sabe-se, no entanto, que o mercado de produtos farmacêuticos está em franco desenvolvimento, sendo que na União Européia, por exemplo, são catalogadas mais de 3000 substâncias diferentes que são utilizadas na medicina humana. Em relação ao mercado de fármacos, o Brasil é um dos maiores consumidores do mundo, juntamente com Estados Unidos, França e Alemanha (CHRISTEN *et al*., 2010; FENT; WESTON; CAMINADA, 2006; STUMPF *et al*., 1999).

Também se estima, segundo a Organização Mundial de Saúde (OMS), que aproximadamente 80% dos medicamentos produzidos no mundo são consumidos pela população de países ricos, enquanto que no Brasil, a classe mais favorecida economicamente é responsável por 48% do consumo de fármacos. Estima-se também que em países em desenvolvimento como o Brasil, cerca de 30% dos recursos em saúde são destinados à aquisição de medicamentos demonstrando que o medicamento é tido hoje como elemento de primeira ordem (OLIVEIRA; XAVIER; ARAÚJO, 2012).

Como esperado, produtos farmacêuticos estão presentes em maior concentração em efluentes hospitalares em relação ao esgoto municipal (KÜMMERER, 2009a; SCHUSTER; HÄDRICH; KÜMMERER, 2008).

Quanto à eliminação caseira de medicamentos, muitas vezes as sobras são eliminadas no lixo doméstico ou descartadas no ralo, em pias ou banheiros, o que pode também ser uma rota importante de eliminação de fármacos, o que requer

maior atenção, especialmente ambiental (AN *et al.*, 2009; BOUND; VOULVOULIS, 2005; COMEAU *et al.*, 2008; GOTZ; KEIL,2007).

Se os medicamentos forem descartados em pias ou banheiros, estes vão diretamente para as estações de tratamento de esgoto. No entanto, se eles forem descartados no lixo doméstico, os compostos vão acabar em aterro e também podem chegar aos efluentes (AHEL; JELICIC, 2001; METZGER, 2004), sendo uma fonte de contaminação de águas superficiais ou subterrâneas (COMEAU *et al.*, 2008, KÜMMERER, 2009a). As possíveis rotas de fármacos no meio ambiente estão ilustrada na Figura 1.

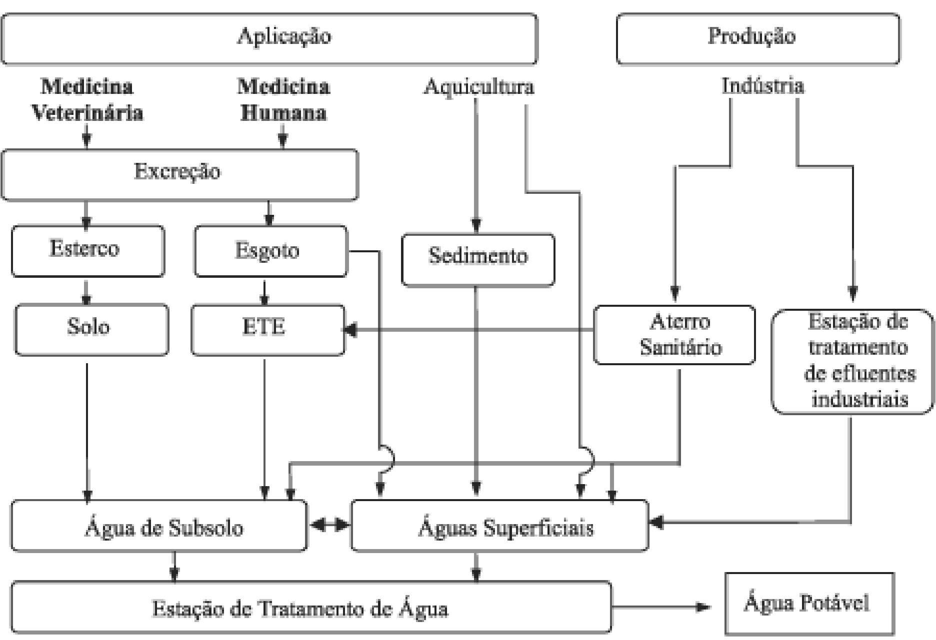

FIGURA 1 - POSSÍVEIS ROTAS DE FÁRMACOS NO MEIO AMBIENTE
FONTE: BILA; DEZOTTI, 2003. ETE – Estação de tratamento de esgoto.

Estudos reportam a presença de mais de 90 tipos de drogas diferentes no ambiente aquático, incluindo os seus metabólitos ativos e inativos, especialmente encontrados em águas superficiais, na União Européia, Estados Unidos, Brasil e Canadá (COMEAU *et al.*, 2008; POMATI *et al.*, 2007).

Devido aos medicamentos e seus metabólitos não serem eliminados durante o tratamento do esgoto (BUERGE *et al.*, 2006; KÜMMERER, 2009a; VERPLANCK *et al.*, 2005), estudos realizados até o momento descrevem a ocorrência desses compostos em efluentes de unidades de esgotos e de estações de tratamento, em

águas superficiais, subterrâneas e até mesmo na água potável, tais como a carbamazepina, o fenobarbital e o diclofenaco (COMEAU *et al.*, 2008; HEBERER, 2002; KALLENBORN *et al.*, 2008; KUMAR; XAGORARAKI, 2010; MORLEY, 2009;STÜLTEN *et al.*, 2008). No entanto, pouco se sabe sobre os efeitos ecotoxicológicos de medicamentos em animais. Especialmente importantes são os organismos aquáticos, que são expostos a águas com resíduos durante toda a sua vida, o que pode afetá-los durante todas as fases de seu desenvolvimento (FENT; WESTON; CAMINADA, 2006; POMATI *et al.*, 2007).

O risco de efeitos adversos em seres humanos através da ingestão de produtos farmacêuticos presentes na água potável parece ser insignificante. Assim, os resíduos de produtos farmacêuticos no ambiente parecem ser mais uma preocupação ambiental do que necessariamente humana (CHRISTENSEN, 1998, KÜMMERER, 2009a). Porém há escassa informação sobre a toxicidade crônica ou o potencial de bioacumulação de produtos farmacêuticos na biota e na cadeia alimentar (CHRISTEN *et al.*, 2010; OAKS *et al.*, 2004).

No entanto, idosos ao administrarem concomitantemente vários medicamentos diferentes já sofrem, frequentemente, os efeitos colaterais destes durante o tratamento. Somado a isso, ao receberem medicação via água potável, podem originar ou mesmo agravar os efeitos colaterais já ocasionados. Infelizmente, dados a este respeito são escassos e os poucos trabalhos nessa área relatam a avaliação de uma substância isoladamente e não de uma mistura, o que é a realidade no ambiente (KÜMMERER, 2009a).

Estudos verificaram que os efeitos negativos dos produtos farmacêuticos contidos no ecossistema aquático podem ser transferidos no interior da cadeia alimentar (CHRISTEN *et al.*, 2010; OAKS *et al.*, 2004). Esses estudos relatam que existe uma correlação direta entre resíduos contendo diclofenaco e insuficiência renal e visceral, o que pode implicar fortemente em mortalidade causada pela ingestão de resíduos de drogas antinflamatórias não esteróides. Outros medicamentos associados à transferência trófica podem ser os antibióticos (SWAN, *et al.*, 2006; TAGGART *et al.*, 2007).

Um fator adicional da toxicidade de um composto farmacêutico no ambiente aquático é a sua persistência. Uma vez liberado no ambiente, o composto é transportado e distribuído para as águas superficiais, sedimentos e biota. As ações e concentrações em cada um destes compartimentos são determinadas por uma

série de fatores e processos, incluindo a concentração da droga e suas propriedades físico-químicas, a separação de sedimentos, a degradação, as características ambientais e as condições climáticas do habitat. A degradação das substâncias pode ocorrer de forma biótica por organismos aeróbios ou anaeróbios, ou de forma abiótica via fotodegradação e/ou hidrólise (BOXALL *et al*., 2004; MORLEY, 2009).

Outro problema relativo a esses compostos é que muitos deles, especialmente os fármacos pouco solúveis, têm um potencial muito grande de bioacumulação. Isso representa um risco ainda maior para a saúde dos organismos aquáticos e da população humana em geral (CHRISTEN *et al.*, 2010; CRANE; WATTS; BOUCARD, 2006; DELÉPÉE; POULIQUEN; LE BRIS, 2004; LUNESTAD, 1992; MIGLIORE *et al*., 1993).

Apesar da existência de uma ampla variedade de classes de fármacos que são usados na terapêutica humana e veterinária, apenas alguns são considerados de real importância para o meio ambiente e para a saúde pública, por causa de seus volumes de consumo, toxicidade e persistência no ambiente. Entre esses, destacam-se os beta-bloqueadores, os quimioterápicos, os hormônios esteróides, os compostos neurológicos, os antiparasitários, os hipolipemiantes, os antibióticos e especialmente os analgésicos e antinflamatórios (CHRISTEN *et al.*, 2010; FENT; WESTON; CAMINADA, 2006; HERNÁNDEZ *et al*., 2007; MORLEY, 2009).

2.1 ANTINFLAMATÓRIOS

A classe dos antinflamatórios pode ser dividida em dois grupos: os antinflamatórios não esteroidais (AINEs) e os antinflamatórios esteroidais (AIEs).

Os AINEs são medicamentos amplamente utilizados e, consequentemente, são frequentemente detectados em esgotos e águas superficiais (GOMEZ *et al*.,2007; MORLEY, 2009). Muitos AINEs, tais como ibuprofeno, diclofenaco e paracetamol (acetaminofeno), são encontrados em corpos d’água, além do ácido salicílico (derivado do ácido acetilsalicílico) e naproxeno (FENT; WESTON; CAMINADA, 2006).

Os AINEs constituem um grupo de compostos muito heterogêneos com várias estruturas químicas, podendo ser distribuídos em diversas classes, de acordo com o grupo químico a que pertencem. Como mecanismo, os AINEs atuam no bloqueio da síntese de prostaglandinas, através da inibição da ciclo-oxigenase (COX). As

prostaglandinas são obtidas através do metabolismo do ácido araquidônico, que se encontra esterificado nos fosfolipídeos das membranas celulares. Uma vez liberado pela ação das fosfolipases, o ácido araquidônico é metabolizado através de duas vias enzimáticas distintas. A via das ciclo-oxigenases, que desencadeia a síntese de prostaglandinas, prostaciclinas e tromboxanos, e a via das lipo-oxigenases, responsável pela síntese de leucotrienos, lipoxinas e outros compostos. A ciclo-oxigenase é encontrada em duas isoformas, denominadas ciclo-oxigenase-1 (COX-1) e ciclo-oxigenase-2 (COX-2). A COX-1 é expressa constitutivamente, ou seja, está presente nas células em condições fisiológicas, principalmente nos vasos sanguíneos, plaquetas, estômago e rins. A COX-2 pode ser induzida na presença de interleucina-1, interleucina-2 e fator de necrose tumoral α (TNF-α), ésteres de forbol, fatores de crescimento e endotoxinas, e é expressa por células envolvidas no processo inflamatório (BURKE; SMYTH; FITZGERALD, 2006; CARVALHO, 2010; KHALAF, 2009).

Em 2002, uma variante da COX-1 em níveis elevados foi isolada no córtex cerebral e no tecido cardíaco de cão. A variante tem sido chamada, desde então, por várias denominações, tais como ciclo-oxigenase-3 (COX-3), COX-1b ou COX-1v. É possível que essa via possa ser o principal mecanismo central pelo qual drogas como paracetamol e fenacetina exercem seus efeitos analgésicos e antipiréticos, estando, talvez, relacionada imunologicamente a COX-2 (Hinz; Brune, 2012; KAM; SO, 2009; LI *et al.,* 2008; MATSUNAGA *et al.*, 2007).

As prostaglandinas estão envolvidas em diversos processos fisiológicos e patológicos, incluindo, por exemplo, vasodilatação ou vasoconstrição; contração ou relaxamento da musculatura brônquica ou uterina; hipotensão; ovulação; metabolismo ósseo; aumento do fluxo sanguíneo renal; proteção da mucosa gástrica e regulação do fluxo sanguíneo local; inibição da secreção gástrica; crescimento e desenvolvimento nervoso; resposta imunológica; hiperalgesia; regulação da atividade quimiotática celular; resposta endócrina; angiogênese; progressão metastásica, entre outros (CARVALHO, 2010; SMYTH; BURKE; FITZGERALD, 2010).

Os AINEs não são efetivamente removidos da água por tratamento convencional sendo, portanto, detectados em afluentes que abastecem vários municípios na União Européia, Estados Unidos e Canadá. Os AINEs atuam de forma sinérgica, com efeitos aditivos em relação à metabolização e bioacumulação em

organismos aquáticos, sendo essa bioacumulação tecidual especialmente produzida por diclofenaco e ibuprofeno (BRUN *et al*., 2006; COMEAU *et al.*, 2008; SANDERSON *et al*., 2003).

Os AINEs apresentam como mecanismo de ação o bloqueio ou retardo do processo inflamatório, reduzindo a permeabilidade do endotélio capilar, inibindo a marginalização e migração leucocitária, reduzindo a cascata que leva a produção de certas prostaglandinas e leucotrienos, através da diminuição de oferta de ácido araquidônico. Essa inibição explica grande parte da ação antinflamatória dos glicocorticóides, devido à importância do ácido araquidônico na produção dos mediadores humorais da inflamação. Os antinflamatórios esteroidais, além de reduzirem a inflamação, suprimem reações alérgicas e a atividade do sistema imune (CHARMAN; WILLIAMS, 2003; MACEDO; OLIVEIRA, 2010; MORLEY, 2009; SCHUERHOLZ *et al*., 2007; TOMLINSON *et al*., 1997).

Os AINEs, tais como os glicocorticóides também são amplamente aplicados na medicina veterinária para restaurar a força muscular, e como promotores de crescimento, para aumento do tamanho muscular em animais.Assim, certa quantidade de glicocorticóides, excretados principalmente na urina de mamíferos, é liberada para o ambiente aquático através de efluentes de estações de tratamento de esgoto ou do escoamento, podendo assim tornar-se potencial contaminante em ambientes aquáticos (CHANG *et al.*, 2007).

Os AINEs empregados no presente estudo toxicológico foram o ibuprofeno, diclofenaco e paracetamol.

2.1.1 Ibuprofeno

.

O ibuprofeno (Figura 2) é frequentemente detectado no ambiente aquático. Assim, os efluentes de águas residuais municipais constituem importante fonte deste fármaco, especialmente em córregos e rios. Devido à sua ocorrência generalizada em ambientes aquáticos, seu potencial de impacto ecológico tem sido uma crescente preocupação (BRUN *et al*., 2006; GOMEZ *et al*., 2007; CHRISTENSEN *et al*., 2009; HAN *et al.*, 2010; KASPRZYK-HORDERN; DINSDALE; GUWY, 2008; KIM *et al*., 2007).

FIGURA 2 - ESTRUTURA QUÍMICA DO IBUPROFENO
FONTE: GROSSER; SMYTH; FITZGERALD (2012).

O ibuprofeno classificado como “perigoso para o ambiente aquático” (CARLSSON *et al.*, 2006) é encontrado no ambiente em uma faixa que varia de 0,9 a 27,25 µg/L (WHO, 2012) mas geralmente ele é encontrado em concentração médias de 1,0 µg/L (BILA; DEZOTTI, 2003).

A maioria das avaliações da ecotoxicidade de ibuprofeno realizadas não foi suficiente para compreender o potencial de efeitos crônicos que esse composto pode produzir nos organismos aquáticos nem seu mecanismo de toxicidade (HAN *et al.*, 2010).

Estudos revelam que a exposição ao ibuprofeno no meio aquático leva a um aumento na atividade da aromatase em uma concentração dose-dependente, levando a alterações na reprodução (FLIPPIN; HUGGETT; FORAN, 2007; HECKMANN *et al.*, 2007; HAN *et al.*, 2010). Outros trabalhos relatam que a exposição ao ibuprofeno e outros AINEs pode modular a biossíntese estrogênica, inibindo a sua produção em peixes (BRUEGGEMEIER; HACKETT; DIAZ-CRUZ, 2005; HAN *et al.*, 2010; TERRY, 2004).

2.1.2 Diclofenaco

O diclofenaco (Figura 3) é amplamente utilizado devido ao seu efeito antinflamatório, analgésico e às suas propriedades antitérmicas. Estas propriedades terapêuticas são baseadas na inibição da ciclo-oxigenase e inibição subsequente da síntese de prostaglandinas. No entanto, as prostaglandinas não desempenham apenas um papel na mediação da dor, mas estão envolvidas na permeabilidade vascular e função renal. Alguns dos efeitos adversos associados à terapêutica com diclofenaco, como nefropatia, se devem à inibição da síntese das prostaglandinas. No entanto, outros efeitos, incluindo a formação de aductos de proteínas e dano oxidativo também foram sugeridos como causadores de sintomas adversos, por exemplo, ulceração gastrointestinal, nefropatia e hepatotoxicidade idiossincrática

(HARGUS *et al.*, 1995; HOEGER *et al.,* 2005; SALI, 2005; SANCHEZ *et al.*, 2002; SCHWAIGER *et al.*, 2004).

FIGURA 3 - ESTRUTURA QUÍMICA DO DICLOFENACO
FONTE: GROSSER; SMYTH; FITZGERALD (2012).

Entre os produtos farmacêuticos utilizados em humanos, o diclofenaco é uma das substâncias mais encontradas no ambiente aquático. Ele é encontrado em concentrações médias que variam de 0,2 a 2,3 µg/L (BILA; DEZOTTI, 2003; WHO, 2012). Após o uso terapêutico em humanos, apenas 15% do diclofenaco é excretado de forma inalterada, sendo a maior parte eliminada após biotransformação e excreção via renal. Como consequência, o diclofenaco ou seus metabólitos atingem o ambiente aquático (SCHWAIGER *et al.*, 2004; STÜLTEN *et al.*, 2008).

A exposição ao diclofenaco tem sido apontada como causa de alterações estruturais e de necrose em células específicas dentro das brânquias em trutas, ou seja, as células pilares e as células de cloreto (STÜLTEN *et al.*, 2008; TRIEBSKORN *et al.*, 2004). Essas células estão envolvidas tanto nos processos respiratórios como na regulação iônica e osmótica (EADES; WARING, 2009; FREIRE; ONKEN; MCNAMARA, 2008).

Outros estudos indicam que a exposição de truta arco-íris durante 4 semanas a diclofenaco em concentrações variáveis produziu alterações histopatológicas nos rins e nas brânquias, além deste se biocumular e reduzir a síntese de prostaglandina E2 (SCHWAIGER *et al.*, 2004; STÜLTEN *et al.*, 2008).

2.1.3 Paracetamol

Paracetamol (acetaminofeno) (Figura 4) é um analgésico e antipirético considerado seguro, sendo comprado livremente na maioria dos países sendo, por esse motivo, considerado a droga mais amplamente utilizada no mundo. Apesar da venda livre, esse fármaco não pode ser considerado uma droga absolutamente segura, pois pode causar necrose hepática, nefrotoxicidade, lesões extra-hepáticas e até mesmo a morte em seres humanos e animais experimentais, quando administrado em *overdose* (AN *et al.*, 2009; OLALEYE; ROCHA, 2008; RAY *et al.*, 1996).

FIGURA 4 - ESTRUTURA QUÍMICA DO PARACETAMOL
FONTE: GROSSER; SMYTH; FITZGERALD (2012).

O mecanismo de ação do paracetamol ainda não é completamente elucidado, mas estudos demonstram que ele tem a capacidade de inibir a produção de prostaglandina em nível do sistema nervoso central e tecidos periféricos. Seus efeitos adversos ocorrem principalmente devido à formação de metabólitos hepatotóxicos, principalmente imina *n*-acetil-*p*-benzoquinona, sintetizada quando a disponibilidade de glutationa nas células do fígado é diminuída (FENT; WESTON; CAMINADA, 2006; HINZ, BRUNE, 2012).

O paracetamol é o fármaco mais utilizado ao redor do mundo para alívio da dor. Em efluentes, o paracetamol chegou a ser determinado a uma concentração máxima de 6,0 µg/L (JONES; VOULVOULIS; LESTER, 2001), porém ele foi encontrado nos corpos hídricos em média de 0,25 µg/L (FENT; WESTON; CAMINADA, 2006).

Estudos *in vitro* mostraram que o paracetamol, quando comparado ao ibuprofeno e ao diclofenaco, tem um maior potencial de dano celular, especialmente por sua facilidade de entrada na célula e mudança do pH, o que pode ser um dos responsáveis pelos referidos danos no DNA (PAROLINI *et al.*, 2009).

O paracetamol é excretado principalmente na forma conjugada. Durante o tratamento das águas residuais, a reação de conjugação é degradada, levando a liberação do composto original (KASPRZYK-HORDERN; DINSDALE; GUWY, 2008).

2.2 BIOMARCADORES

Biomarcadores podem ser definidos como qualquer resposta biológica que pode ser representada e mensurada por alterações moleculares, celulares, fisiológicas e até comportamentais no indivíduo ou parte dele. Estas podem ser relacionadas e detectadas devido à exposição a um agente tóxico ou seus efeitos no organismo (PEAKALL, 1994; 1999).

O uso de biomarcadores bioquímicos, genéticos, hematológicos e imunológicos em programas de monitoramento oferece vantagens, pois normalmente estes são os primeiros a apresentar alterações, mostrando boa sensibilidade e especificidade relativa, podendo ser considerados até mesmo sistemas de aviso precoce, indicando a contaminação do ambiente antes que danos mais graves ocorram aos organismos e, possivelmente, ao ecossistema em que estes se encontram inseridos (MCCARTHY; SHUGART, 1990; NUNES *et al.*, 2008).

Os biomarcadores bioquímicos são utilizados, por exemplo, para detectar o estresse oxidativo que se dá quando a geração de radicais livres provocados por substâncias poluentes, ou xenobióticos, é maior que a capacidade antioxidante da célula, promovendo assim a oxidação dos constituintes celulares, tais como os lipídios das membranas, as proteínas e o DNA (AVCI; KAMAZ; DURAKA, 2005). Entre os biomarcadores mais comumente utilizados para se avaliar o estresse oxidativo (Figura 5) estão a atividade enzimática da catalase (CAT), da superóxido dismutase (SOD), e da glutationa S-transferase (GST) que atua na conjugação de substâncias eletrofílicas (KEEN; HABIG; JAKOBY, 1976).

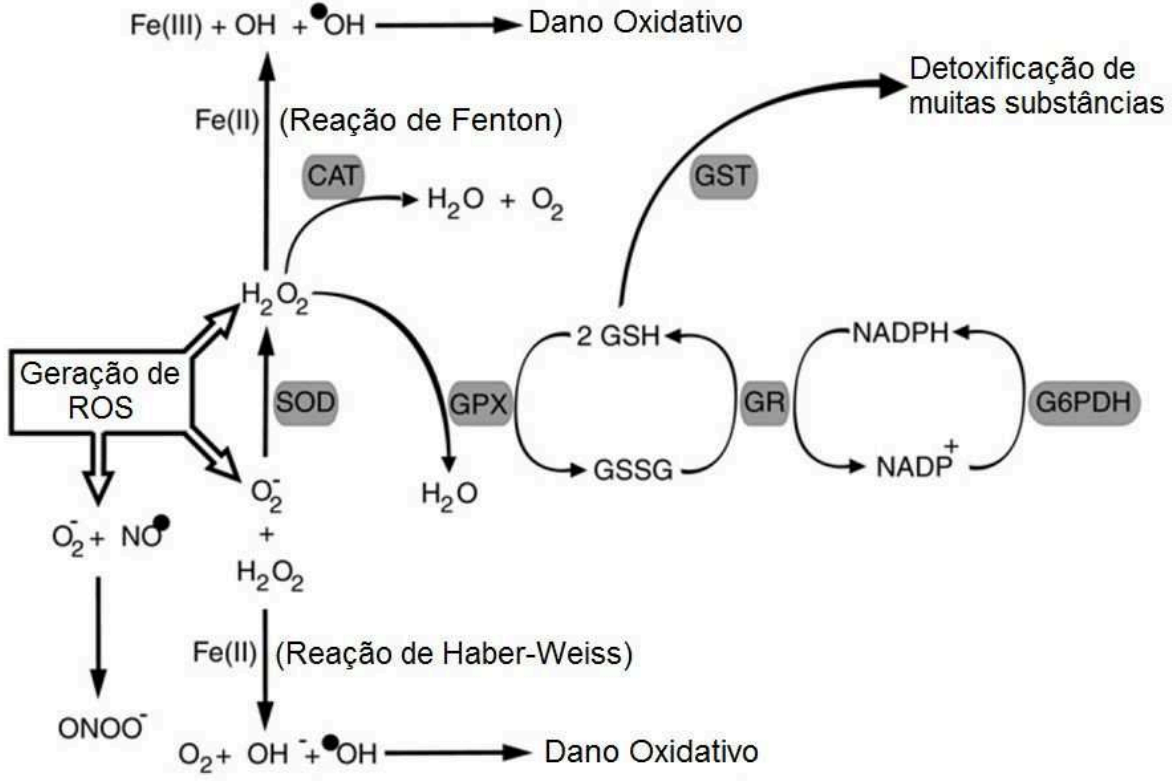

FIGURA 5 - RESPOSTA AO ESTRESSE OXIDATIVO
LEGENDA: CAT: catalase; SOD: superóxido dismutase; GST: glutationa S-transferase; GPX: glutationa peroxidase; GSH: glutationa reduzida; GSSG: glutationa oxidada; GSTs: glutationa S-transferase; GR: glutationa redutase; G6PDH: glucose-6-fosfato desidrogenase; O_2^-: superóxido; H_2O_2: peróxido de hidrogênio.
FONTE: ADAPTADO DE RITTIÉ; FISHER, 2002; KARADAG, 2014

As catalases (CAT) são enzimas que catalisam a eliminação de peróxido de hidrogênio (H_2O_2), liberando como produtos água e oxigênio molecular. São também utilizadas como biomarcadores, embora estejam localizadas nos peroxissomos de muitas células e envolvidas no metabolismo de ácidos graxos, apesar de que mudanças na sua atividade possam ser de difícil interpretação (FASHIMI; CAJARAVILLE, 1995; VAN DER OOST; BEYER; VERMEULEN, 2003).

A superóxido dismutase (SOD) desempenha um papel fundamental na defesa contra os efeitos de agentes tóxicos no organismo. Em eucariontes, ela está presente no citoplasma, núcleo e peroxissomos, além da mitocôndria. Modificações oxidativas em suas isoformas são geradas pela reação com espécies de oxigênio ou de aldeídos derivados da peroxidação lipídica. Essas isoformas da SOD podem ser usadas como bioindicadores de estresse oxidativo provocado por poluentes (LÓPEZ-CRUZ; ZENTENO-SAVÍN; GALVÁN-MAGAÑA, 2010; PASCUAL *et al.,* 2003; PEDRAJAS *et al.,* 1998; VEGA-LÓPEZ *et al.*, 2007). A principal função da SOD é

catalisar a dismutação do radical superóxido (O_2^-) levando à formação do peróxido de hidrogênio (H_2O_2) que por sua vez é degradado pela catalase ou pela glutationa peroxidase (HALLIWELL; GUTTERIDGE, 2007).

A glutationa peroxidase (GPx) é comumente encontrada nas mitocôndrias, sendo utilizados para reduzir diversos tipos de peróxidos, empregando como co-fator a glutationa (GSH), gerando como produto a glutationa oxidada (Gssg) (HAYES *et al.*, 1997; HALLIWELL; GUTTERIDGE, 2000). Devido a essa característica, ela é utilizada como importante ferramenta de indicação de estresse oxidativo (VAN DER OOST; BEYER; VERMEULEN, 2003).

Outra glutationa que faz parte dos sistemas oxidantes não enzimáticos que reduz hidroperóxidos é a glutationa reduzida (GSH), sendo responsável por sequestrar espécies reativas de oxigênio e proteger as membranas do estresse oxidativo (ORUC; SEVGILER; UNER, 2004). Ela desempenha papel fundamental na proteção celular contra danos oxidativos causadas por oxidantes, atuando essencialmente como sequestradora de radicais, na homeostase tiólica, manutenção do balanço redox e defesa contra agentes eletrolíticos, como os xenobióticos (PEIXOTO *et al.*, 2013; SEVCIKOVA *et al.*, 2011).

As glutationa S-transferases (GSTs) são uma superfamília de enzimas diméricas, multifuncionais e primariamente solúveis que catalisam a conjugação de compostos eletrofílicos (ou metabólitos de fase I) com a glutationa reduzida (GSH). Essa reação faz parte do metabolismo de fase II, que envolve a conjugação de um composto xenobiótico ou seu metabólito a um ligante endógeno. As enzimas dessa fase têm um importante papel na homeostase, detoxificação e eliminação de diversas substâncias exógenas, e os níveis de cofatores de fase II podem ser afetados após exposição a poluentes ambientais. Além de seu papel no transporte intracelular e biossíntese de leucotrienos e prostaglandinas, o papel crítico das GSTs é a defesa do DNA e lipídios contra o dano oxidativo e produtos peroxidativos. O processo de lipoperoxidação (LPO), ou oxidação de lipídios tem grande potencial como biomarcador de estresse oxidativo (LIVINGSTONE, 2001; NUNES *et al.*, 2008; VAN DER OOST; BEYER; VERMEULEN, 2003).

Alguns biomarcadores podem ainda ser utilizados para verificar a oxidação dos lipídios, como a lipoperoxidação (LPO), e a oxidação de proteínas, tal como a carbonilação de proteínas (PCO) (QUINLAN; GUTTERIDGE, 2000).

A LPO tem como característica causar essencialmente a integridade da membrana celular, podendo resultar em um ambiente favorável ao ataque do DNA, além de potencialmente poder alterar todo o processo de transporte, transdução de sinais mediada por receptores e o gradiente iônico e metabólico (HIGUCHI, 2003).

A PCO tem atraído grande atenção devido a sua natureza irreversível e irreparável. Essa característica é devido ao escape da degradação e formação de agregados de alto peso molecular, que se acumulam e levam ao aumento da produção de ROS, a redução da capacidade de remoção de proteínas oxidadas ou aumento da susceptibilidade de proteínas para o ataque oxidativo (NYSTRÖM, 2005).

Os biomarcadores genéticos, diferentemente dos biomarcadores bioquímicos avaliam uma série de mudanças no material genético, induzido nesse contexto, por substâncias tóxicas encontradas no ambiente, caracterizadas por alterações estruturais do DNA e, consequentemente, o processamento e expressão desse dano como produtos de genes mutantes. A detecção e quantificação desses eventos podem ser empregadas como biomarcadores, como o teste do micronúcleo e o ensaio cometa (VAN DER OOST; BEYER; VERMEULEN, 2003).

A exposição de organismos a substâncias genotóxicas pode induzir uma série de eventos que resultam em mudanças em seu material genético,

O princípio do teste do micronúcleo está baseado no fato de que, durante a anáfase, as cromátides e fragmentos cromossômicos acêntricos não são transportados pelas fibras do fuso para pólos opostos, ao contrário dos fragmentos com centrômero. Após a telófase os cromossomos sem dano são incluídos no núcleo de cada uma das células filhas. Elementos que não foram transportados pelo fuso também podem ser englobados pelos núcleos recém formados. No entanto, alguns destes elementos, normalmente muito pequenos, não são incluídos nos núcleos recém formados e permanecem no citoplasma, constituindo as estruturas caracterizadas como micronúcleos (ALBERTINI *et al.*, 2000; FERRARI, 1991; SCHMID, 1975; UDROIU, 2006).

Desta maneira, os efeitos de substâncias que provoquem quebras cromossômicas ou ainda afetem os componentes do fuso ou da região centromérica podem ser detectados a partir da presença de micronúcleos (ALBERTINI *et al.*, 2000; HEDDLE *et al.*, 1991).

O ensaio cometa é uma técnica capaz de detectar dano ao DNA em células individualizadas (SPEIT; HARTMANN, 1999). O DNA contido em células de organismos eucariotos possui alguns centímetros de comprimento. Para que o DNA seja acomodado no interior do núcleo, que possui entre 5 μm e 10 μm de largura, este DNA tem que ser fortemente condensado. Danos impostos à molécula de DNA provocam um relaxamento desta condensação e ocasionalmente quebras na estrutura molecular (ROJAS; LOPEZ; VALVERDE, 1999).

Os locais do DNA que são susceptíveis ao processo de alquilação são mais sensíveis à degradação. Estes pontos, onde a depurinação está aumentada, transformam-se em pontos de quebras da fita de DNA sendo, portanto, visíveis através do ensaio cometa (HAHN; HOCK, 1999).

Tão importante quanto os biomarcadores bioquímicos e genéticos, os biomarcadores hematológicos conjuntamente agregados aos imunológicos na interface da determinação leucocitária, se destacam na avaliação dos efeitos de um determinado poluente sobre o organismo,

Muitos parâmetros hematológicos em peixes também podem ser empregados como biomarcadores, os quais podem ser sensíveis a certos poluentes (VAN DER OOST; BEYER; VERMEULEN, 2003).

Estudos hematológicos das diferentes espécies de peixe são de interesse ecológico e fisiológico, uma vez que auxiliam na compreensão da relação entre as características sanguíneas, a filogenia, a atividade física, o habitat e a adaptabilidade dos peixes no ambiente. Os valores hematológicos podem ser influenciados não apenas pelo crescimento, mas também pelas condições ecofisiológicas (TAVARES-DIAS; MORAES, 2004). O sangue dos peixes teleósteos é formado basicamente por eritrócitos, trombócitos e leucócitos.

Os eritrócitos são as células mais numerosas que contem essencialmente a hemoglobina, pigmento respiratório que tem por função transportar oxigênio (RANZANI-PAIVA; SILVA-SOUZA, 2004).

Os trombócitos diferentemente das plaquetas nos mamíferos são células completas que participam ativamente tanto da homeostasia quanto na participação no mecanismo de defesa orgânica, demonstrado pela sua presença nos processos de coagulação, inflamação e atividade fagocitária nos processos de infecções (TAVARES DIAS; MORAES, 2004; MARTINS *et al.*, 2004).

Os leucócitos são células que atuam ativamente na defesa do organismo, participando da resposta imunológica com seus principais componentes: linfócitos, monócitos, neutrófilos, eosinófilos e basófilos (FERNANDEZ *et al*., 2002). Os linfócitos são responsáveis pela resposta imune humoral e celular específica, atuando tanto na produção de anticorpos quanto em sua atividade citotóxica (TIZARD, 2002). Os neutrófilos por sua vez, são as primeiras células envolvidas nos estágios iniciais de um processo de inflamação em peixes, com capacidade de fagocitar e gerar radicais livres (FERNANDEZ *et al*., 2002; FALCON, 2007). Outra célula de importância no contexto dos biomarcadores hematológicos são os monócitos. Os monócitos são considerados as células sanguíneas mais importantes no papel que desempenham na resposta imune, como na produção de citocinas e apresentação de antígenos em teleósteos (VELLEJO *et al*., 1992; SHOEMAKER *et al*., 1997; FALCON, 2007). Os monócitos podem ser encontrados isolados no sangue, no rim anterior e na cavidade peritoneal (SHOEMAKER *et al*., 1997; SIWICKI *et al*., 2009). Dentre as principais características dessas células cabe ressaltar a capacidade de secretarem radicais livres e destruir diferentes tipos de patógenos (FALCON, 2007).

Além de situações adversas serem capazes de diminuir a atividade do sistema imunológico de peixes, a presença de certos poluentes na água tem efeitos supressores sobre a atividade dos fagócitos dos peixes (BOLS *et al.,* 2001; SECOMBES; FLETCHER, 1992).

A caracterização dos principais mecanismos e vias do sistema imune especialmente relacionados a imunotoxicologia, vem preenchendo importantes lacunas no conhecimento a respeito dos numerosos mecanismos imunes relacionados aos peixes (ALVAREZ-PELLITERO, 2008; CHAGAS *et al*., 2009).

O óxido nítrico é uma molécula de sinalização gasosa que regula diversos processos fisiológicos, incluindo a função imune, exibindo atividade citostática ou citotóxica contra diversos microorganismos. A produção de óxido nítrico por macrófagos de peixes pode, assim, ser utilizada como parâmetro funcional dessas células (SARMENTO *et al.*, 2004).

Os peixes são organismos complexos em relação a organização do seu sistema imunológico. Assim como os mamíferos, apresentam dois tipos básicos de sistema de reação imunológica: a imunidade mediada por células e a imunidade humoral (MAGNADOTTIR, 2006). A imunidade celular específica está basicamente

relacionada com os linfócitos T e sua capacidade de reconhecer antígenos com capacidade de ligação à epítopos de certas células. Os linfócitos B, por sua vez, desenvolvem seu papel na produção de anticorpos específicos e células de memória (FALCON, 2007; SECOMBES, 1994). A imunidade humoral está relacionada a produção de imunoglobulina e fatores solúveis como produção e liberação de citocinas (MEDZHITOV, 2007). Além das citocinas, outros mediadores solúveis estão presentes nas reações imunes dos peixes, como as proteínas envolvidas nas reações do sistema complemento. O sistema complemento pode ser ativado pela superfície do patógeno por meio de sua via alternativa, reação inata e inespecífica, por meio da formação de complexo antígeno anticorpo através da via clássica ou ainda pela ligação do complexo protéico de lectina ligada a manose em uma reação antígeno anticorpo, através da ativação da via da lectina (CLAIRE; HOLLAND; LAMBRIS, 2002). As três vias de ativação foram identificadas e descritas em peixes, exceto nos não mandibulados, que não apresentam a forma clássica de ativação (NONAKA, 2001).

A principal diferença do sistema complemento (FIGURA 6) de peixes e mamíferos está no fato de que peixes apresentam uma grande variedade das proteínas expressas para o sistema complemento, com característica de possuir múltiplas isoformas, contribuindo provavelmente para aumentar significativamente sua capacidade de reação e combinação (CLAIRE; HOLLAND; LAMBRIS, 2002).

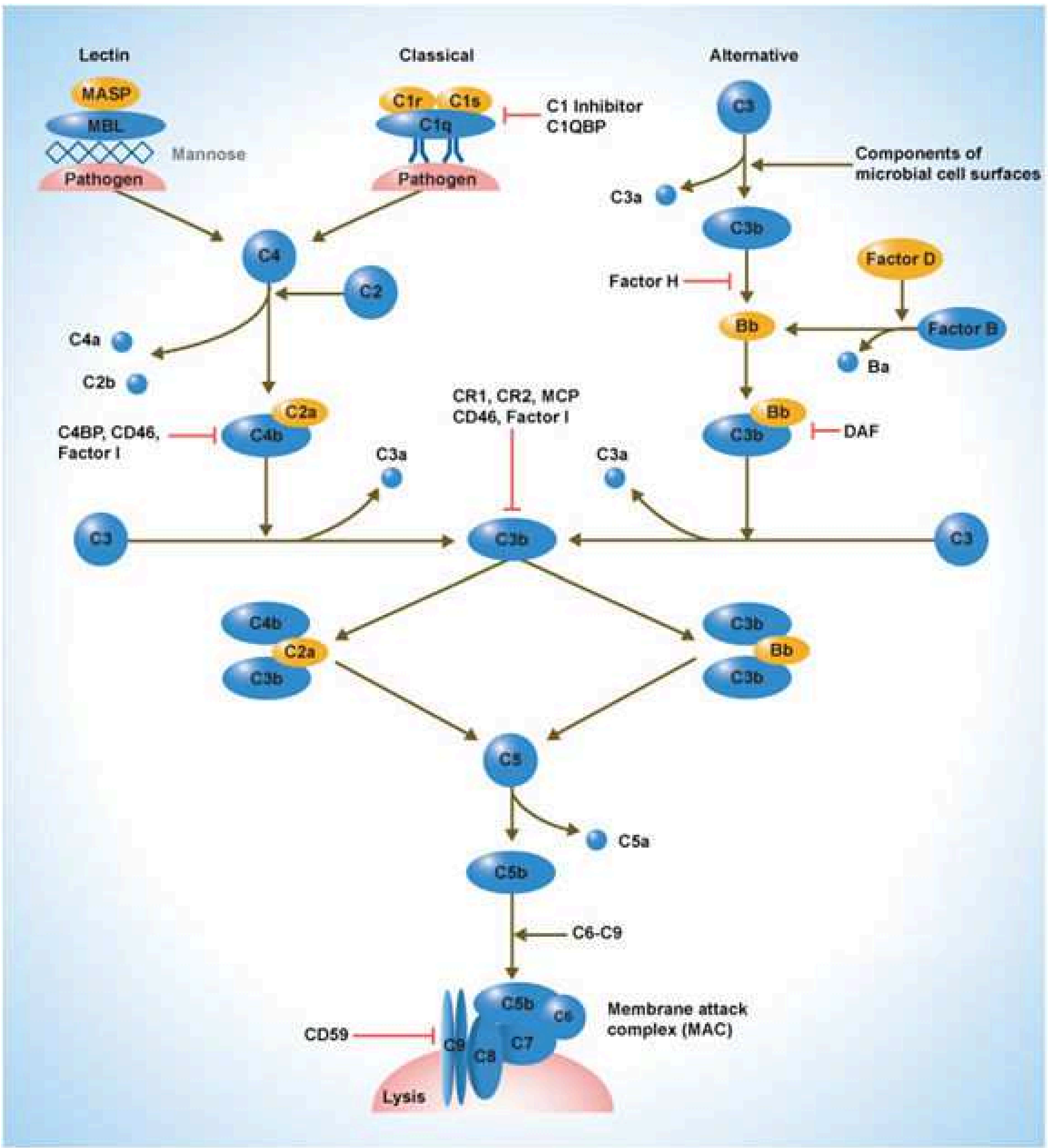

FIGURA 6 - VISÃO GERAL DO SISTEMA COMPLEMENTO
FONTE: http://www.sinobiological.com/Complement-Activation-Pathways-a-1511.html

Outro ponto chave no processo de defesa e proteção do organismo é a migração celular (FIGURA 7), mais especificamente a migração leucocitária. Os macrófagos, por exemplo, migram comumente através dos tecidos, com a principal função de remover células apoptóticas ou necróticas e proteínas desnaturadas. Essa migração depende de uma série de eventos iniciando pela adesão celular e finalizando pela transmigração (COOK-MILLS; DEEM, 2005). Em geral, a migração de leucócitos ocorre por um processo de multi-passos em um processo envolvendo recrutamento,

rolagem, ativação, adesão ao endotélio, diapedese e finalmente migração transendotelial (SCHUBERT *et al*., 2011).

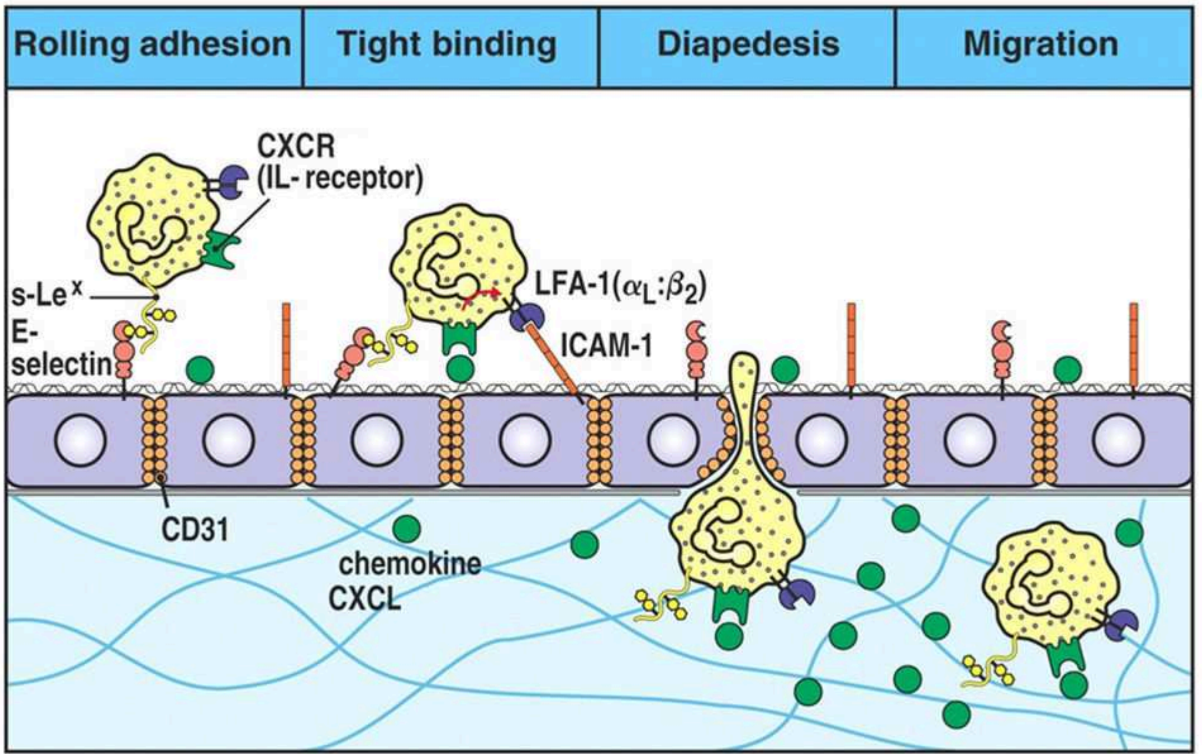

FIGURA 7 - VISÃO GERAL DO PROCESSO DE MIGRAÇÃO CELULAR
FONTE: ADAPTADO DE http://www.bio.davidson.edu/courses/immunology/students/spring2006/latting/home%20copy.html

Os macrófagos residentes são, portanto considerados como sistema de alarme, responsáveis pela primeira sinalização que visa iniciar o processo de migração de neutrófilos (ZHU; PAUL, 2008). O grande problema é que com a supressão do sistema imunológico aumenta a susceptibilidade do peixe à patógenos, um aspecto importante quando consideramos a presença de xenobiótico no ecossistema aquático que pode levar a essa reação (DALAKAS *et al*., 2005; WEDEMEYER *et al*., 1990).

Uma forma de se avaliar o processo migratório e consequentemente a modulação imunológica em peixes expostos a poluentes ambientais é através da aplicação de elementos flogísticos na cavidade peritoneal (bexiga natatória), simulando a resposta leucocitária à inflamação produzida na cavidade peritoneal (AFONSO *et al*., 1999; KAIKO *et al*., 2008; ZHU *et al*., 2010). Dentre os elementos flogísticos mais utilizados para promover a migração leucocitária em peixes, estão o lipopolissacarídeo (LPS) e a carragenina (MARTINS *et al*. 2001; 2004; 2008; MATUSHI; MARIANO, 1996).

2.3 PROTEÔMICA

O termo proteômica foi inicialmente proposto em 1995 e foi basicamente definido como sendo a caracterização de larga escala do conjunto de proteínas expressas em uma célula ou tecido e o estudo de suas funções (TANVETYANON; CREELAN; CHIAPPORI, 2014; WILKINS *et al*., 1997).

A abordagem proteômica tem permitido estudos da expressão de proteínas em diferentes tecidos e fluidos dos organismos. Agregado a isso os recentes progressos em metodologias nessa área possibilita novas oportunidades de análises e obtenção de informações relevantes sobre os processos fisiológicos e patológicos. Cabe salientar que as proteínas de um organismo podem se modificar dependendo da condição e do estímulo a que esse organismo está exposto. Sendo assim a proteômica reflete a expressão de proteínas que influenciam mais diretamente todo o sistema, sendo por isso uma metodologia de grande importância e aplicação no contexto ambiental (BARBOSA, 2012; SILVA; CORREA; REIS, 2007).

As técnicas proteômicas estão inseridas em diversas áreas da biologia, bioquímica e toxicologia, incluindo a toxicologia ambiental. Nesse contexto, a proteômica busca biomarcadores e alvos de poluentes que demonstrem em termos moleculares o que de fato está ocorrendo com o organismo e elucide os possíveis mecanismos moleculares da ação desses poluentes nos organismos aquáticos (BELLGARD, 2013; KUHNER *et al*., 2009; SANTOS *et al*., 2004). No entanto, a falta de informação genética anterior na maioria das espécies de peixes tem sido um grande problema para uma aplicação mais geral e irrestrita das diferentes tecnologias proteômicas disponíveis (FORNÉ; ABIÁN, CERDÁ, 2010).

O mesmo tipo celular pode apresentar diferentes proteínas e rotas metabólicas em relação, por exemplo, a ação de drogas e/ou poluentes ambientais. Por esse motivo, os dados gerados por uma análise proteômica permitem alcançar objetivos diferentes tais como esclarecer as principais proteínas envolvidas em rotas metabólicas relacionados aos diferentes processos celulares, identificar novos alvos biológicos, caracterizar moléculas bioativas e caracterizar as respostas celulares a determinadas drogas, doenças ou mudanças ambientais (SILVA; CORREA; REIS, 2007).

Apesar de sua relevância, a abordagem proteômica em peixes ainda mostra-se tímida, com um baixo número de espécies pesquisadas. Essas espécies muitas vezes estão restritas a algumas de interesse em aquicultura e modelos experimentais laboratorialmente já estabelecidas, como o *Danio rerio* (zebrafish). Mesmo com essa restrição, a proteômica em peixes tem emergido como uma ferramenta importante para o estudo dos sistemas biológicos e para abordar diferentes questões relacionadas com a biologia do peixe e suas variações quando expostos a alguma substância específica. A possibilidade de combinar os dados de proteômica com os resultados de outros biomarcadores fazem dessa metodologia um grande avanço na abordagem ambiental (AMATRUDA *et al*., 2008; FORNÉ; ABIÁN, CERDÁ, 2010; LOVE *et al*., 2004).

2.4 ANIMAIS DE ESTUDO

Hoplias malabaricus (Bloch) ou traíra (Figura 8) é um peixe de água doce, carnívoro, que devido ao seu comportamento predador ocupa um alto nível trófico na cadeia alimentar aquática, sendo, por isso, um valioso modelo biológico, podendo sofrer bioacumulação. A traíra não tem comportamento migratório, sendo encontrada em toda América do Sul. Esta espécie é um modelo biológico interessante para fins experimentais, devido ao seu comportamento e capacidade de se adaptar às condições experimentais e à sua posição na cadeia alimentar (ALVES COSTA *et al*., 2007; FILIPAK NETO *et al.*, 2007; 2008; MOL *et al*., 2001; OLIVEIRA RIBEIRO *et al*., 2006; OLIVERO-VERBEL, 2006; RABITTO *et al*., 2005; SILVA DE ASSIS *et al*., 2013).

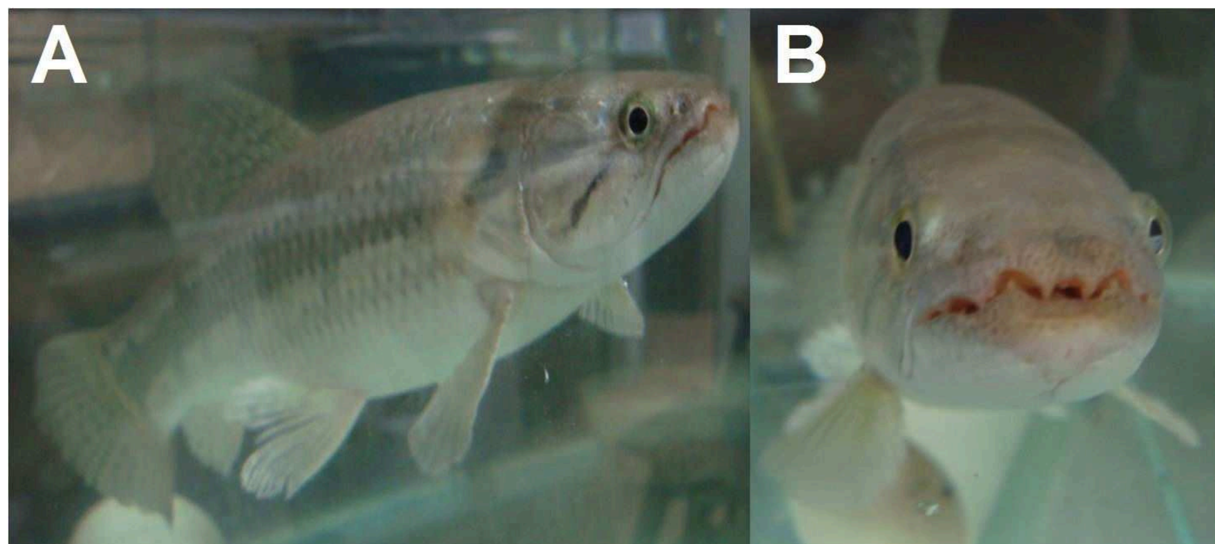

FIGURA 8 - EXEMPLAR DE *H. malabaricus*, CONHECIDO POPULARMENTE COMO TRAÍRA. (A) VISTA LATERAL; (B) VISTA FRONTAL.
FONTE: O AUTOR.

Além disso, *H. malabaricus* tem uma das maiores tolerâncias de privação de alimento, sobrevivendo por períodos de até 180 dias sem redução da taxa metabólica (consumo de oxigênio) (ALVES COSTA *et al.*, 2007; RIOS *et al.*, 2005).

Rhamdia quelen, ou jundiá (Figura 9), é um peixe que tem distribuição neotropical, sendo encontrado desde o centro da Argentina até o sul do México, e cujo cultivo está aumentando no sul do Brasil. O jundiá tem como característica viver em lagos e rios, e preferir os ambientes de águas mais calmas, com fundo de areia e lama (GOMES *et al.,* 2000). É uma espécie de grande aceitação no mercado consumidor, especialmente por sua carne agradável ao paladar e ausência de espinhos intramusculares. Devido a sua fácil domesticação e adaptação às condições de cultivo, vem atraindo a atenção de pesquisadores, com o qual a espécie tem sido utilizada como modelo de estudos ecotoxicológicos (BARCELLOS *et al.*, 2001; BENADUCE *et al.*, 2008; BIBIANO MELO *et al.*, 2006; COLDEBELLA *et al.*, 2011; HERNÁNDEZ *et al.*, 2012; PAMPLONA *et al.*, 2011; PRETTO *et al.*, 2010; 2011), e produtores, por suas características que vêm permitindo sua inclusão na lista de espécies de peixes criadas comercialmente no Brasil (CARNEIRO; MIKOS, 2005).

FIGURA 9 - EXEMPLARES DE *R. quelen*, CONHECIDO POPULARMENTE COMO JUNDIÁ.
FONTES: http://www.scotcat.com/heptapteridae/rhamdia_quelen4.htm; http://planetacuario.com/showthread.php/17693-Rhamdia-quelen

Ambas as exposições, a trófica com o Hoplias malabaricus e a hídrica com o Randia quelen foram escolhidas porque representam avaliações diferentes, mas complementares à avaliação dos riscos ecológicos. A hídrica demonstrando os possíveis efeitos na exposição direta e a trófica que sob condições controladas é reprodutiva e interpreta como age um ou mais agentes ao longo da cadeia alimentar. Ambas com especial poder de demostrar cada um a seu modo possível bioacumulação e biomagnificação (SIMON *et al.*, 2013).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os efeitos toxicológicos de antinflamatórios não esteiroidais por via trófica e hídrica em espécies de peixe nativo através de diferentes biomarcadores de contaminação em doses/concentrações ambientalmente relevantes.

3.2 OBJETIVOS ESPECÍFICOS

- Padronizar o cultivo primário de rim anterior de *Hoplias malabaricus*
- Avaliar a produção de óxido nítrico e danos genotóxicos em cultivo primário de rim anterior de *Hoplias malabaricus* expostos ao diclofenaco, ibuprofeno e paracetamol
- Avaliar o estresse oxidativo em fígado de *Hoplias malabaricus* após exposição trófica ao diclofenaco
- Avaliar o potencial de migração celular do diclofenaco após desafio com a carragenina
- Avaliar proteomicamente proteínas do sistema imunológico relacionados à produção de óxido nítrico, migração celular e sistema complemento, em machos e fêmeas de *Rhamdia quelen* expostos via hídrica ao diclofenaco

Todos os estudos foram aprovados pela Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná sob os números 453 (*Hoplias malabaricus*) e 652 (*Rhamdia quelen*). Os procedimentos também foram realizados de acordo com os princípios éticos estabelecidos pelo Colégio Brasileiro de Experimentação Animal (COBEA) e as exigências estabelecidas no *Guide for the Careand Use of Experimental Animals (Canadian Councilon Animal Care)*.

CAPÍTULO I

PADRONIZAÇÃO DO CULTIVO PRIMÁRIO DE MACRÓFAGOS DO RIM ANTERIOR DE *Hoplias malabaricus* E SUA UTILIZAÇÃO EM ENSAIOS TOXICOLÓGICOS

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Effects of anti-inflammatory drugs in primary kidney cell culture of a freshwater fish

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Abstract

The toxicity of widely used non-steroidal anti-inflammatory drugs (NSAIDs) was evaluated on primary culture of monocytic lineage of *Hoplias malabaricus* anterior kidney. The effects of diclofenac, acetaminophen and ibuprofen in cell viability, lipopolysaccharide (LPS)-induced NO production and genotoxicity were evaluated. Cytometry analysis CD11b^{+}cells showed 71.5% of stem cells, 19.5% of macrophages and 9% of monocytes. Cell viability was lower in the Ficoll compared to Percoll separation. LPS-induced NO production by these cells was blocked after treatment with dexamethasone and L-NMMA. Exposure of the cells to diclofenac (0.2-200 ng/mL), acetaminophen (0.025-250 ng/mL) ibuprofen (10-1000ng/mL) reduced basal NO production and inhibited LPS-induced NO production at all concentrations after 24 h of exposure. Genotoxicity occurred at the highest concentration of diclofenac and at the intermediary concentrations of acetaminophen. Genotoxicity was also observed by ibuprofen. In summary, the pharmaceuticals influenced NO production and caused DNA damage in monocytic cells suggesting that these drugs can induce immunosuppression and genotoxicity.

Keywords: Pharmaceuticals, cell culture, immunotoxicity, genotoxicity, macrophage.

1. Introduction

Some studies have shown that the concentration of pharmaceutical drugs has increased in water bodies all over the world and has became a growing environmental problem and a major source of pollution. These compounds come especially from urban, industrial and hospital wastewater and have therefore been identified as emerging contaminants in aquatic ecosystems (ZUCCATO *et al.*, 2006). The main pharmaceutical group detected is nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, ibuprofen and acetaminophen (ZUCCATO *et al.*, 2006; KHETAN; COLLINS, 2007). Some studies reports that trace NSAIDs concentrations in environment elicit toxic effects, including immunological changes (KHETAN; COLLINS, 2007). Diclofenac, widely used in the world especially for chronic inflammatory conditions, is one the most prevalent water contaminants. It has been identified as one of the most import pharmaceutically active compounds present in the aquatic environment (LETZEL; METZNER; LETZEL, 2009). Diclofenac is found at concentrations ranging from 0.2 to 2.3 µg/L (BILA; DEZOTTI, 2003; WHO, 2012). Similarly to diclofenac, ibuprofen, based on inherent properties, is classified as "dangerous for the aquatic environment" (CARLSSON *et al.*, 2006). The concentration of ibuprofen found in the environment varies from 0.9 to 27.25 µg/L (WHO, 2012), but it has been found at concentrations around 1.0 µg/L (BILA; DEZOTTI, 2003). Acetaminophen is the most widely used over-the-counter common pain reliever. In sewage effluent, the acetaminophen reported maximum concentration is 6.0 µg/L (JONES; VOULVOULIS; LESTER, 2001). However, it has been found at concentrations around 0.25 µg/L (FENT; WESTON; CAMINADA, 2006).

Among aquatic organisms, fish species have been used for detection of the effects of environmental pollution on aquatic ecosystem. The alterations on this organism can be useful to diagnose functional status of the tissue and cells exposed to the toxicant and thereby it is an indirect way to monitor the aquatic environment quality (SARAVANAN *et al.*, 2012).

Functional monocytic lineage and macrophage cells are essential for the establishment of a properly working immune system. They constitute a major, widely dispersed system of cells that regulate homeostasis in the host and respond to tissue

injury by contributing with essential functions during inflammation and repair (BARREDA; BELOSEVIC, 2001).

Several pharmaceuticals have been investigated in *in vitro* systems such as fish cell lines and especially in primary fish cell cultures. Among the advantages of these procedures, based on fish cells or reporter gene systems are their potential for toxicity screening, besides of course, they are important alternatives to animal testing (FENT; WESTON; CAMINADA, 2006). In the present work, the freshwater fish *Hoplias malabaricus* was chosen as a model to establish a lineage of monocytic primary cultures since this species has a large ecological plasticity, with a wide distribution in Brazilian rivers and reservoirs (SILVA *et al.*, 2011).

The aim of the present study was to develop and test a protocol of primary culture of a lineage of monocytic cells from anterior kidney of *H. malabaricus* and expose these cells to different NSAIDs to investigate possible effects on its viability and functionality (NO production at non-stimulated and stimulated conditions). We also evaluated the genotoxic effects of the NSAIDs in these cells.

2. Material and methods

All procedures and protocols were approved by the Institution’s Ethical Committee for Animal Use and are in accordance with international guidelines for Animal Use.

Hoplias malabaricus (100±20g) were purchased from a commercial farm. Fish were kept at 24±1ºC with filtered water on a simulated natural photoperiod and fed twice a week *ad libitum* with fish (*Astyanax* sp) (30±5g). The fish were acclimated in the lab for at least three weeks prior to use in experiments.

2.1 Primary anterior kidney cultures

The monocytic lineage of *H. malabaricus* anterior kidney was obtained, as previously described (NEUMANN; BARREDA; BELOSEVIC, 1998) with some modifications. Briefly, for each experiment, three fishes were anesthetized with benzocaine 0.02% (v/v) and killed by medullar section. The anterior kidneys were removed, pooled and transferred to L-15 medium (Leibovitz-; supplemented with 20U of heparin and 1% antibiotic penicillin/streptomycin) under sterile conditions. The

tissue was homogenized in the same medium, transferred to a 15mL tube and suspended with a sterile Pasteur pipette for 1 min. For a better suspension this procedure was repeated once using a Pasteur pipette of minor diameter. Cellular suspension (in the proportion 2:1) was transferred to a new 15 mL tube containing Percoll gradient 50/50 or 50:40 (v/v) or 60:40 (v/v) or 60:40:10 (v/v) or Ficoll-Pack and centrifuged at 400 x g for 30 min at 22 ºC. Cells at the Percoll or Ficoll interface were removed, washed twice in serum-free medium and centrifuged at 400 x g for 10 min. Viable cells (1.10^6) were placed in 96 wells plates with L-15 medium supplemented with fetal bovine serum 2% and incubated at 20-22 °C, 1.7% CO_2 for 24h for adherence. After this period, non-adherent cells were washed away with phosphate-buffered saline (PBS). The remaining adhered cells were used for subsequent experiments. Cells were detached using ice-cold PBS. For cytometry analysis, the cells were incubated for 40 min with anti-CD11b antibody. Data acquisition was performed in a BD FACS calibur equipped with an argon ion laser tuned to 488 nm. For all measurements, 100,000 cells were collected from each sample tube using Cell Quest software (Becton Dickinson, San Jose, CA, USA). Data analyses were performed using Cell Quest and WinMDI 2.9 software.

2.2 Monocytic stimulation

To establish the optimal LPS concentration and stimulation time for nitric oxide (NO) production, monocytic lineage adherent cells in 96 well plates (1.10^6 cells/well) were stimulated with *Escherichia coli* serotype 0111:B4 lipopolysaccharide (LPS) at different concentrations (0.1,1.0,10.0,100.0,1,000.0 and 10,000.0 ng/mL) diluted in L-15 medium supplemented with fetal bovine serum 2%,at 20-22 °C, 1.7% of CO_2 for 24, 48, 72 and 96 h. At the end of the experiment, cell viability was determined by modified MTT method (MOSMANN, 1983).

2.3 Monocytic cells treatment and stimulation

In subsequent tests, to characterize NO production in monocytic lineage of this species, adherent cells were treated with dexamethasone 0.3 and 0.03 µg/mL (positive control), L-arginine or D-arginine 2 mM or with the inducible NO synthase inhibitor L-NMMA (N^G-Methyl-L-arginine) 1 mM immediately before the stimulation

with LPS 0.1 and 1.0 ng/mL. Cells were incubated at 20-22°C, 1.7% CO_2 and nitrite concentration measured as described below.

2.4 Exposure of the cells to NSAIDs

Adherent cells were treated with Diclofenac (0.2; 2.0; 20.0; 200.0 and 2,000.0 ng/mL), Ibuprofen (0.1; 1.0; 10.0; 100.0 and 1,000.00 ng/L), Acetaminophen (0.025; 0.25; 2.5; 25.0 and 250ng/L) or L15 medium (control) immediately before the stimulation with LPS (1ng/ml). The effect of the NSAIDs in the same concentrations used above was also evaluated in non-stimulated cells. NO concentration was evaluated after 24h in the supernatant of the cultures and the genotoxicity assay was performed in the resulting control cell monolayer (without LPS).

The tested concentrations were selected based on the concentrations found in the environment, especially in water. The average concentration of diclofenac, ibuprofen and acetaminophen usually found in the environment is approximately 2.0 ng/mL, 1.0 ng/mL (BILA; DEZOTTI, 2003) and 0.25 ng/mL (FENT; WESTON; CAMINADA, 2006), respectively. Concentrations ten times lower and ten, one hundred and one thousand times higher were also tested.

2.5 Nitrite assay

NO production was assayed using a modification of the method described by Neumann et al, 1998. This method is based on the Griess reaction (GREEN *et al.,* 1982) that quantifies the nitrite content, since NO is an unstable molecule and degrades to nitrite and nitrate. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

2.6 Alkaline Comet Assay

To evaluate the genotoxic effects of the NSAIDs at 24 h after the treatment, cells were washed and removed from the plate with ice-cold PBS, harvested, centrifuged and ressuspended in 150 µL of PBS. Only the wells that presented cell

viability above 70% were used for the further tests according to the International Workshop on Genotoxicity Test Procedures (TICE *et al.*, 2000).

The comet assay was performed according to Speit and Hartmann (2005). The cell suspension (13 µl) were diluted in low melting point agarose 0.5% (LMP) and placed on a microscope slide pre-coated with a layer of normal melting point agarose (1.5%). The slides were placed for 24 h at 4 °C in lysis working solution (triton X100 1% v/v, DMSO 10% v/v and lysis stock solution 89% v/v (NaCl 2.5 M, EDTA 0.1 M, Tris 0.01 M, NaOH 0.2 M, N-lauroylsarcosine sodium salt0.034 M, pH 10).

An electrophoresis was run at 300 mA and 1V/cm for 25 min. The slides were washed three times for 5 min each with Tris-HCl (0.4 M), pH 7.5 at 4°C and fixed for 5 min in absolute ethanol and stained with 0.02 µg/ml ethidium bromide. DNA strand breaks were scored using one Leica epifluorescence microscope at a magnification of 400×. DNA damage was determined in each slide (three replicates for treatment) and one hundred nucleoids were scored visually according to tail intensity and given a value of 0, 1, 2, 3 or 4 (from undamaged 0, to maximally damage 4). The total score of 100 nucleoids could range from 0 (all undamaged) to 400 (all maximally damage) (COLLINS; AI-GUO; DUTHIE, 1995).

The comets with small or non-existent head and large, diffuse tails (i.e. so-called hedgehogs) are not considered because they are associated with cell death (FAIRBAIRN *et al.*, 1996; FRENZILLI; NIGRO; LYONS, 2009).

2.7 Viability assessment of cell cultures

Different cell viability between Percoll and Ficoll separation was evaluated after 24h, 48h, 72h and 96h of incubation. The cell viability was also evaluated in the same incubation times of exposure to LPS or NSAIDs (or both) by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MOSMANN, 1983). After cells incubation, were added 100µL of MTT and the plate was incubated again. After 24h the reaction was blocked with an acid solution of 10% SDS. The measure was carried out at 550 nm.

2.8 Drugs and reagents

Penicillin/streptomycin, Ficoll-Pack, LPS, MTT, L-arginine, D-arginine, L-NMMA, dexamethasone, diclofenac, acetaminophen and ibuprofen were purchased from Sigma Chemicals & Co., U.S.A. The L-15 medium and Percoll were purchased from Cultilab, Brazil. Trypan Blue dye was from Gibco and anti-CD11b antibody from BD Biosciences. Other reagents used were of analytical grade.

2.9 Statistical Analysis

Data are presented as mean ± SEM. Kolmogorov-Smirnov normality test was used and comparisons between groups were done by One-Way ANOVA followed by Bonferroni's post-hoc test (cells viability and NO) or Kruskal-Wallis test followed by Dunn's post hoc tests (comet assay). Differences of $p < 0.05$ were considered to be significant. The statistics tests were performed using GraphPad Prism version 5.00 for windows (Graph Pad Software, USA).

3. Results and Discussion

3.1 Primary anterior kidney cultures

Isolated anterior kidney cells of *H. malabaricus* were obtained using Percoll or Ficoll separation, and then were plated on culture plates. After 24 h non-adherent cells were washed away using phosphate-buffered saline (PBS) and the viability and predominant adherent cell type were analyzed.

Different concentrations of Percoll were tested and the quantity of isolated cells changed. The gradient proportion 60:40% showed higher cell number after separation of the anterior kidney cells. It has been shown that the density gradient of Percoll for isolation of macrophage cells is variable. Results in anterior kidney cells isolation were achieved with density combinations of 51% in goldfish (HANINGTON *et al.*, 2009), 51:34% in rainbow trout (STAFFORD *et al.*, 2001) and 41:35% in sea bass (SARMENTO *et al.*, 2004). It seems that the best density to be used depends on the fish species.

The anterior kidney was evaluated at light microscopy and it was observed red cells, monocytic lineage as well as steam cells (Figure 1A). In culture plates, after percoll or ficoll separation, the different populations were not evident (Figure 1B and C) and the cells were stained for monocytic lineage with anti-CD11b. Positive cells were evaluated both in relation to size (SSC – side scatter) and in relation to the cellular complexity (FSC – Forward scatter). The resulting plot is shown in Figure 1D. The lineage was composed of 71.5% of stem cells, 19.5% of macrophages and 9% of monocytes.

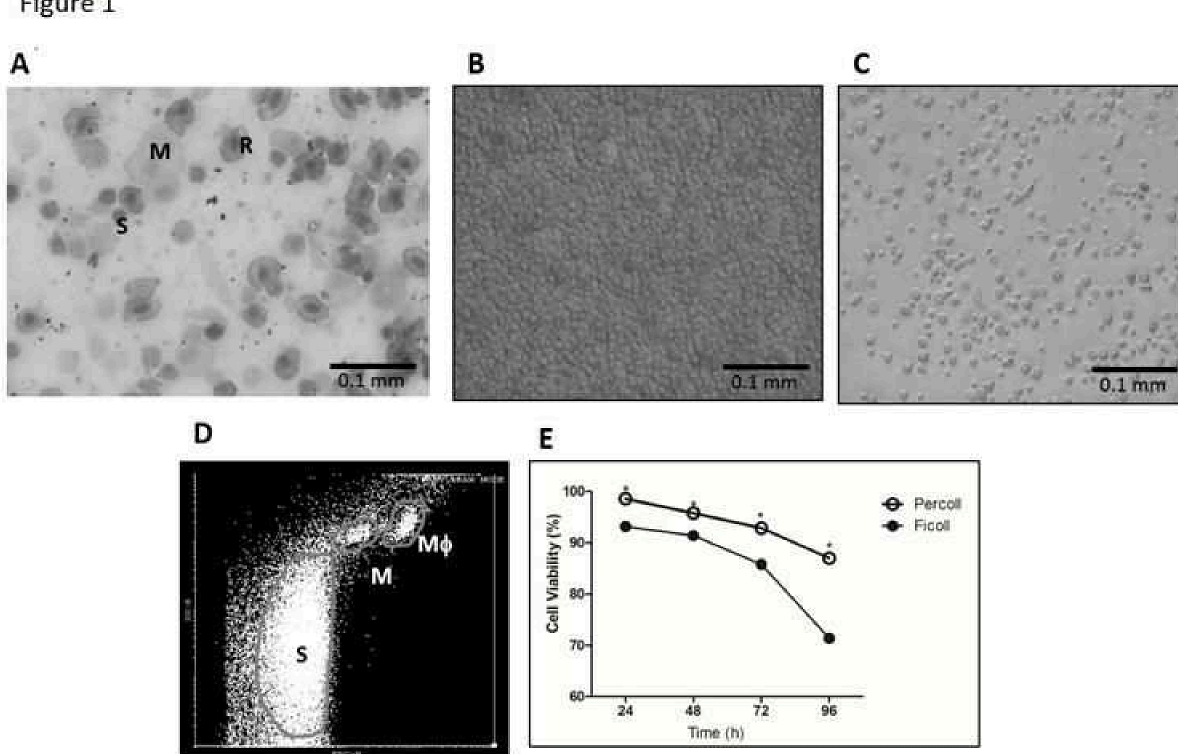

Figure 1 - CULTURE ANTERIOR KIDNEY CELLS OF *Hoplias malabaricus*. Overview of previous anterior kidney cells (A); Placed cells after 24h of incubation in 96 well plates (B); Placed cells after 24h of incubation in 24 well plates (C); Cytometry analysis, using FSC and SSC parameters of cells labeled with $CD11b^+$ antibody (D); Comparison cell viability with percoll and ficoll at different times (E), One-Way ANOVA followed by Bonferroni's post-hoc test $p<0.001$. R-red cells; M-cell monocytic lineage (M – Monocyte; Mϕ – Macrophages); S–stem cells (monocytic lineage).

Similar results were reported before for other species; 12% of macrophage after 24 hours of culture in goldfish (HANINGTON *et al.*, 2009) and rainbow trout (STAFFORD *et al.*, 2001) and 10.4%-58.0% in goldfish (NEUMANN; BARREDA; BELOSEVIC, 1998; NEUMANN; BARREDA; BELOSEVIC, 2000), using a similar protocol.

Cell viability reduced in both separation processes over the time (Figure 1E). However, Percoll separation showed a less pronounced reduction in cell viability when compared to Ficoll separation.

In the present study the cell viability reduced over time. Similar results were described especially after 48 hours. Some studies suggest that this decrease in viability does not seem to be related to nutrient consumption, but with cell senescence (SARMENTO *et al.*, 2004).

3.2 NO production by monocytic cells

Viability and NO production by monocytic lineage from anterior kidney of *H. malabaricus* after stimulation with different concentrations of LPS for 24, 48, 72 and 96h of incubation at 20-22 °C, 1.7% CO_2 are shown in Figure 2.

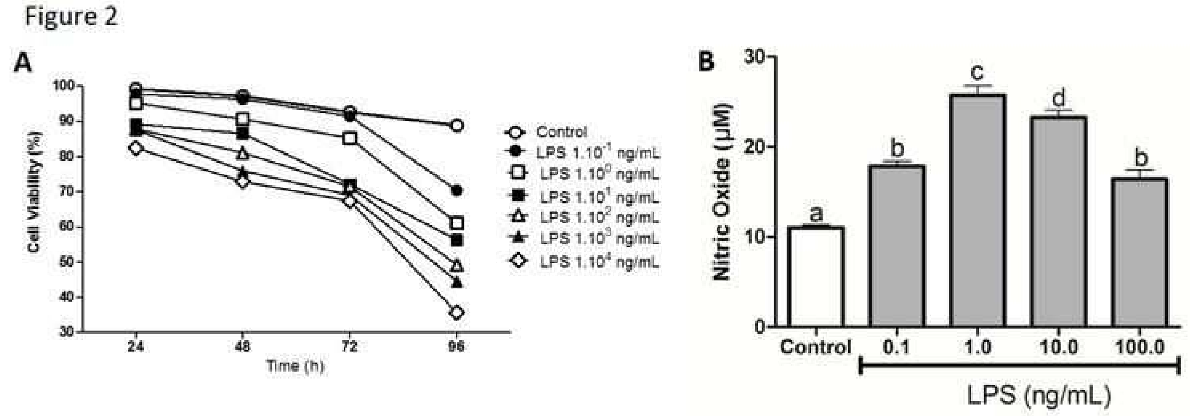

Figure 2 - MONOCYTIC STIMULATION BY LPS. Viability cells in different LPS concentration (A). Nitric oxide production after 24h stimulation by LPS (B); One-Way ANOVA followed by Bonferroni´s post-hoc test $p<0.001$. Different letters represent statistically different values.

Cells viability significantly decreased with incubation time and reductions were evident 48, 72 and 96 h after LPS stimulation. For this reason NO production was analyzed only after 24 hours of incubation (Figure 2B).

NO production blockers and stimulators were tested in order to better characterize the NO production by monocytic/macrophage fish cells after LPS stimulation and its similarities to other species. When the cells were stimulated with LPS at 1 ng/mL NO production significantly increased, which was reverted by the treatment of the cells with dexamethasone at both concentrations 0.3 and 3 µg/mL (Figure 3A). In addition, L-NMMA, an inhibitor of iNOS (inducible nitric oxide synthase)

also abolished NO production induced by LPS (Figure 3B). When monocytic cells were treated with NO precursor L-arginine, NO production increased significantly (Figure 3C) and this increase was more prominent after L-Arg treatment concomitantly with the stimulation of low LPS concentrations (0.1 ng/mL, Figure 3D). In this case, the amount of NO produced was similar to the stimulation of LPS 1 ng/mL (Figure 3D). The effect of L-arginine on NO production was completely blocked with simultaneous exposure to L-NMMA, as expected (Figure 3C). The treatment of the cells with the inactive isomer D-arginine was not significant in the NO production (Figure 3C and 3D).

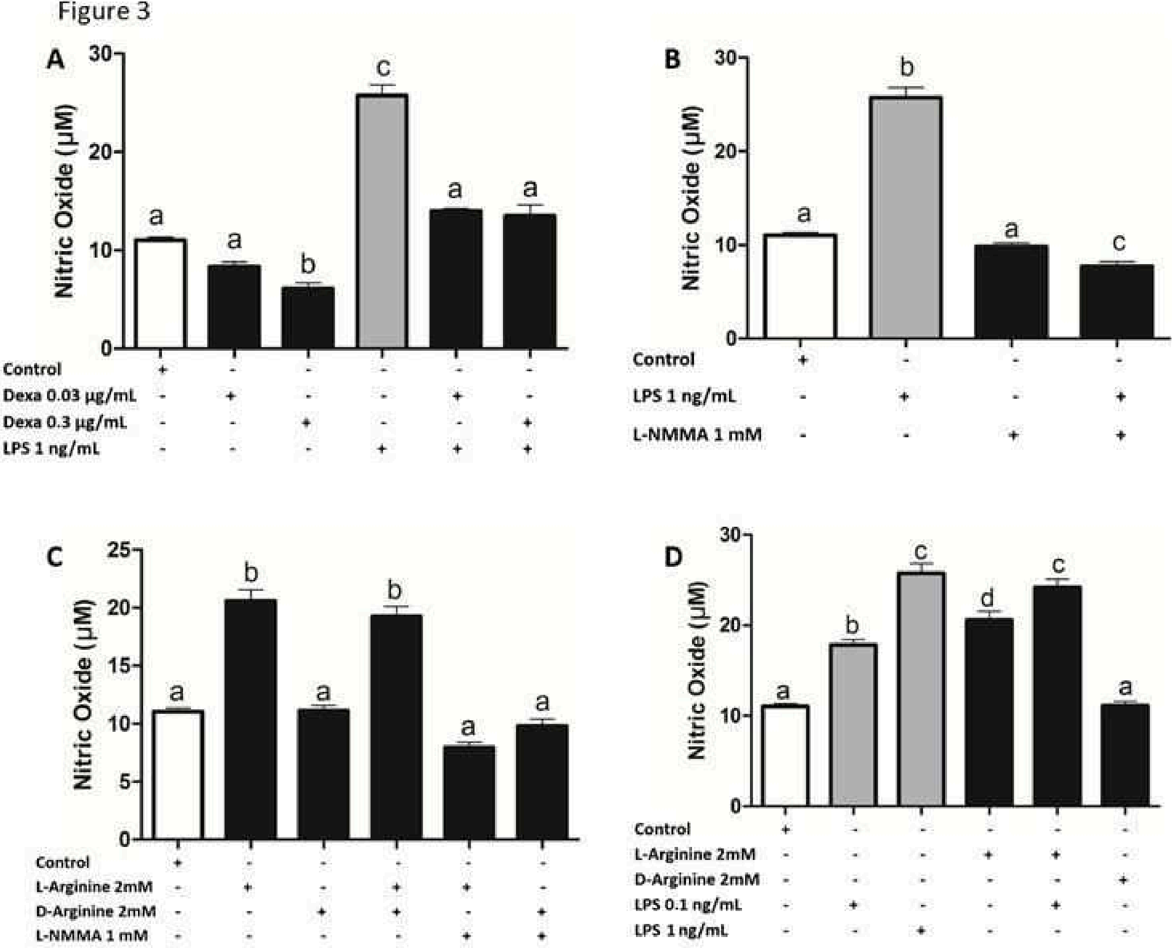

Figure 3 - MONOCYTIC LINEAGE CHALLENGE FROM ANTERIOR KIDNEY CELLS OF *Hoplias malabaricus*. Dexamethasone and LPS challenge (A); LPS and L-NMMA challenge (B); L-arginine, D-arginine and L-NMMA challenge (C); L-arginine, D-arginine and LPS challenge (D). One-Way ANOVA followed by Bonferroni´s post-hoc test $p<0.001$. Different letters represent statistically different values.

LPS has been extensively used for *in vitro* stimulation of fish macrophages. Important molecules associated with the production of the enzyme inducible nitric

oxide synthase (iNOS) (FORLENZA *et al.*, 2011). In our study the lower production of NO at higher LPS concentrations was due to the decrease in cell viability associated with low capacity of monocytic lineage progenitor cells to produce NO. Optimal LPS concentration for NO production by monocytes/macrophages differs among studies. Previous studies showed that optimal LPS concentration required for NO production in monocytes/macrophages isolated form carp and trout was from 0.1 to 50 µg/mL (FIERRO-CASTRO *et al.*, 2012). In a different way, the murine/human macrophage can be stimulated with low LPS concentrations (ng/ml) (REBL; GOLDAMMER; SEYFERT, 2010). The monocytes/macrophages from *H. malabaricus* seem to be very sensitive to LPS stimulation and may represent a good model to study xenobiotics effects.

The activation status of monocytic lineage is important in determining the response to a harmful stimulus. Arginine is an immunomodulator that acts as the unique precursor for NO (LI *et al.*, 2007). Pohlenz *et al.* (2012) showed that the supplementation of L-arginine increased the NO production by monocytic cells stimulated or not by LPS. We confirmed these results using monocytic cells from *H. malabaricus*.

The production of NO in monocytic lineage in response to LPS alone was inhibited by L-NMMA as would have been predicted, but it not occurred with the cells treated with L-NMMA alone. In this case, L-NMMA was not able to decrease spontaneous NO production but only when stimulated by 1 ng/mL of LPS. These results suggested that the production of NO by monocytic/macrophages from *H. malabaricus* is due to *de novo* synthesis of iNOS which transforms L-Arg in NO as usually occurs in other species.

3.3 Effect of NSAIDs on cell viability, NO production and genotoxicity

Diclofenac at 0.2, 2.0, 20.0 and 200.0 ng/mL significantly reduced basal NO production (Figure 4A). Similar results were observed for acetaminophen at 0.025, 0.25, 2.5, 25.0 and 250 ng/mL (Figure 4C), while ibuprofen only reduced basal NO production at the higher concentrations used (10.0, 100.0 and 1000.0 ng/mL) (Figure 4E).

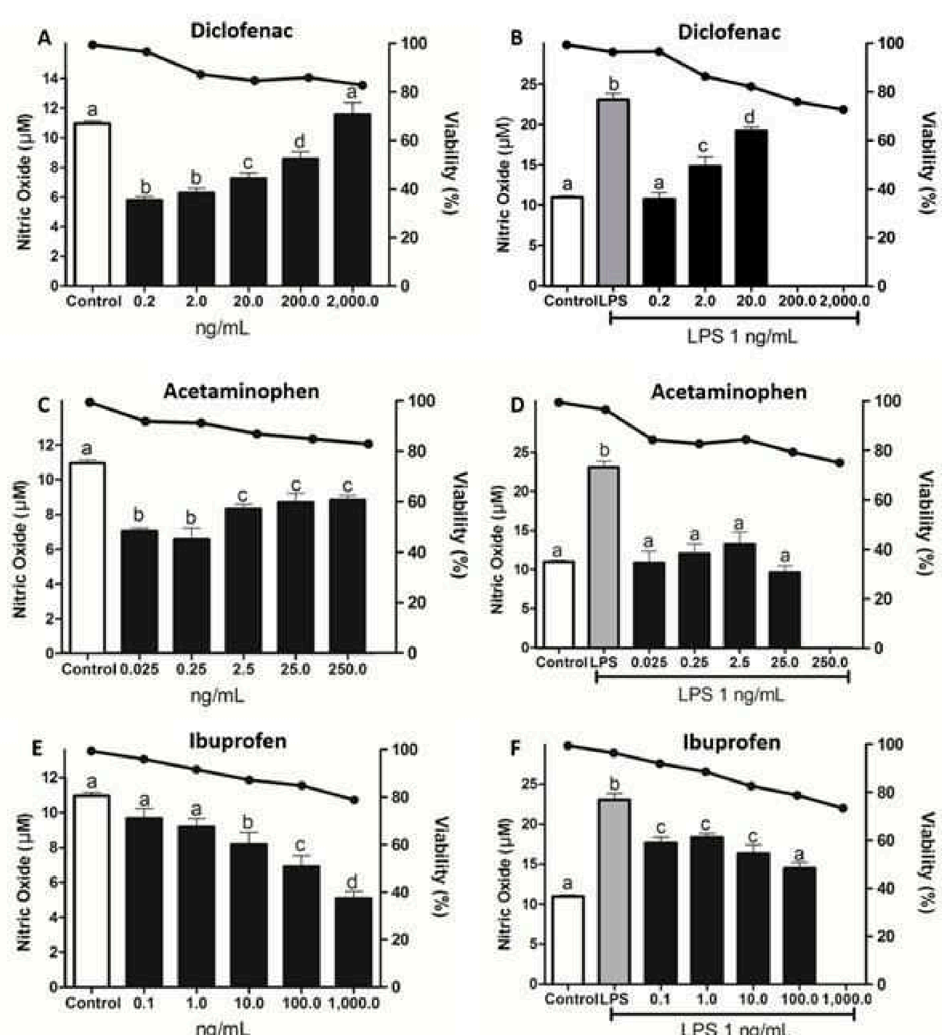

Figure 4 - MONOCYTIC LINEAGE CHALLENGE FROM ANTERIOR KIDNEY CELLS OF *Hoplias malabaricus*. NO production after exposure to diclofenac (A); NO production after exposure to diclofenac with LPS-stimulus (B); NO production after exposure to acetaminophen (C); NO production after exposure to acetaminophen with LPS-stimulus (D); NO production after exposure to ibuprofen (E); NO production after exposure to ibuprofen with LPS-stimulus (F); One-Way ANOVA followed by Bonferroni´s post-hoc test $p<0.001$. Different letters represent statistically different values.

The cells were exposed to NSAIDs and LPS-stimulus, diclofenac, acetaminophen and ibuprofen inhibited LPS-induced NO production in all concentrations tested compared with LPS control (Figure 4B, 4D and 4F). NO production by monocytic cells treated with the highest concentrations of diclofenac (200.0 and 2000.0 ng/mL), acetaminophen (250.0 ng/mL) and ibuprofen (1000.0

ng/mL) and stimulated with LPS was not evaluated, since the cell viability was below 80% (figure 4B, 4D and 4F).

The genotoxicity was observed at the highest concentration of diclofenac (20 ng/mL) (Figure 5A), at the concentrations of acetaminophen (0.25; 2.5 and 25.0 ng/mL) (Figure 5B) and at the ibuprofen concentrations (except 100.0 ng/mL) (Figure 5C).

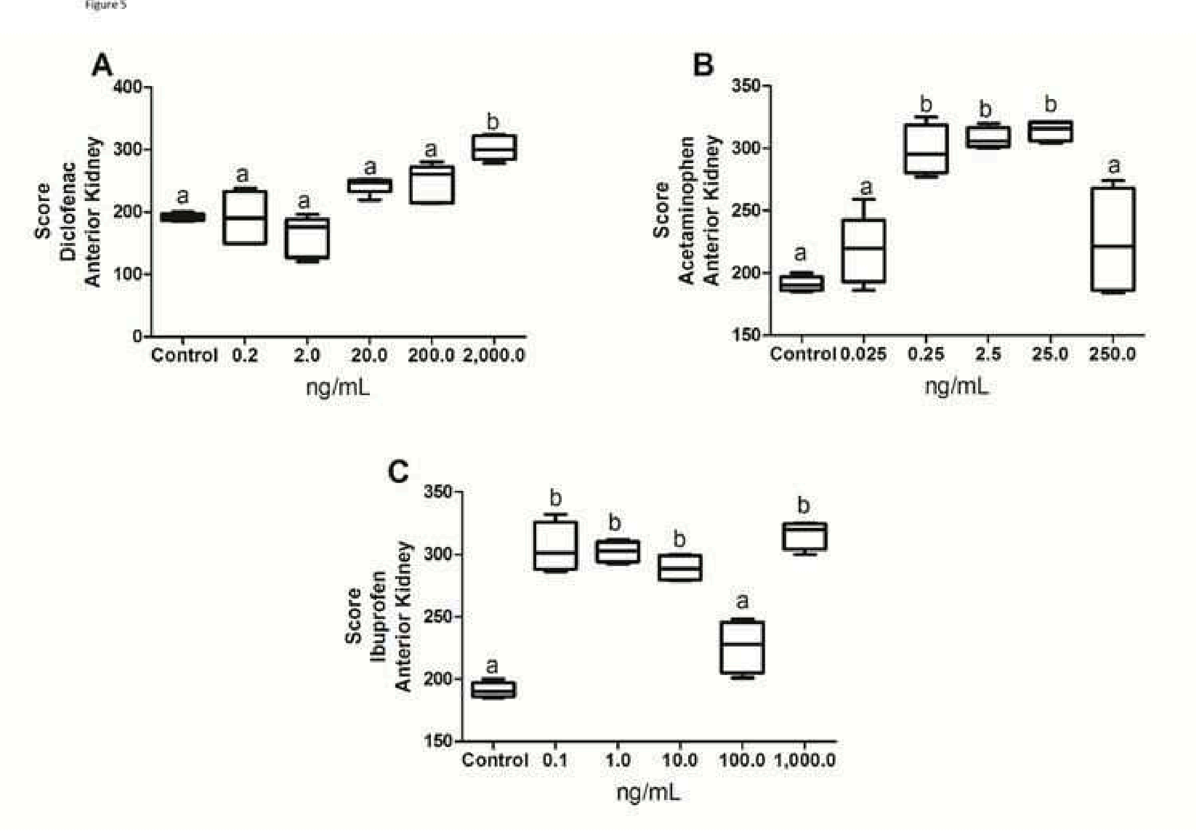

Figure 5 - COMET ASSAY. DNA strand breaks. (A); Score DNA strand breaks after exposure to diclofenac (B); Score DNA strand breaks after exposure to acetaminophen (C); Score DNA strand breaks after exposure to ibuprofen; Kruskal-Wallis test followed by Dunn’s post hoc tests $p<0.001$. Different letters represent statistically different values.

The NSAIDs tested in this studies reduced basal NO production. Previous studies, after 24h of incubation the murine macrophage culture with 1 and 100µg/mL of diclofenac, demonstrated an inhibition in the NO production (CIRINO *et al.*, 1996).

In mouse macrophage cells exposed to 10 µmol/mL for 2 and 18h of acetaminophen was also able to decrease the NO production (AL-BELOOSHI *et al.*, 2010). In fish monocytic lineage, acetaminophen inhibited basal NO production.

Ibuprofen also inhibited basal NO production. In other literature studies the inhibition on phagocytosis in haemolymph and catecholase-type phenoloxidade activity in the plasma of the Pacific oyster *Crassos trea gigas* was related with the 5 ng/mL of ibuprofen concentration mixed with herbicides (LUNA-ACOSTA *et al.*, 2012).

In rat glial cell, ibuprofen 178 µg/mL decreased iNOS protein levels in 50%, possibly due to an inhibition of post transcriptional processing of this enzyme, as well as decreased the production of pro-inflammatory cytokines (COMBS *et al.*, 2000).

Furthermore, in the cells from freshwater mussel *Dreissena polymorpha* ibuprofen increased the percentage of haemocytes showing apoptosis and lysosomal membrane destabilization (PAROLINI *et al.*, 2009) suggesting that ibuprofen was toxic for these cells.

In fish monocytic lineage, diclofenac inhibited NO production and probably iNOS induced by LPS. In previous works, Cirino *et al*. (1996) demonstrated that diclofenac was able to inhibit directly iNOS activity, once expressed by LPS-stimulus. Diclofenac (0.38 mM) also decreased activation of NF-kB from KBM5 cells in culture and the levels of iNOS in Raw 264.7 cells, impairing their activation in response to LPS (TAKADA *et al.*, 2004).

Acetaminophen inhibited NO production by LPS-stimulus. In mouse macrophage cells stimulated by 1µg/mL LPS for 12 h prior to treatment with 1 and 10 µmol/mL of acetaminophen for 2 and 18 hours, decreased NO production (concentration dependent) and the expression of the iNOS (AL-BELOOSHI *et al.*, 2010). The acetaminophen (0.5, 3.0 and 10 mM) was able to decrease activation of mouse NF-kB complex in Hepa1-6 cells (BOULARES *et al.*, 2000).

Ibuprofen is also involved in down regulation of iNOS and decreased the expression of iNOS protein activating by LPS-stimulus (HENEKA; KLOCKGETHER; FEINSTEIN, 2000) in granule cells from rat cerebellum. It also decreased the activation of NF-kB in Pc 3, LNCaP and DU145 cells and in nuclei KBM5 cells (TAKADA *et al.*, 2004) previously stimulated.

The production of basal NO or NO LPS-induced by monocytic/macrophages of *H. malabaricus* can be affected by anti-inflammatory drugs, as demonstrated in this work.

The most commonly prescribed NSAIDs, acetaminophen, diclofenac and ibuprofen, were genotoxic to cells of lineage monocytic of *H. malabaricus.*

In lineage DT40 culture cells NSAIDs also induce the DNA damages (LIU *et al.*, 2012) similarly to the results of the present work. MEHINTO, HILL and TYLER (2010) observed a decreasing of cyclooxygenases (COX 1 e COX 2) gene expression in kidney leading to impairment of immune system with diclofenac in trout. Furthermore, affected other enzymes related to metabolism of xenobiotics (CyP) and cell cycle (p53). The genotoxic mechanism of diclofenac is related to oxidative stress.

The hydric fish exposure to 0.18 $ng.mL^{-1}$ of diclofenac (similar to the low concentration used in this study), caused DNA fragmentation, apoptosis and genomic alterations of polymorphic patterns (ROCCO *et al.*, 2010). Diclofenac could induce specific types of DNA lesions. At high concentration diclofenac caused replication blockage leading to chromosomal aberrations as well as translesion DNA synthesis, measured through proliferation of DT40 mutant cells lines (LIU *et al.*, 2012). Induction of the p53 gene and CYP1A gene was observed in fish exposure to diclofenac indicating carcinogenic and/or apoptotic potential effects (HONG *et al.*, 2007).

Studies demonstrated that acetaminophen can covalently bind to DNA and cause chromosomal aberrations in mammalian and zebrafish cells. The genotoxic effects of acetaminophen can be a consequence of cytotoxic events (BLANSET; ZHANG; ROBSON, 2007).

The genotoxicity and carcinogenicity of acetaminophen is widely discussed. For the International Agency for Research on Cancer (IARC) the acetaminophen is non-classifiable as carcinogenic in humans, however this compound might be considered genotoxic and carcinogenic. Studies have demonstrated that acetaminophen can covalently bind to DNA and cause chromosomal aberrations, teratogenesis and inactivation of ribonucleotide reductase with consequent disturbances of DNA repair and replication (BLANSET; ZHANG; ROBSON, 2007; PENG *et al.*, 2010).

The ibuprofen, *in vivo* studies was associated with induction of micronuclei frequency, general DNA damage and punctual genomic alterations (RAGUGNETTI *et al.*, 2011). In Chinese hamster ovary (CHO) cells was reported an increase in the frequencies of micronucleated cells with and without kinetochores indicating aneugenic and clastogenic activity of ibuprofen (DOPPALAPUDI *et al.*, 2012). There is no report of *in vitro* genotoxicity of ibuprofen using fish cells.

Ibuprofen was genotoxic to monocytic cells of *H. malabaricus* including at the environmental reference concentration. The risk to aquatic organisms increases

because ibuprofen is a hydrophobic compound with ability to pass through cell membranes and to accumulate in living organisms (VICQUELIN *et al.*, 2011).

In fish, ibuprofen is associated with induction of micronuclei (RAGUGNETTI *et al.*, 2011); general DNA damage and punctual genomic alterations (ROCCO *et al.*, 2010). In mammals ibuprofen induce sister chromatid exchange (SCE) (PHILIPOSE *et al.*, 1997) and increased micronucleated and binucleated cells with and without kinetochores indicating aneugenic and clastogenic activity (DOPPALAPUDI *et al.*, 2012). There is no report of *in vitro* genotoxicity of ibuprofen using fish cells.

4. Conclusion

In the present study, diclofenac, acetaminophen and ibuprofen reduced NO production and induced DNA damage in the lineage monocytic cells from anterior kidney of *H. malabaricus*. The results suggested potential immunosuppressive action and induction of genotoxicity. The results implied in a better understanding on the toxic effects of these specific pharmaceutical drugs and the methodology can be used as potential immunological biomarkers of freshwater fish toxicity.

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CAPÍTULO II

EFEITOS DA EXPOSIÇÃO TRÓFICA DO DICLOFENACO EM *Hoplias malabaricus*

Effects of trophic exposure to diclofenac in freshwater fish

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceutical prescribed in human medicine and have the potential to contaminate water and sediments via inputs from sewage treatment plants. Their impacts on humans and ecosystems are emerging issues in environmental health. The aim of the present work was to evaluate the effects of diclofenac in the fish species Hoplias *malabaricus* after trophic exposure. Fish were fed twice every week with *Astyanax sp*. submitted to intraperitoneal (IP) inoculation with diclofenac (0; 0.2; 2.0 or 20.0 µg/Kg). After 12 doses, half of fish received 1 mg/Kg of carrageenan IP and after 4 hours they were anesthetized and euthanized for cells migration evaluation. In the other fish (without carrageenan) the hematological parameters, nitric oxide (NO) basal production and after LPS-stimulate in head kidney were measured. In the liver hepatosomatic index (HSI) and biochemical analysis, such as activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), Ethoxyresorufin-O-deethylase (EROD) and catalase (CAT) were analyzed. The reduced glutathione (GSH) and lipoperoxidation (LPO) was also evaluated. The results showed an increase in red blood cells count and in the hematocrit at the low dose Therefore, the hemoglobin reduced at the highest dose. The thrombocytes count increased in all groups exposed to diclofenac and the total blood leukocyte counts decreased due to the neutrophils reduction. Monocytes were also decreased at the highest dose. The number of resident peritoneal cells did not differ among the groups, but the cell migration reduced after carrageenan-with a significant decrease in the migration of polymorphonuclear cells. The basal NO synthesis of anterior kidney cell cultures from diclofenac-treated animals was significant lower in the cells from the group 2 and 20 µg/kg. LPS-stimulated NO production was reduced in all the diclofenac-treated groups. Diclofenac also reduced HSI at the 0.2 µg/Kg. In liver, diclofenac caused oxidative stress with increased GPx activity and LPO. The GST activity decreased by diclofenac in liver. The results suggest that a trophic exposure to diclofenac can lead to potential toxic effects, including hematological, immunological and biochemical changes.

Keywords: diclofenac, trophic exposure, oxidative stress, migration cells, hematological parameters, culture cells, *Hoplias malabaricus*

1. Introduction

Pharmaceuticals are considered a new class of pollutants and negative effects on terrestrial and aquatic environments have been attributed recently to their presence in the ecosystems. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently detected pharmaceuticals in treatment plants and surface waters worldwide, due to their volume of consumption and incomplete removal during the wastewater treatment processes (ISLAS-FLORES *et al*., 2013). Diclofenac is a

NSAIDs generally used as an analgesic to reduce pain and to treat inflammatory disorders (ISLAS-FLORES *et al*., 2013). Diclofenac was identified as one of the most important pharmaceutically active compounds present in the water cycle. It was found in groundwater samples and even in treated drinking water (PRASKOVA *et al*., 2014). In the present study antioxidant defense parameters (superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and reduced glutathione) were investigated as biomarkers of cellular oxidative stress. Lipid peroxidation is also associated with oxidative stress resulting in the oxidation of polyunsatured lipids (QUINN *et al*., 2011). Oxidative stress is also considered an important factor affecting reproductive performance (COSTANTINI *et al*., 2011). The biotransformation of some pharmaceuticals in the aquatic environment may modify enzymatic pathways mediated by CYP450 and cause physiological effects and toxicity (BURKINA *et al.*, 2013). The hematological parameters studies of fish are the ecological and physiological interest, since that assist in understanding the relationship between blood characteristics, physical activity, habitat and adaptability of fish in the environment. Hematologic values can be influenced not only by growth but also by the ecophysiological conditions (TAVARES-DIAS; MORAES, 2004).

Several pharmaceuticals have been investigated in *in vitro* systems such as fish cell lines and especially in primary fish cell cultures. Among the advantages of these procedures, based on fish cells or reporter gene systems are their potential for toxicity screening, besides of course, they are important alternatives to animal testing (FENT; WESTON; CAMINADA, 2006).The functional monocytic lineage and macrophage cells constitute a major, widely dispersed system of cells that regulate homeostasis in the host and respond to tissue injury by contributing with essential functions during inflammation and repair. In order to study the immune response in fish cells exposed to diclofenac the analysis of functional monocytic lineage and macrophage cells are essential for the establishment of a properly working immune system. They constitute a major, widely dispersed system of cells that regulate homeostasis in the host and respond to tissue injury by contributing with essential functions during inflammation and repair (BARREDA; BELOSEVIC, 2001).

The experimental model used in this study was *Hoplias malabaricus* (Characiformes, Erythrinidae), a carnivorous fish with large distribution in tropical rivers and lakes and very consumed by human populations (FERREIRA *et al.,* 2003). This

fish has the advantage that it is easily fed under laboratory and has been explored in several biological studies (MONTEIRO *et al.*, 2013; SILVA DE ASSIS *et al.*, 2013).

Since diclofenac has been found in aquatic environment, the aim of the present work was to evaluate the biochemical, hematological and immunological effects in the fish *Hoplias malabaricus* after trophic exposure.

2. Material and methods

The present work was approved by the Animal Experimentation Ethics Committee of Federal University of Parana, under number 456 and all protocols were realized in accordance with International Guidelines for Animal Use.

2.1 Chemicals

Diclofenac sodium salt (D6899) was obtained from Sigma Aldrich. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Corporation (USA) and Merck.

2.2 Experimental design

Adult *Hoplias malabaricus* males weighing 171.4 ± 15.7 g (standard length: 25.3± 4.4 cm) were purchased from Santa Candida Commercial Farm, Santa Cruz da Conceição, São Paulo, Brazil. Fish were kept at 24±1°C in glass aquaria (120 L capacity) for 30 days in filtered and dechlorinated tap water on a simulated natural photoperiod (12 hours dark:12 hours light). After acclimation to laboratory conditions, fish were randomly divided into four groups (n=20 fish in each group) and was transferred to test aquarium. During the experiment, each fish were maintained in a 30L aquarium and were fed twice every week with fish (*Astyanax sp*. 30 ± 5g) as pray vehicle prior injected intraperitoneally with diclofenac (0.2; 2.0 or 20.0 μg/Kg simulating a trophic contamination. These doses were based on the concentration of diclofenac (2 μg/L) usually found in the environment (SANTOS *et al*., 2010). After 12 doses, half of fish (n=10) received carrageenan (Cg) 1mg/kg intraperitoneal. The fish were anesthetized and killed by medullar section 4 hours later and the intraperitoneal was washed with PBS. The fluid was removed to determinate the cell migration induced by

Cg. The fish which did not receive Cg were anesthetized in water with benzocaine 1 % (Sigma) and blood was taken from caudal vein for hematology and biochemical analyses. The fish were killed by medullar section weighed and the liver was removed for hepatosomatic index (HIS) calculation and biochemical analysis. The HIS was determined as HSI= (Liver weight/Whole body weight) x 100. The head kidney was used to primary macrophage culture cell.

2.3 Hematological parameters

The erythrocyte count or RBC (Red Blood Cells) was performed with the technique of formaldehyde - citrate, modified by Oliveira-Júnior et al. (2009). Hemoglobin (Hb) contents were determined spectrophotometrically at 540 nm using the cyanomethemoglobin method (COLLIER, 1944; DRABKIN, 1946). The hematocrit (Hct) was determined by the volume occupied by erythrocytes in heparinized microhematocrit (HINE, 1992; NELSON; MORRIS, 1989). The hematimetric indices - MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration) were determined using the following standard formulas described by WINTROBE (1934).

The leukocyte (WBC - White Blood Cells) and the thrombocyte count were realized according to Tavares-Dias and Moraes (2006, 2003). The leukocyte differentiation was realized like described by Tavares-Dias *et al.* (2002, 1999).

2.4 Head kidney primary macrophage culture cell and nitric oxide determination

The monocytic lineage of *H. malabaricus* anterior kidney was obtained, as previously described by Ribas *et al* (2014). Briefly, the head kidney were removed, pooled and transferred to L-15 medium (Leibovitz-; supplemented with 20U of heparin and 1% antibiotic penicillin/streptomycin) under sterile conditions. The tissue was homogenized in the same medium, transferred to a 15mL tube and suspended with a sterile Pasteur pipette for 1 min. For a better suspension this procedure was repeated once using a Pasteur pipette of minor diameter. Cellular suspension (in the proportion 2:1) was transferred to a new

15 mL tube containing Percoll gradient 60:40 (v/v) and centrifuged at 400 x g for 30 min at 22 ºC. Cells at the Percoll interface were removed, washed twice in serum-free medium and centrifuged at 400 x g for 10 min. Viable cells (1.10^6) were placed in 96 wells plates with L-15 medium supplemented with fetal bovine serum 2% and incubated at 20-22 °C, 1.7% CO_2 for 24h for adherence. After this period, the supernatant was removed for Nitric Oxide (NO) determination. Non-adherent cells were washed away with phosphate-buffered saline (PBS). The remaining adhered cells were used for subsequent experiments to determine NO production induced by 1ng/mL of LPS by 24 hour.

NO production was assayed using a modification of the method described by Neumann *et al*. (1998). This method is based on the Griess reaction (GREEN, 1982) that quantifies the nitrite content, since NO is an unstable molecule and degrades to nitrite and nitrate. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

Different cell viability was evaluated in the same incubation times of basal exposure or after stimulus of LPS by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MOSMANN, 1983). After cell incubation, 100μL of MTT were added and the plate was incubated again. After 24h the reaction was blocked with an acid solution of 10% SDS. The measurement was carried out at 550 nm.

2.6 Biochemical analysis

For the biochemical analysis the samples were homogenized in a phosphate buffer (0.1 M) at pH 7.0, and centrifuged at 15,000 *x g* for 30 min, at 4°C.

SOD activity was assayed by measuring its ability to inhibit the reduction of nitrobluetetrazolium (NBT), which was determined by the method described by Crouch *et al.* (1981). The reduction of NBT by O_2^- to blue formazan was measured spectrophotometrically at 560 nm during 30 min. One unit of SOD was defined as the enzymatic activity necessary to inhibit the reduction of NBT to 50% of the blank and was expressed U/mg protein.

CAT activity was assayed by direct measurement of H_2O_2 degradation and absorbance decrease was measured at 240 nm for 1 min at 27°C and the activity was expressed as μmol/ min/mg protein.

GPx activity was measured based on the decrease of absorbance of NADPH at 340 nm, encouraged by the reduction of GSSG, catalyzed by GR, in the presence of NADPH (PAGLIA; VALENTINE, 1967). Absorbance was monitored at 340 nm and the activity was expressed as nmol/min/mg protein.

GSH was measured according Sedlak and Lindsay (1968). Absorbance was determined at415 nm and GSH concentration was calculated by comparison with the standard curve for GSH and the activity was expressed as µg/mg protein.

The analysis of LPO was carried out using the ferrous oxidation–xylenol assay (JIANG *et al.*, 1992). After 30 min of reaction at room temperature, the absorbance was measured at 570 nm and the activity was expressed as µmol hidroperoxides/min/mg protein.

EROD activity was evaluated according to Burke and Mayer (1974). The fluorimeter measurement was at a wavelength of 530 nm (excitation) and 590 nm (emission) for 10 min at 27 °C and the activity was expressed as pmol/min/mg protein.

GST activity was measured using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (KEEN *et al*., 1976). The absorbance increase was measured at 340 nm for 3 min at intervals of 15 s and the activity was expressed as nmol/min/mg protein.

The protein concentration was determined by using Bradford's method (1976), with bovine serum albumin as the standard at 620 nm.

2.7 Statistical analysis

The data analysis was preceded by the Kolmogorov–Smirnov normality test. Data were analyzed using the One-way Analysis of Variance (ANOVA), followed by the Bonferroni *post hoc* tests. All data were statistically analyzed by the GraphPad Prism 5.00 (GraphPad Software, Inc.). All tests were regarded as statistically significant when $p < 0.05$.

3. Results

3.1 Hematological parameters

In the present work, *H. malabaricus* exposed to diclofenac showed an increase in RBC and Hct determination in the group treated with diclofenac at 0.2 µg/Kg. The Hb determination significantly decreased in the 20.0 µg/Kg exposed group. The hematimetric indices MCV, MCH and MCHC did not change in all groups exposed to diclofenac (Table 1). The leukocyte count decreased significantly in a dose-dependent manner (Figure 1A). Decreased polymorphonuclear leukocytes (0.2, 2.0 and 20.0 µg/Kg) (Figure 1B) and mononuclear leukocytes (2.0 and 20.0 µg/Kg) (Figure 1C) were observed in the present study. In all groups thrombocytes count increased (Anova, followed by Bonferroni post-hoc, $\alpha = 0.05$).

TABLE 1 - *Hoplias malabaricus* HEMATOLOGICAL PARAMETERS AFTER DICLOFENAC EXPOSURE

	Diclofenac Doses			
	Control	0,2 µg/Kg	2 µg/Kg	20 µg/Kg
RBC (10^6/ µL)	1.347 ± 0.06	1.580 ± 0.03*	1.410 ± 0.02	1.410 ± 0.08
Hemoglobin (g/dL)	6.790 ± 0.48	7.070 ± 0.40	6.850 ± 0.40	5.800 ± 0.13*
Hematócrit (%)	23.76 ± 0.51	27.67 ± 0.58*	26.20 ± 0.82	22.66 ± 0.87
MCV (*f*L)	178.2 ± 5.28	175.9 ± 5.88	186.3 ± 6.60	166.6 ± 12.31
MCH (ρg)	51.11 ± 4.24	44.63 ± 2.11	48.74 ± 3.04	42.78 ± 3.16
MCHC (g/dL)	28.68 ± 2.13	25.81 ± 1.81	26.75 ± 2.31	25.76 ± 0.56
Thrombocytes (10^3/ µL)	20.10 ± 1.16	45.16 ± 1.31*	33.34 ± 2.21*	42.40 ± 3.15*

Values are expressed as mean ± standard error of mean. * indicate statistically significant differences ($p<0.05$). ANOVA, Bonferroni, N=10. RBC - Red Blood Cells; MCV - mean corpuscular volume; MCH - mean corpuscular hemoglobin; MCHC - mean corpuscular hemoglobin concentration.

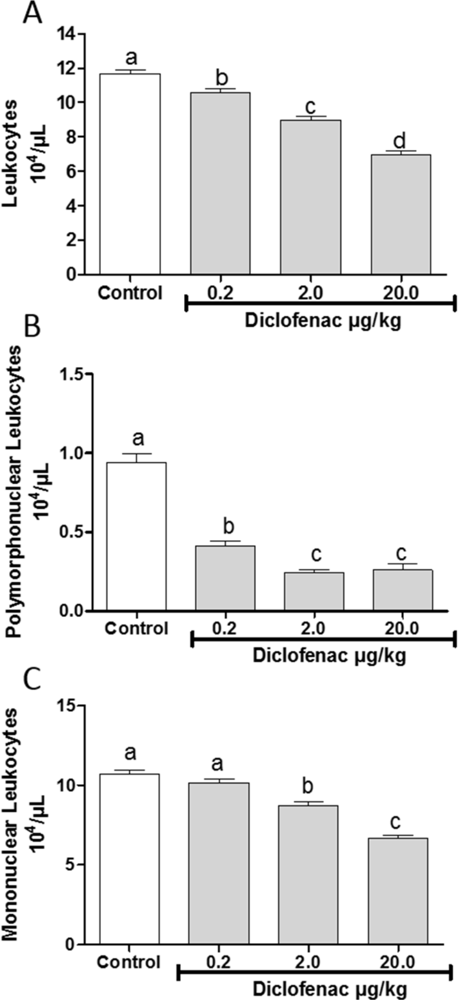

Figure 1 - LEUCOGRAM OF *Hoplias malabaricus* THROPHICALLY EXPOSED TO DICLOFENAC. (A): Leukocyte total number (B): Polymorphonuclear differential leukocytes count (C): Mononuclear differential leukocytes count (Anova, followed by Bonferroni post hoc test ($p<0.0001$). Different letters represent statistically different values.

3.2 Intraperitoneal migration cells induced by carrageenan

In the present study, was not observed difference in the quantity of leukocytes which migrated to peritoneal cavity in *Hoplias malabaricus* exposed to diclofenac. However, after the carrageenan stimulation, the total cell number decreased significantly in all groups, especially in the dose 0.2 µg/Kg (Anova, followed by Bonferroni post-hoc, α = 0.05) (Figure 2A). The differential count of the polymorphonuclear leukocytes increased in the 20.0 µg/Kg diclofenac group, but decreased in the other ones (Anova, followed by Bonferroni post-hoc, α = 0.05) (Figure 2B). The mononuclear cell counts increased in fish exposed to diclofenac and

challenged with carrageenan (Figure 2C) (Anova, followed by Bonferroni post-hoc, α = 0.05).

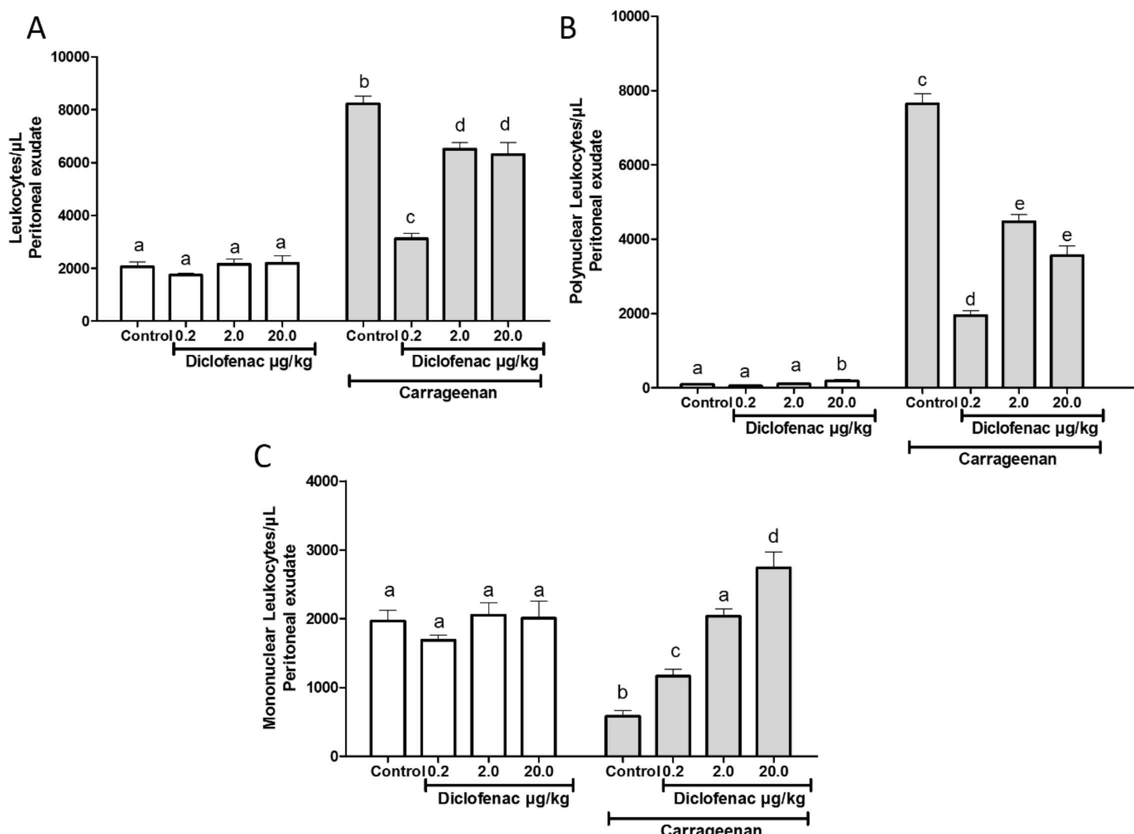

Figure 2 - *Hoplias malabaricus* EXPOSED TO DICLOFENAC AND CHALLENGED WITH CARRAGEENAN. (A) Total cells in fish exposed to diclofenac with and without carrageenan polymorphonuclear differential count in fish exposed to diclofenac with and without carrageenan. (B) Mononuclear differential count in fish exposed to diclofenac with and without carrageenan. (C) One-Way ANOVA followed by Bonferroni's post-hoc test p<0.001. Different letters represent statistically different values.

3.3 Head kidney primary macrophage culture cell and nitric oxide determination

Macrophages cultured from the head kidney of *H. malabaricus* exposed to diclofenac had basal NO production significantly reduced at 2.0 and 20.0 µg/Kg (Figure 3A). After to the LPS-stimulus, the diclofenac inhibited LPS-induced NO production in all the groups compared with LPS control group (Figure 3B) (Anova, followed by Bonferroni post-hoc, α = 0.05).

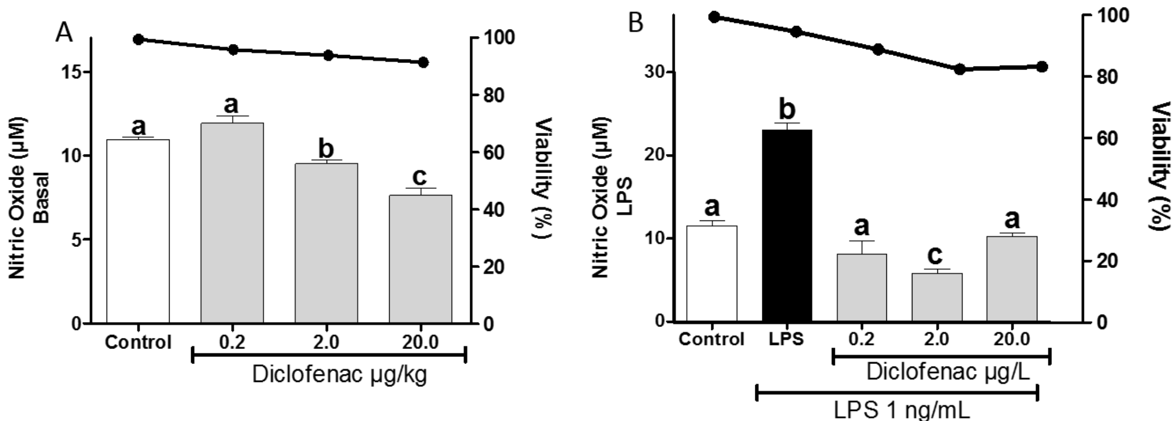

Figure 3 - MACROPHAGE CHALLENGE OF *H. malabaricus* ANTERIOR KIDNEY CELLS. A) Nitric Oxide production after exposure to diclofenac; B) Nitric Oxide production after exposure to diclofenac with LPS-stimulus; One-Way ANOVA followed by Bonferroni´s post-hoc test $p<0.001$. Different letters represent statistically different values.

3.4 Hepatosomatic Index

Diclofenac reduced the HSI only at the low dose (Figure 4).

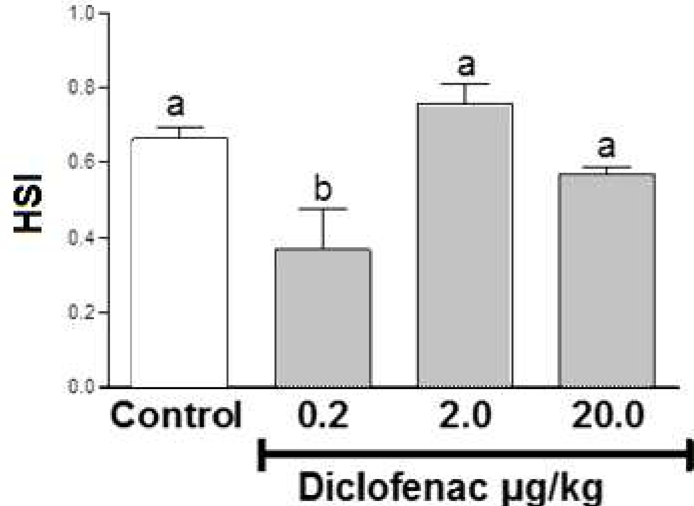

Figure 4 - HEPATOSOMATIC INDEX OF *H. malabaricus* AFTER DICLOFENAC EXPOSURE. One-Way ANOVA followed by Bonferroni's post-hoc test $p<0.05$. Different letters represent statistically different values.

3.5 Biochemical analysis

In the liver the diclofenac significantly increased the SOD activity, at 2 and 20 µg/Kg doses, the GSH concentration at the 20 µg/Kg and the GPx activity in all doses in relation to the control group, suggesting the generation of free radicals. The LPO was also induced by diclofenac in all exposed groups. Although CAT did not change,

diclofenac reduced the GST activity, demonstrating that inhibited the metabolism of phase II. The EROD activity was not altered by diclofenac (Table 2).

TABLE 2 - BIOCHEMICAL ANALYSIS IN LIVER OF *H. malabaricus* AFTER DICLOFENAC EXPOSURE

	Diclofenac			
	Control	0.2 µg/Kg	2 µg/Kg	20 µg/Kg
SOD (U/mg protein)	68.42 ± 1.50[a]	75.63 ± 4.55[a]	94.70 ± 6.07[b]	114.70 ± 2.57[c]
CAT (µmol/min/mg protein)	84.09 ± 6.22[a]	93.74 ± 9.17[a]	85.38 ± 10.61[a]	99.06 ± 6.45[a]
GPx (nmol/min/mg proteín)	107.50 ± 8.50[a]	161.10 ± 5.58[b]	164.40 ± 6.79[b]	213.40 ± 17.92[c]
EROD (pmol/min/mg proteín)	11.46 ± 2.12[a]	14.83 ±3.76[a]	17.60 ± 3.04[a]	7.56 ± 1.01[a]
GST (nmol/min/mg proteín)	259.00 ± 18.09[a]	41.00 ± 6.09[b]	35.37 ± 2.80[b]	37.90 ± 2.55[b]
GSH (µg/mg protein)	8.78 ± 0.66[a]	8.01 ± 0.86[a]	10.30 ± 1.25[a]	13.07 ± 0.48[b]
LPO (µmol hidroperoxides/mg protein)	7.52 ± 0.57[a]	11.23 ± 0.32[b]	12.15 ± 0.45[b]	8.71 ± 0.60[a]

Values are expressed as mean ± standard error of mean. Different letters indicate statistically significant differences ($p<0.05$). ANOVA, Bonferroni, n=10.

4. Discussion

After a trophic exposure using low doses of diclofenac was observed an increase in level of blood RBC and hematocrit in exposure of 0.2 µg/Kg. Only the hemoglobin reduced at the high dose. There are few studies about the effects of diclofenac on aquatic vertebrates. An increase in the RBC number even at the lowest dose, suggests an increase in the ability of cells to carry oxygen (MARIAPPAN *et al*., 2011), which is corroborated by the increased hematocrit in the same group (0.2 µg/Kg). The decrease of hemoglobin at the highest dose may indicate an anemia process as observed by Saravanan *et al*. (2011) in the exposure of *Cyprinus carpio* to 1, 10 and 100 µg/L of diclofenac during 96 h. This effect also was observed with other toxicants such as heavy metals (LAVANYA *et al*., 2011; SUVETHA *et al*., 2010).

The number of blood leukocytes is a sensitive indicator of stress in fish. Decreased leukocytes counts are indicative of immunosuppression (PIMPÃO *et al*., 2007, SARAVANAN *et al*., 2011). There are several studies that suggest which xenobiotics are capable of inducing immunosuppression in fish. This feature can be detected from decreased leukocyte counts (BARTON, 1991, FISCHER *et al*., 2006, MUÑOZ *et al*., 2014). The polymorphonuclear cells are the most important leukocytes in fish and show great sensitivity to changes in environmental parameters (MUÑOZ *et*

al., 2014). The lymphocytes are the first-line of defense in fish therefore a decrease in the number of mononuclear leukocytes represents a decreased immune response, ultimately predisposing fish to diseases (WEDEMEYER *et al*., 1990; FISCHER *et al*., 2006). In the present study we observed a decreased leukocyte total count and also a decline in the number of polymorphonuclear and mononuclear cells. It is likely that these decreases represent an important change in the fish physiology, contributing to immunosuppression.

Other result that may contribute to immunosuppression is the incapacity of the *H. malabaricus* exposed to diclofenac and challenged with carrageenan for promoting the adequate cell migration especially in an inflammatory process. Inflammatory reactions frequently occur in fish but the mechanisms involved in this process are poorly understood (PIMPÃO *et al*., 2007) include fish exposed to pharmaceutical compounds.The inflammatory reaction enables an organism to defend itself against microbe infection. The migration of leukocytes from the vascular system to sites of pathogenic exposure is a key event in the process of inflammation (WAGNER; ROTH, 2000; COOK-MILLS; DEEM, 2005). Migration of leukocytes is initiated by the process of cell adhesion, followed by transmigration. In general, leukocytes extravasation is a multi-step process that involves the tethering, rolling and activation, firm adhesion to the endothelium, diapedesis and finally transendothelial migration (APLIN *et al*., 1998; SCHUBERT *et al*., 2011). In this work the migration cells decrease in fish exposed to diclofenac, include the polymorphonuclear count demonstrated a possible immunosuppression.

Nitric oxide is an important molecule involved in diverse physiological processes, including vasorelaxation, neuronal communications, inhibition of cell proliferation, and intracellular signaling. Nitric oxide also has potent toxic effects and, as such, is an important component of the arsenal available to animal hosts for effective antimicrobial defenses (RIEGER; BARREDA, 2011); so an imbalance in the NO defense mechanism could predispose the animal to infections.

In the present study, diclofenac decreased NO production in macrophage culture. Other study employing macrophage cell culture also demonstrated an inhibition in the NO production after incubation during 24h with 1μg/mL and 100μg/mL of diclofenac (CIRINO *et al.*, 1996). In the same study, Cirino *et al*. (1996) demonstrated that diclofenac directly inhibited iNOS activity, after induction by LPS-stimulus. Diclofenac (0.38 mM) also decreased activation of Factor Nuclear Kappa B

(NF-kB) in KBM5 cell (leukemic cell line) culture, and levels of iNOS in Raw 264.7 cells, which impaired NO activation in response to LPS (TAKADA *et al.*, 2004). In trout exposed to diclofenac, Mehinto *et al*. (2010) observed a decrease in gene expression of cyclooxygenases (COX 1 and COX 2) in kidney, leading to impairment of immune system.

The production of basal NO or NO LPS-induced by monocytic/macrophages of *H. malabaricus* can be affected by diclofenac, as demonstrated in the present work.

Diclofenac caused a reduction in liver size (HSI) at 0.2 µg/Kg. Exposure of rainbow trout to diclofenac caused a depletion of glycogen in hepatocytes (TRIEBSKORN *et al*., 2004), which could cause reduction of HSI. The hepatocyte growth can be affected by changes in the function of these cells when exposed to NSAIDs (FLIPPIN *et al*., 2007). In exposure for three months at concentrations of 1 to 10,000 µg/L of diclofenac, the fish species *Orizyas latipes*, and Japanese medaka, presented no change in liver somatic index (LEE *et al*., 2011).

Furthermore, diclofenac significantly increased SOD activity at 2 and 20 µg/Kg, GSH concentration at 20 µg/Kg and GPx activity in all doses in relation to the control group, suggesting the generation of free radicals, but not in a dose-dependent manner. There are not many studies of oxidative stress on fish caused by pharmaceuticals. Stepanova *et al.* (2013) observed an increasing of GST of juvenile carp exposed to high concentrations of diclofenac (3 mg/L). The GST activity of fish *Danio rerio* exposed to 40, 120 and 250 mg/L of acetylsalicylic acid also increased (ZIVNA *et al*., 2013). The GST provides cellular protection against toxic effects of a variety of xenobiotics (BARTOSKOVA *et al*., 2014). Dipyrone, other NSAID, diminished the GST activity in *Rhamdia quelen* sub-chronically exposed (PAMPLONA *et al*., 2011).

The superoxide dismutase is the first mechanism of antioxidant defense, converts O_2^- to H_2O_2 (VAN DER OOST *et al.*, 2003). Islas-Flores *et al.* (2013) reported SOD inhibition on brain of *Cyprinus carpio* exposed to diclofenac. In other study, *Cyprinus carpio* exposed to industrial effluent containing NSAIDs had an increase in SOD activity (SAN JUAN-REYES *et al*., 2013).

Glutathione peroxidase is an enzyme which transforms hydroperoxides to hydroxyl compounds using a reduced glutathione as a substrate. As well as diclofenac, the NSAID ibuprofen increased GPx in fish exposed to 0.05; 1; 8 and 25 mg/L (BARTOSKOVA *et al*., 2014).The present study data also showed a decrease in GSH levels in the same concentrations that GPx was diminished. GPx uses GSH as a

cofactor to remove the H_2O_2. Increased of levels of GSH is due to induction of antioxidant system. Several authors previously described the importance of GST, GPx, and GSH in preventing cellular damages (SILVA *et al*., 2011).

Lipid peroxidation has been a major contributor to the loss of cell function under oxidative stress. Diclofenac caused hepatic LPO in all groups exposed. Bivalves exposed to a concentration of 1 to 1000 µg/L diclofenac during 96 h, increased LPO (QUINN *et al.*, 2011) and amphipods *Hyalella azteca* suffered oxidative stress by increasing the LPO and SOD when exposed to artificial sediment containing diclofenac (OVIEDO-GÓMEZ *et al*., 2010).

5. Conclusion

This study highlighted that trophic exposure to diclofenac caused alterations in hematological parameters, migration cells, NO productions in kidney primary culture cell and in liver cause oxidative stress to *H. malabaricus*. These effects may cause negative impacts in aquatic organisms.

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CAPÍTULO III

EFEITO IMUNOSSUPRESSOR DO DICLOFENACO EM PEIXES NEOTROPICAIS

Immunosuppressive effects of diclofenac in a freshwater fish: a proteomic approach

Abstract

Drugs for human and veterinary use, such as anti-inflammatory drugs, have been frequently found in water bodies. In the present study, the effects of diclofenac in some components of the immune system of a tropical fish species were evaluated after hydric exposition. *Rhamdia quelen* fish (male and female) were exposed to 3 concentrations of diclofenac (0.2; 2.0 and 20.0 µg/L) for 14 days. After exposure, the fish were anesthetized, blood was taken from caudal vein and the anterior kidney was collected. Plasma and kidney proteins were analyzed using liquid chromatography tandem mass spectrometry in a shotgun proteomic approach to focusing on proteins involved in nitric oxide production, migration cell and complement system activation. In plasma of *R. quelen*, the expression toll like receptor 2 (Tlr2), phospholipase Cγ (Plcγ), kinase kinase kinase 3 (Mekk), 1-phosphatidylinositol 3-kinase (Pi3k), activator protein-1 (Ap-1), nuclear factor of Kappa light polypeptide (Nf-kb), and the NO synthase inducible protein (iNOS) were significantly inhibited by exposure to diclofenac. In the head kidney, the expression of Tlr2, Plcγ, Mekk, Pi3k, Ap1 and Nf-kb were also significantly inhibited. Various proteins involved in cell migration were detected in the plasma. In male fish, the expression of Chemokine receptor 4 protein (Cxcr4), Integrin α1 (ITα1), Radixin (Rdx) and Matrix Metallopeptidase (Mmp)-17 was inhibited. In female fish, the expression of Cxcr4, Itga1, Rdx, Mmp17 and Mmp1 decreased. In the present study, the expression of complement component 3 protein (C3), complement factor B (Cfb) and mannan-binding lectin serine peptidase 1 (Masp1) changed as well as C1q and complement component 7 (C7). In addition, MHC1 in plasma significantly decreased. In summary, the diclofenac exposure inhibited the expression of many proteins involved in NO synthesis, cell migration and activation of the complement system in fish which may compromise innate immune defense mechanisms in these animals.

Keywords: Emerging contaminants, diclofenac, migration cell, Nitric Oxide production, Complement system, immunosuppression

1. Introduction

The contamination of freshwater with a wide range of pollutants has become a matter of concern over the last few decades (ABOUD, 2010). Numerous environmental chemicals like pharmaceuticals drugs have been identified as emerging and persistent

toxicants. These chemicals are known by their potential to modulate immune system components with adverse consequences to individual's resistance (SEGNER, 2011). The suppression of immune system by these pollutants is not fully understood (ABOUD, 2010).

Studies have shown that the amount of pharmaceutical drugs found in water bodies has increased all the world, becoming a growing environmental problem and a major source of pollution (OVIEDO-GÓMEZ, 2010; KOLPIN, 2004) especially from urban, industrial and hospital wastewater (PAROLINI, 2009). These compounds have been identified as emerging contaminants in aquatic ecosystems (KIM *et al.*, 2007; ZUCCATO, 2006). The most frequently detected pharmaceuticals groups detected are the nonsteroidal anti-inflammatory drugs (NSAIDs), a class of pain relievers which blocks cyclooxygenase (COX) enzymes. The COX enzyme family catalyzes the synthesis of prostaglandins from arachidonic acid (KHETAN; COLLINS, 2007; VAN HECKEN *et al*., 2000). Some studies report that traces of NSAIDs in environment may elicit toxic effects, including immunological changes (KHETAN; COLLINS, 2007).

Diclofenac is a widely used NSAID all over the world, especially in chronic inflammatory conditions and is one of the most prevalent in aquatic environment (MYCEK, 2004). It has been identified as one of the most important pharmaceutically active compounds present in the aquatic environment (LETZEL; METZNER; LETZEL, 2009). Low levels of diclofenac (µg/L) have been detected in the influents and effluents of municipal sewage treatment plants and also in surface waters (ng/L - µg/L) (LETZEL; METZNER; LETZEL, 2009). Global concentrations of diclofenac in the water bodies range from 0.2 to 2.3 µg/L (BILA; DEZOTTI, 2003; HONG *et al*., 2007; WHO, 2012).

Despite these high levels of contamination, few studies have attempted to characterize the health risks associated with exposure to pharmaceutical products in water. Kumar and Xagoraraki (2010) showed a quantitative pharmaceutical risk assessment after accidental contamination of stream water by fish consumption and direct ingestion of finished drinking water in children and adults (KUMAR; XAGORARAKI, 2010). Results of their studies have identified none potential risk of adverse effects for human populations. However, fish are particularly sensitive to water contamination and pollutants including drugs for human and veterinary use, which may impair many physiological and immunological parameters (OAKES; VAN DER KRAAG, 2003; SILVA DE ASSIS *et al*., 2013a). In addition, it should be considered

that the fundamental immune molecules and mechanisms are similar in fish and mammals (RAUTA, 2012) and therefore, even though some adverse effects are not observed in humans, long-time exposure can also affect our species in the future.

In fish, nonspecific immunity is a fundamental defense mechanism (URIBE, 2011). The innate response has been considered an essential component in combating pathogens due to limitations of the adaptative immune system (WHYTE, 2007; URIBE, 2011). However, the intensity of this immunologic reaction has been shown to vary between different species and environmental conditions (WHYTE, 2007). Fish health depends on the interrelationship of some major components of the fish and the environment in which they live (KUM; SEKKIN, 2011). The environment may be the most critical component of the fish health especially because the environment and the pollutants may influence the fish's immune physiology and the ability to maintain natural and acquired resistance and immunity (PLUMB; HANSON, 2011).

Previous studies of our group have shown that fish exposed to diclofenac exhibited a reduction in NO production (RIBAS *et al*., 2014), circulating leukocytes and abnormal cell migration after challenge with carrageenan (data not published).

Thus, the aim of the present study was evaluate the effects of diclofenac exposure on the immune system of *Rhamdia quelen*, in proteins related to NO production, cell migration and complement system activation using a proteomic approach.

2. Material and methods

All procedures and protocols were approved by the Institution's Ethical Committee for Animal Use and are in accordance with international guidelines for Animal Use.

2.1 Bioassay

In the present study, exposure concentrations were selected because they are representative of concentrations detected in the aquatic environment (typical concentrations of diclofenac are 2.0 μg/L) (BILA; DEZOTTI, 2003; HONG *et al*., 2007).

Thus, a range of concentrations ten times lower and ten times higher (0.2, 2.0 and 20.0 µg/L) were selected for waterborne test exposures.

Rhamdia quelen fish were selected because exhibits a large ecological plasticity, with a wide distribution in Brazilian rivers and reservoirs (HENSLEY; MOODY, 1975). It is a omnivorous fish, endemic from South America and of economical importance in the Southern Brazil (BARCELLOS *et al*., 2001). The responses of this species after exposure to environmental pollutants has been studied for some authors (BECKER *et al*., 2009; DOS SANTOS MIRON *et al*., 2004; MELA *et al.*, 2013; MIRON *et al.*, 2005).

2.2 *Rhamdia quelen*

Rhamdia quelen weighing 80±10g were purchased from a Krahu Commercial Farm, Pomerode, Santa Catarina, Brazil. The fish were kept at 28±2°C in filtered water on a simulated natural photoperiod of 12-12 hlight:dark cycle (lights on at 0700 AM), and they were fed *ad libitum* with commercial feed once a day. The fish were acclimated to this environment for at least three weeks prior the use in experiments.

After the acclimation period, fish were divided into 4 experimental groups (0.2 µg/L, 2.0 µg/L and 20 µg/L of diclofenac plus one control group of male and female fish). One third of the water volume in each tank was replaced twice daily in order to maintain the diclofenac concentration. Male (n=20) and female (n=20) fish were exposed to each diclofenac concentration. After 14 days of exposure, the fish were anesthetized in water with benzocaine 1% (Sigma) and blood was taken from caudal vein. The fish were then euthanized by medullar section, and the anterior kidney was removed. All the samples were immediately frozen at -80ºC until analysis.

2.3 Proteomic analyses

Samples were prepared according to previously established methods (SIMMONS *et al*., 2012; SILVA DE ASSIS *et al*., 2013b). Head kidney tissue samples were added to microcentrifuge tubes and 10x the volume (v/w) of Triethylammonium bicarbonate buffer (TEAB) was added to each tube along with two 4mm stainless-steel balls. Tissues were homogenized using a ball mill (Retsch MM300) for 1 min at 20 Hz. Tubes were centrifuged for 15 min at 14,000 x *g* at 4 °C and the supernatant was

collected into low-density microcentrifuge tubes. Protein concentrations were estimated by light absorbance at 280 nm (Thermo Scientific Nanodrop 1000). Approximately 1mg of total protein of each kidney homogenate or 15 µL of plasma were transferred to a low-retention microcentrifuge tube after used for subsequent digestion.

All samples were then diluted with TEAB to a total volume of 50 µL, followed by the addition of 2.65 µL of 100 mM Tris(2-carboxyethyl)phosphine (TCEP) in TEAB to each tube, which were then vortex mixed, and left to incubate for 1 h to reduce disulfide bonds. To acetylate cysteine residues, 2.8 µL of 200 mM Iodoacetamide (IAA) in 50 mM Tris-HCl (pH = 7.8) was added to each tube, mixed by vortex, and then left to incubate for another hour. A 50 µL portion of 20% v/v formic acid was added to each tube, mixed thoroughly by vortex, and then left to digest on a heating block for 30 min at 115 °C. The resulting peptide mixtures in each tube were then evaporated to near dryness using centrifugal evaporation (Savant Instruments, Inc., model AES1010-120) for approximately 45 min and then resuspended by vortex in 20 µL of 5% acetonitrile, 95% water, and 0.1% formic acid. Finally, the tubes were centrifuged for 10 min at 15,000 x *g* to remove debris, and the supernatant was transferred from each tube into glass chromatography vials containing 200 µL polypropylene conical vial inserts for subsequent analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Peptides were separated on a Zorbax, 300SB-C18, 1.0 mm × 50 mm 3.5 µm column, with a thermostat controlled column temperature of 40 °C, using the Agilent 1260 Infinity Binary LC system. The Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) was used as the detector in tandem to the Agilent 1260 system. The pump timetable was the following: 0−2 min 2% solvent B, 2−22 min 2−40% solvent B, 22−27 min 40−60% solvent B, 27−32 min 90% solvent B, 32−50 min 5% solvent B. A blank, peptide standard and BSA digest standard injection was performed every 10 samples in order to monitor baseline, carry-over, drift, and sensitivity during the runtime. Reference mass correction was enabled using the dual-ESI reference nebulizer for m/z 121.051 and m/z 922.010.

Both centroid and profile mass spectral data files were collected using Mass Hunter Data Acquisition Software (Version B.02.00). Data files were pooled into groups by treatment, and database search was performed on each group separately. Spectrum Mill Software (Version A.03.03 SR4) was used to extract good quality

spectra and filter noise spectra and poor quality spectra from raw data files, sequence peptide sequences, and then search protein databases. All Spectrum Mill MS/MS search settings were left to default values except; spectra were searched using reversed database scores and dynamic peak thresholding, and a precursor mass tolerance of ± 20 ppm and product mass tolerance of ± 50 ppm. Mixed acetamide (C), oxidized methionine, pyroglutamic acid (NtermQ), deamidated (N), phosphorylated (S, T, and Y), and ubiquitination-GG (K) were specified as variable modifications. Proteins were searched using a subset database that contained only protein sequences of teleost fish species from within the NCBInr and Swissprot databases. Proteins were validated manually and accepted when matched to multiple members of a protein family or it had a summed score greater than five and a minimum of one peptide with a %SPI of greater than 60% (as recommended for Agilent Q-TOF data). Mean spectral intensity values were calculated by the Spectrum Mill Software and are defined as the sum of intensity for all spectra of peptides belonging to the protein, divided by the number of spectra. Peptide intensities were calculated from the sum of the precursor m/z abundance from the MS scans. The mean spectral intensity from all peptides detected for each protein was used as a relative quantitative measure of protein expression.

Valid protein matches were analyzed through the use of Ingenuity Pathways Analysis (IPA, 2013) Data was pooled for each treatment, and was then uploaded into the application using corresponding gene identifiers and expression values (mean peptide spectral intensity values). Each identifier was mapped to its corresponding molecule in Ingenuity's Knowledge Base. These network eligible molecules were overlaid onto a global molecular network developed from information contained in Ingenuity's Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Functional analysis identified the biological functions and/or diseases that were most significant to the data set based upon p-values obtained by the IPA software using the right-tailed fisher exact test and the Ingenuity Knowledge Base.

2.4 Statistical analysis

Data are presented as mean ± standard error mean (SEM) values for each condition. Kolmogorov-Smirnov normality test was used and comparisons among groups were done by Kruskal-Wallis test followed by Dunn's *post hoc* tests. The χ^2 test was performed to identify significant differences in the number of proteins that were significantly associated with a particular biological function compared to the total number of proteins among treatments. Differences of p-value < 0.05 were considered significant. The statistics tests were performed using GraphPad Prism version 5.00 for windows (Graph Pad Software, USA).

3. Results

3.1 Effects on NO production-related proteins

Since our previous results had shown that diclofenac exposure impairs LPS-induced NO production by macrophages from head kidney of fish, shotgun proteomics was employed in the present study to determine which proteins related to NO production could be affected by diclofenac exposure. Many of these proteins, particularly the kinases and the transcription factors are also involved in other processes. In the plasma of male fish exposed to diclofenac, expression of phospholipase Cγ (Plcγ), kinase kinase kinase 3 (Mekk), 1-phosphatidylinositol 3-kinase (Pi3k), and the NO synthase inducible protein (iNOS) were significantly reduced (Figure 1). Unexpectedly, in females, Plcγ and Mekk expression was not changed by the exposure to diclofenac (Figure 1). In contrast to the obtained results for females, the expression of the enzyme serine/threonine-protein kinase (Akt) was significantly increased in the male fish exposed to 20 µg/L of diclofenac (Figure 1) (Kruskal-wallis, followed by Dunn's post-hoc, α = 0.05).

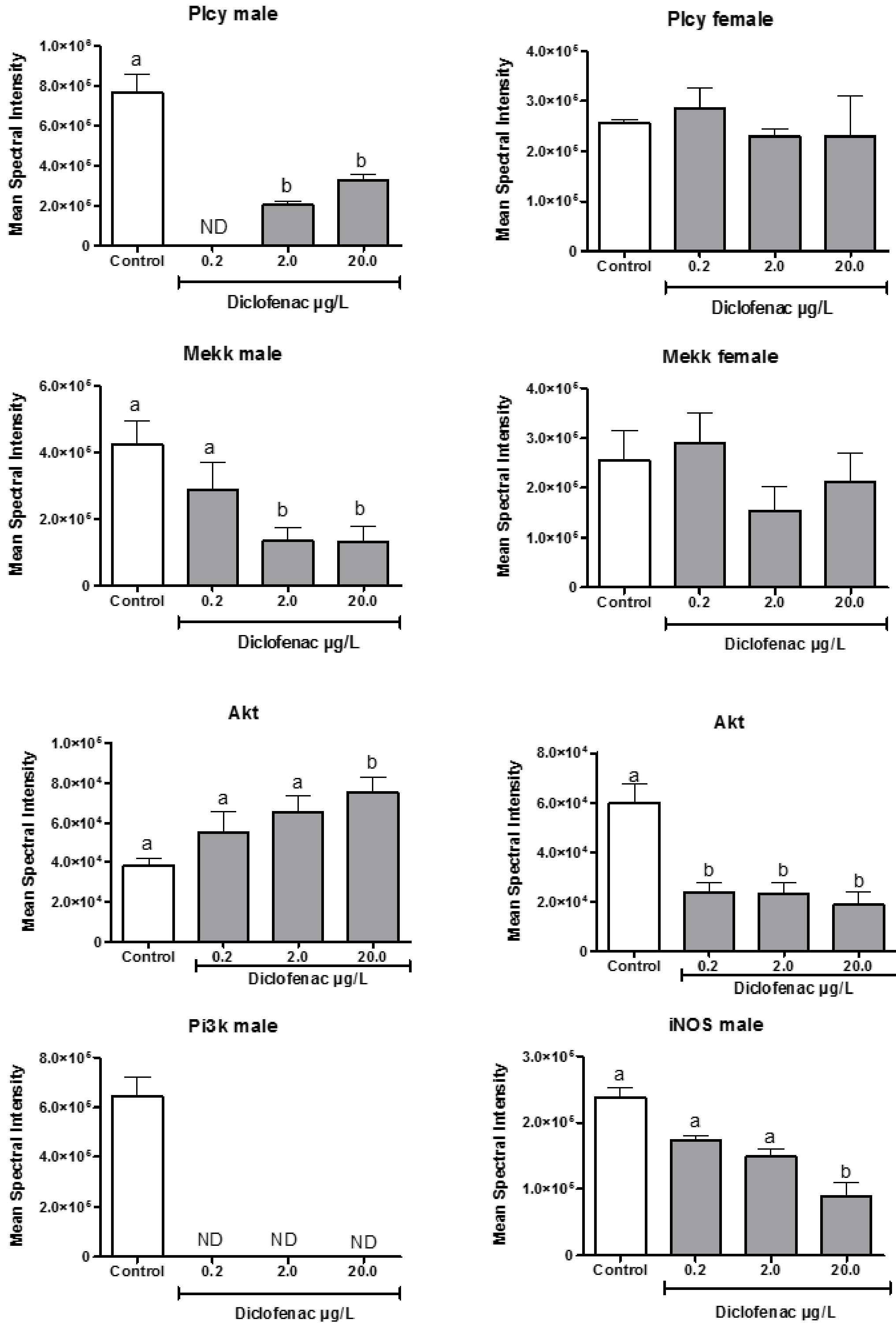

Figure 1 - EXPRESSION OF PROTEINS RELATED TO THE NITRIC OXIDE PRODUCTION IN PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Phospholipase Cγ (Plcγ, male and female), Protein Kinase Kinase Kinase (Mekk, male and female), serine/threonine protein kinase (Akt, male and female), 1-phosphatidylinositol 3-kinase (Pi3k, only in males) and Nitric Oxide Synthase (iNOS, only in males) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

Additionally, in male fish plasma, expression of the transcription factor nuclear factor κB light peptide (Nf-κb) was reduced only in the higher concentration of diclofenac while activator protein-1 (AP1) was roughly reduced in all exposure concentrations tested (Figure 2) (Kruskal-wallis, followed by Dunn's post-hoc, α = 0.05). In contrast with the enzymes showed previously, the reduction observed in the transcription factor Nf-κb in females was more severe than in males (Figure 2).

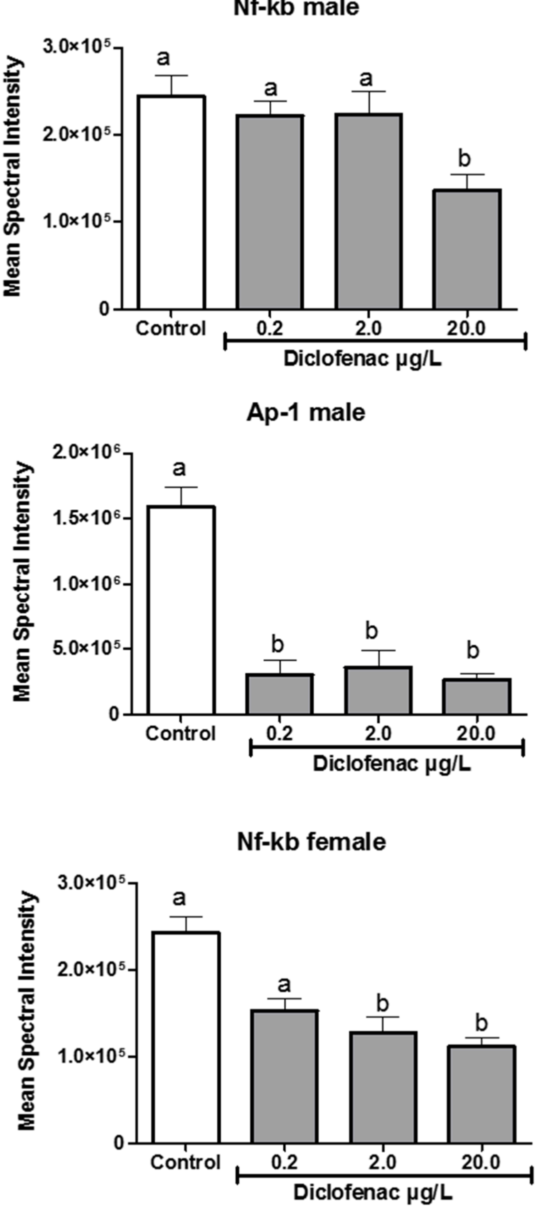

Figure 2 - EXPRESSION OF TRANSCRIPTION FACTORS RELATED TO NITRIC OXIDE PRODUCTION IN THE PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of nuclear factor κB light peptide (Nf-κb, males and females) and Activator protein-1 (males) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

In the head kidney of male *Rhamdia quelen* exposed to diclofenac, the expression of the Plcγ, Mekk, Pi3k were also significantly inhibited for all concentrations tested (Kruskal-wallis, followed by Dunn's post-hoc, α = 0.05) (Figure 3). Similar results were observed for female fishes concerning Plcγ and Pi3k (Figure 3).

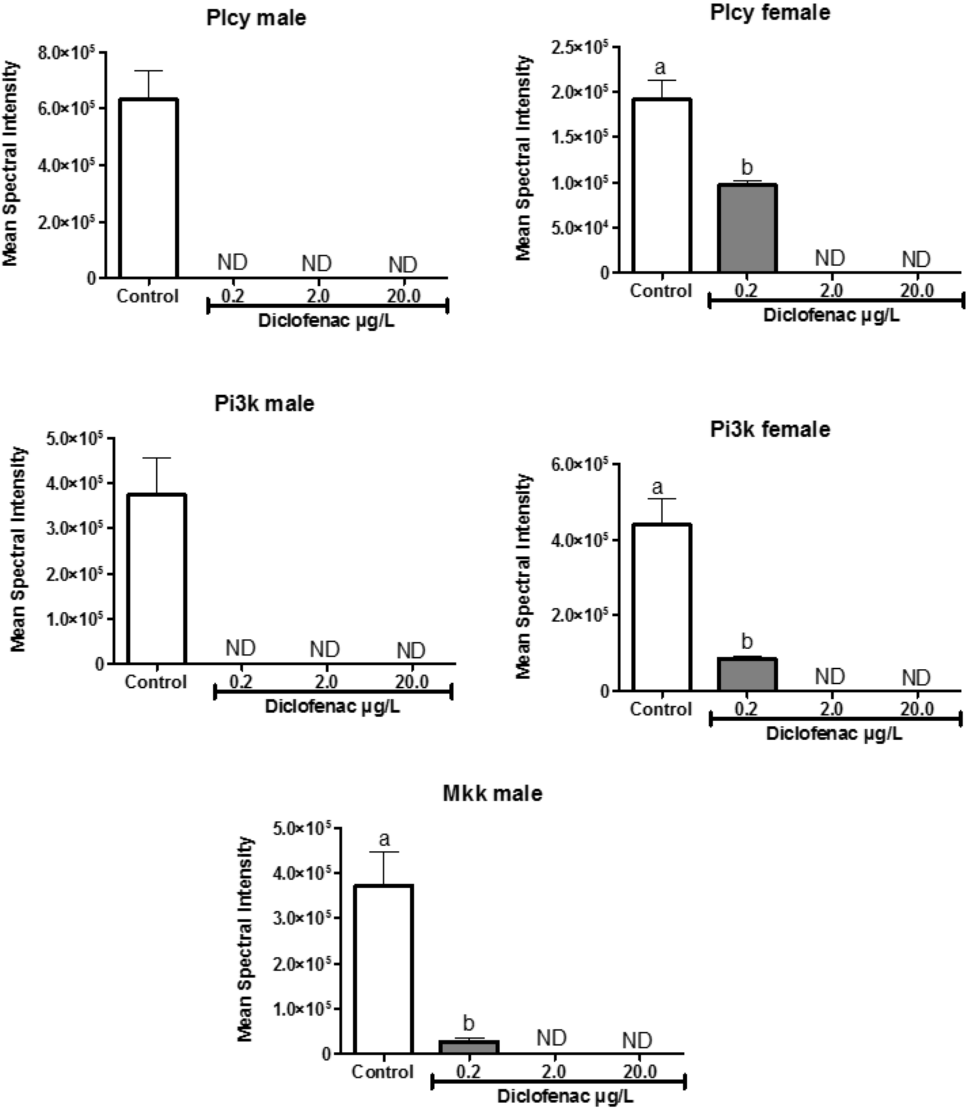

Figure 3 - EXPRESSION OF PROTEINS RELATED TO THE NITRIC OXIDE PRODUCTION IN THE HEAD KIDNEY OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Phospholipase Cγ (Plcγ, male and female), Protein Kinase Kinase Kinase (Mekk, only in male), and 1-phosphatidylinositol 3-kinase (Pi3k, in male and female) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

Similarly to plasma results, the expression of the transcription factor nuclear factor κB light peptide (Nf-κb) reduced at the higher concentration of diclofenac while activator protein-1 (Ap1) was reduced in all exposure concentrations tested (Figure 4). The reduction observed in the transcription factor Nf-κb in females was more intense than in males (Figure 4).

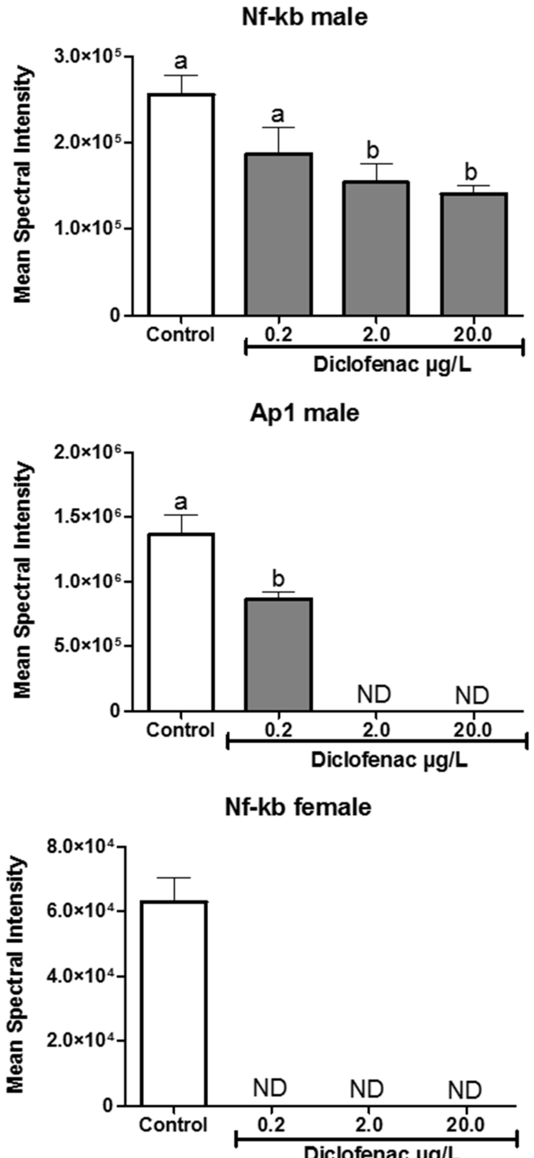

Figure 4 - EXPRESSION OF TRANSCRIPTION FACTORS RELATED TO NITRIC OXIDE PRODUCTION IN THE HEAD KIDNEY OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of nuclear factor κB light peptide (Nf-κb, males and females) and Activator protein-1 (Ap1 - males) is shown. Kruskal-Wallis test followed by Dunn’s post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

The activation of Plcγ and Pi3k is macrophages can be done through the activation of toll-like receptors (TLR). Interestingly we found that in female fish plasma, even though Plcγ was not altered as it was in males, Tlr2 expression was reduced in all doses tested (Figure 5). An even more intense reduction of this receptor expression was seen in the head kidney (Figure 5).

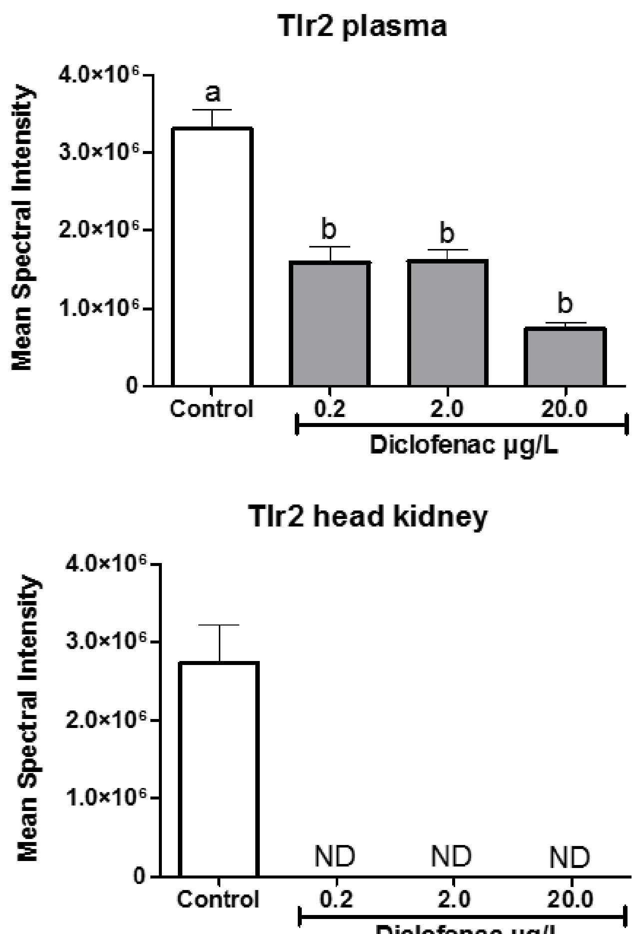

Figure 5 - EXPRESSION OF Tlr2 IN THE PLASMA AND HEAD KIDNEY OF FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Tlr2 is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

A visual pathway containing the proteins involved in NO production that were detected in the present study in male and female fish exposed to diclofenac is presented in Figure 6.

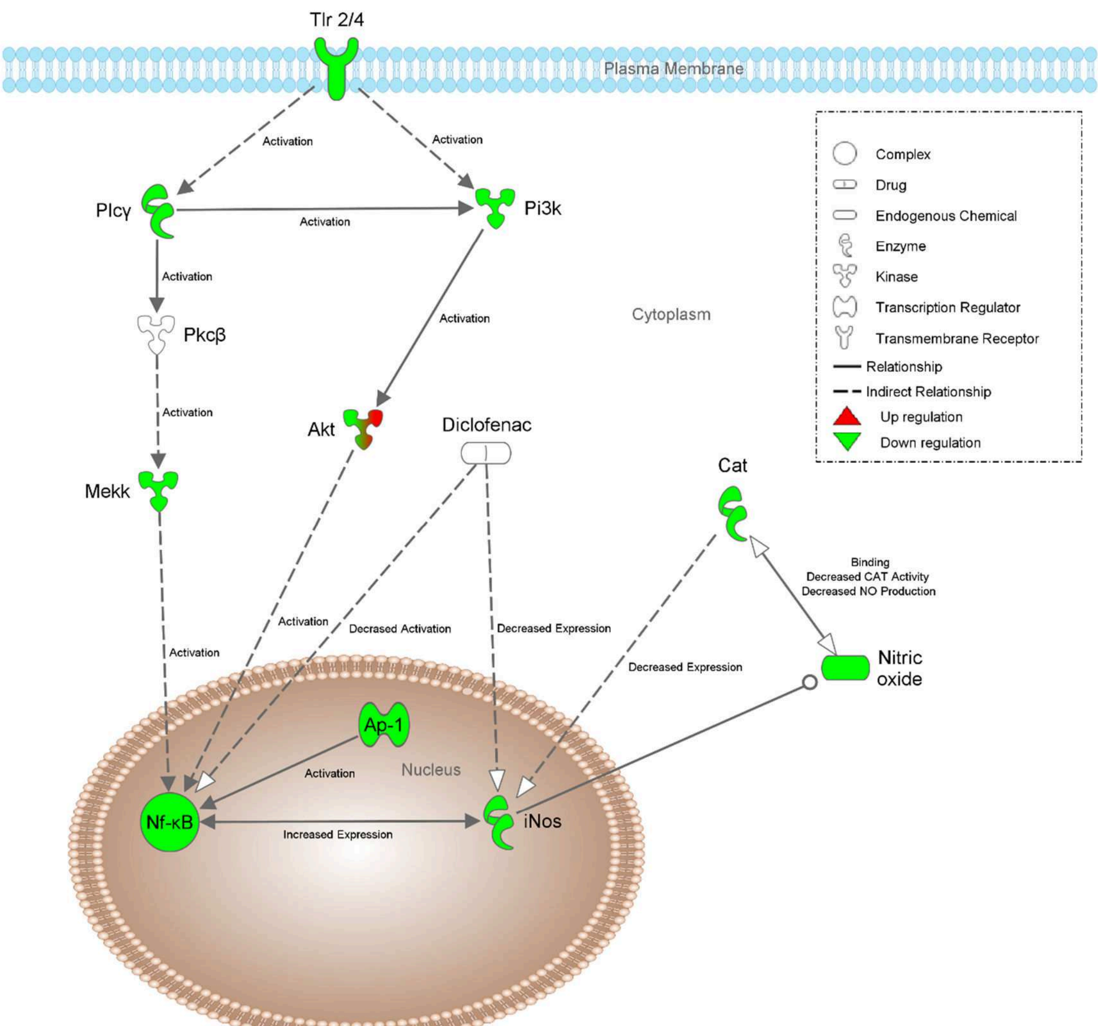

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Figure 6 - VISUAL PATHWAY OF THE PROTEINS INVOLVED IN THE NITRIC OXIDE PRODUCTION FOUND IN MALE AND FEMALE FISH PLASMA THAT ARE KNOWN TO INTERACT WITH OR BE AFFECTED BY DICLOFENAC. Arrows with dotted lines represent indirect associations and arrows with solid lines represent direct relationships. The direction of the arrow indicates which molecule is known to affect the other. Relationship lines that point to the same molecule from which they originate indicate a feedback mechanism. Relationship lines with a flat head instead of an arrow indicate a negative or inhibitory relationship. Legend: Tlr 2/4= Toll Like Receptor 2 and 4; Plcy = phospholipase C gamma; Pkcβ= Protein Kinase C Beta; Mekk = kinase kinase kinase 3; Ikb = Nuclear factor of Kappa light polypeptide inhibitor; Nf-kb = Nuclear factor of Kappa light polypeptide; iNOS = nitric oxide synthase inducible; Pik3 = 1-phosphatidylinositol 3-kinase; Akt = serine/threonine-protein kinase; Cbp=Creb binding protein; Ap-1= activator protein-1.This Figure was generated by the Ingenuity Knowledge Base (IPA, Ingenuity Systems).

3.2 Effects on cellular migration-related proteins

Among the various proteins involved in cellular migration, some proteins were found in plasma of *Rhamdia quelen* in the present study. In male fish exposed to diclofenac, the expression of Chemokine receptor 4 protein (Cxcr4) was inhibited at the higher concentration while the expression of Integrin alpha 1 (Itga1), Radixin (Rdx), and Matrix Metallopeptidase 17 (membrane-inserted, Mmp17) was inhibited was inhibited at all concentrations tested (Kruskal-Wallis test followed by Dunn's post hoc test, α = 0.05) (Figure 7). Matrix Metallopeptidase 1 (interstitial collagenase, Mmp1) was not significantly different in males after exposure to diclofenac (Kruskal-Wallis, followed by Dunn's post-hoc, α = 0.05) (Figure 7). Similar results were obtained in the plasma of female fish (Figure 7).

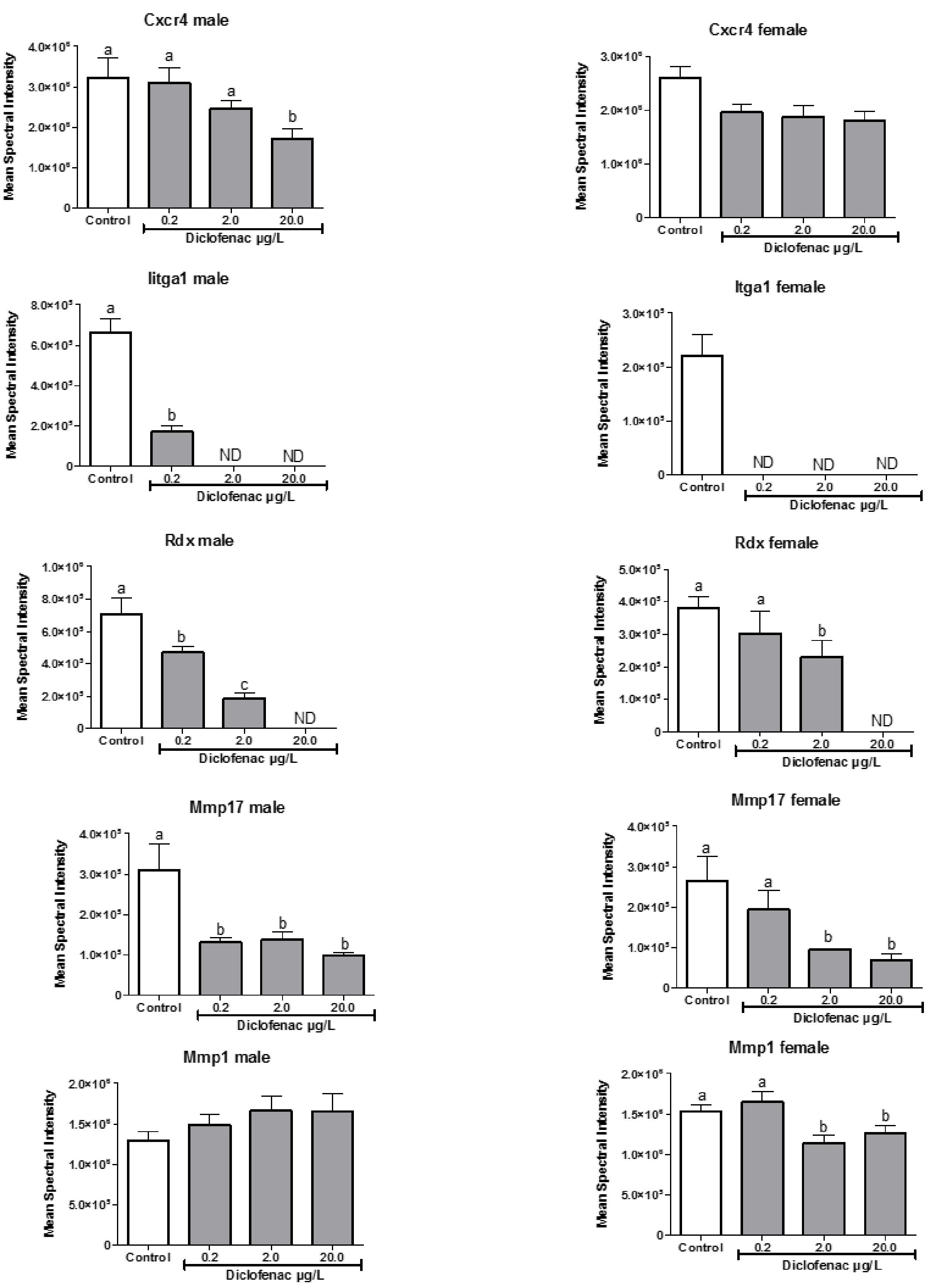

Figure 7 - EXPRESSION OF PROTEINS RELATED TO CELLULAR MIGRATION IN THE PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Chemokine receptor 4 protein (Cxcr4), Integrin alpha 1 (Itga1), Radixin (Rdx), Matrix Metallopeptidase 17 (Mmp17) and Matrix Metallopeptidase 1 (Mmp1) in both male and female fish is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

3.3 Effects on complement system-related proteins

The effects of diclofenac exposure on the complement system-related proteins were more variable and more complex than for the previous ones. In male fish plasma, while the complement component 1 (C1q) and the mannan-binding lectin serine peptidase 1 (Masp1) expression were reduced the expression of complement factor B (Cfb) was increased (Figure 8). The complement component 3 protein (C3) expression was unchanged (Figure 8). For the female fish the expression of C3, C1q and Cfb was reduced (Figure 8).

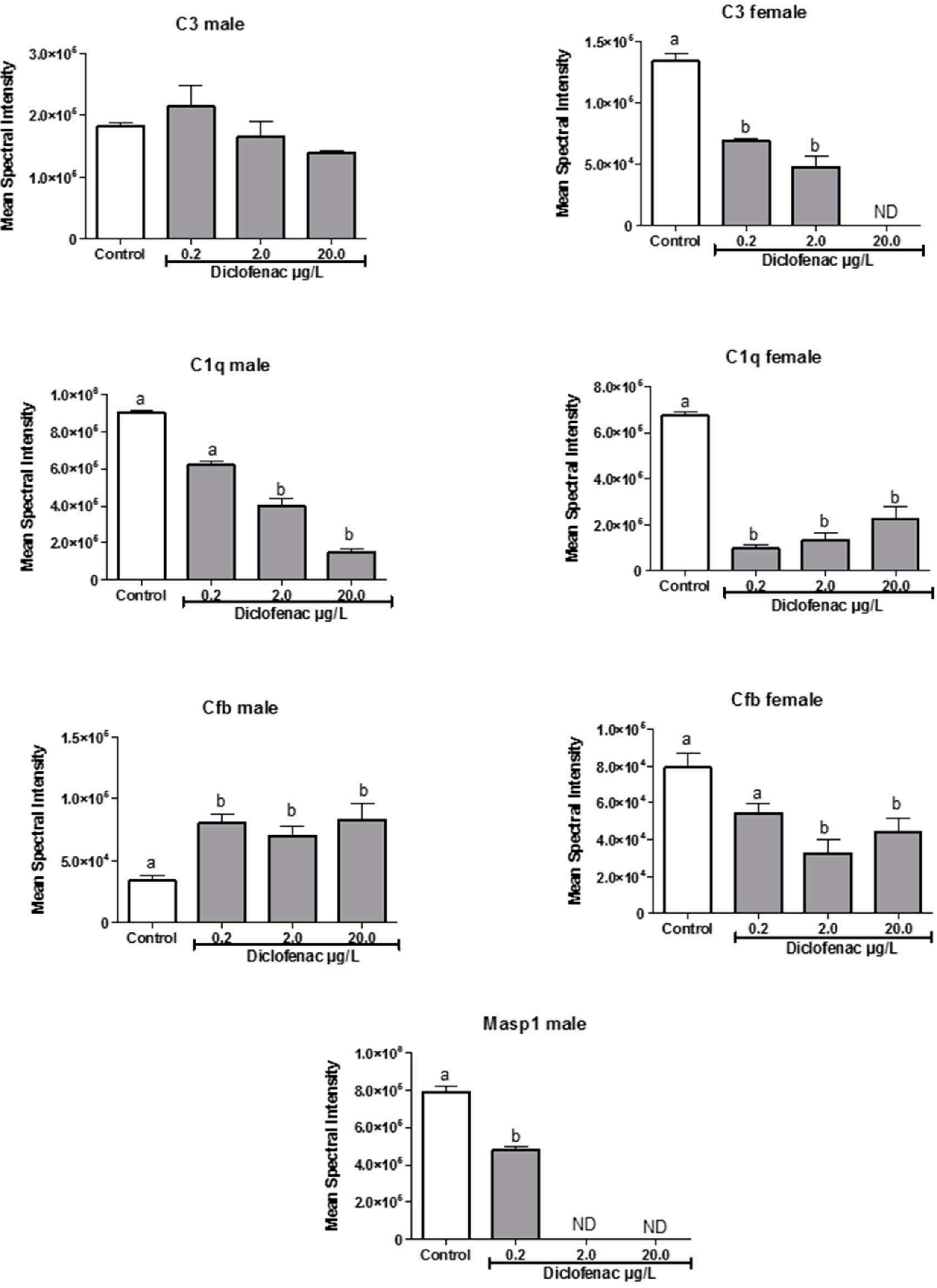

Figure 8 - EXPRESSION OF PROTEINS RELATED TO THE COMPLEMENT SYSTEM IN THE PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Complement component 3 protein (C3, male and female), Complement component 1 protein (C1q, male and female), Complement Factor B (male and female) and Mannan-Bilding Lectin Serine Peptidase 1 (Masp1, only in males) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

3.4 Effects on the Class I Major Histocompatibility complex

In both in male and female fish, the expression of MHC1 in plasma and kidney was significantly reduced in all concentrations of diclofenac tested (Figure 9) (Kruskal-Wallis, followed by Dunn's post-Hoc, α = 0.05).

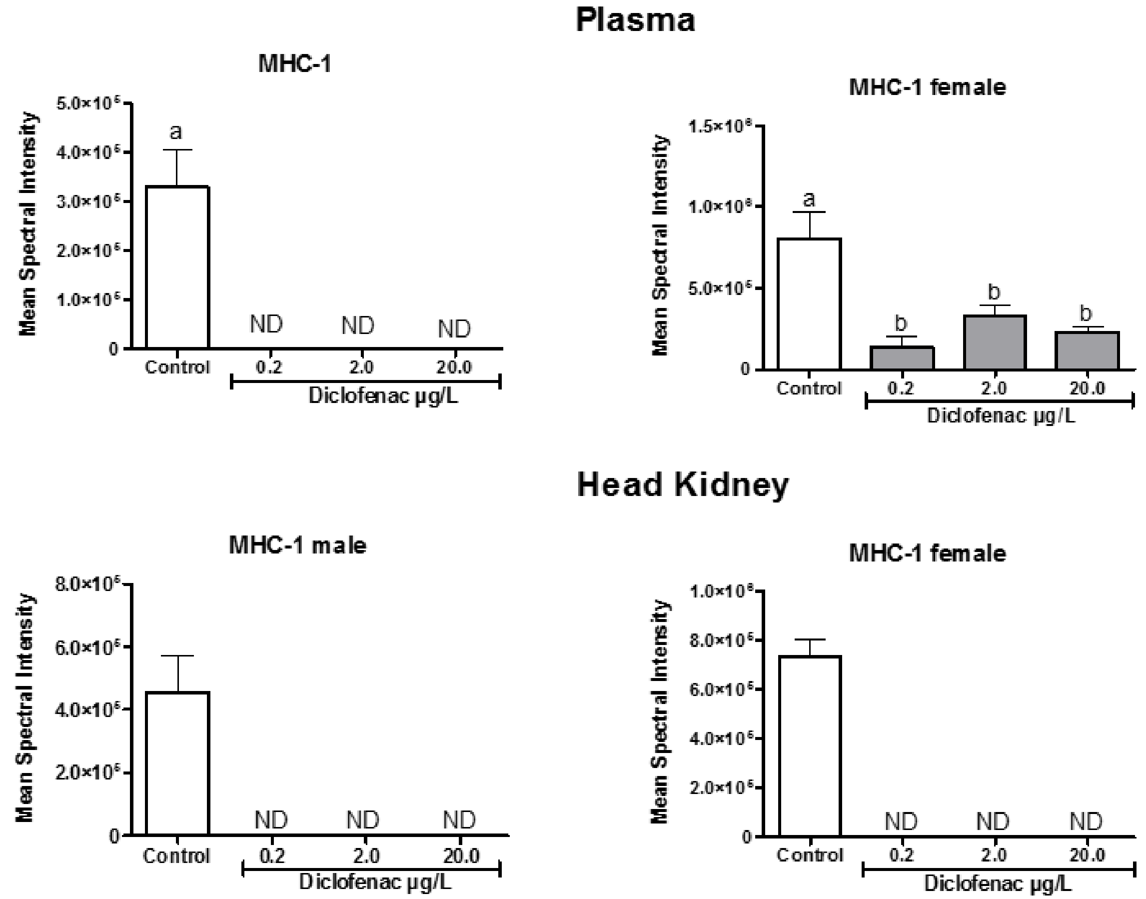

Figure 9 - EXPRESSION OF CLASS I MHC IN THE PLASMA AND KIDNEY OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Class I MHC is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

4. Discussion

4.1 Nitric oxide production

Nitric oxide is an important molecule involved in several physiological processes, including vasorelaxation, neuronal communication, inhibition of cell proliferation, and intracellular signaling. Nitric oxide also has potent toxic effects and,

as such, is an important component of the arsenal available to animal hosts for effective antimicrobial defenses (RIEGER; BARREDA, 2011). For this reason an imbalance in the NO defense mechanism could predispose the animal to infections. In our experiments, we found significant changes in the expression of proteins known to be involved in NO production after diclofenac exposure, especially a decrease in expression of several enzymes which are involved in the final steps of NO production.

The stimulation of Plcγ leads to increased of activity of the protein kinase Cβ, which increases activity of Mekk protein (ZHOU *et al*., 2006). Mekk phosphorylates the Ikk complex, which ultimately increases the phosphorylation of Ikb protein (LI; VERMA, 2002) and, as a consequence, activates Nf-kb (CHAN *et al*., 2001). Increased activity of the Nf-kb protein is thought to increase expression of several proteins including of iNOS protein, and therefore the NO production (MEMET, 2006; ZHOU *et al*., 2008). In an alternative pathway, the phosphorylation of Plcγ causes increased activation of phosphorylated Pi3k complex (CHAO, 2003; CHAO *et al.,* 2006) that increases activation of Akt protein (DARIEVA *et al*., 2004; BOULBES *et al.,* 2011).

In the present study we show that the exposure of male fishes to diclofenac inhibited the expression of Plcγ, even at lower doses and in both plasma and kidney, which could indirectly promote a decrease in Mekk phosphorylation and activity. This decrease could result in decreased Nf-kb activation and consequently reduced iNOS expression. However, diclofenac reduced not only this first component of the NO production mechanism. The expression of the following components such as mkk, Pi3k, Nfκb and Ap-1 were also reduced. Another protein apparently inhibited by diclofenac in the present study was Ap1 (activator protein 1). The expression of Ap1 is known to increase by Pi3k activation (PERON *et al*., 2001; KIM *et al*., 2012). The expression of Akt increased while the expression of Pi3k decreased. Although Pi3k is cited as the main regulator of Akt (CHEN *et al*., 2001), other studies relate that E-cadherin (MUNSHI *et al*., 2002) and Hb-egf protein (MEHTA; BESNER, 2005) can both increase Akt protein expression regardless of Pi3k regulation. In the present study, diclofenac affected Pi3k negatively, but it seems to affect positively other protein intermediaries, leading to an increased expression of Akt. Additionally, the activation of Akt is known to result in the increased activation of Nf-kb complex in nuclei and thus increased expression of iNOS (WRIGHT; WARD, 2000; HSU *et al*., 2007). Even though the Akt expression had augmented at the higher dose of diclofenac, the

addictive effect of all proteins reductions would not be compensated. These results are in accordance with our previous study (RIBAS *et al.*, 2014). In previous studies, diclofenac (0.38 mg/L) decreased activation of Nf-kb in cultured kbm5 cells (TAKADA *et al.*, 2004) and the levels of iNOS in raw 264.7 cells (mouse leukaemic monocyte macrophage cell line), impairing NO production in response to LPS (VILLALONGA *et al.,* 2010).

The changes observed in the plasma of male fishes were similar to that observed in the kidney being more evident in this organ for some protein such as Mkk and Nfκb. These results confirmed the effects of diclofenac in the doses used in this species. Interestingly, some proteins in plasma of female fishes were less affected by the exposure to diclofenac such as Plcγ and Mkk. However, even in plasma reduced levels of Nfκb and Akt were observed. In addition, an important reduction in kidney proteins in female fish was observed in a similar manner that in males. These results suggested that, even though some gender differences may exist, both male and female may have an impaired production of NO after exposure to diclofenac.

The Tlr2 protein, which is located in the plasma membrane of macrophage cells, is known to increase expression of Plc gamma (ZHOU *et al*., 2006) and the expression of Pi3k (LEE; PARK; KIM, 2008). In the present study, diclofenac inhibited the Tlr2 protein in female fish, which likely explains the observed decrease in PI3K expression.

A visual pathway containing the proteins involved in NO production that were detected in the present study in male and female fish exposed to diclofenac is presented in Figure 7.

4.2 Cellular migration

The inflammatory reaction enables an organism to defend itself against microbe infection. The migration of leukocytes from the vascular system to sites of pathogenic exposure is a key event in the inflammatory process (WAGNER; ROTH, 2000; COOK-MILLS; DEEM, 2005). Migration of leukocytes is initiated by the process of cell adhesion, followed by transmigration. In general, leukocytes extravasation is a multi-step process that involves the tethering, rolling and activation, firm adhesion to the endothelium, diapedesis and finally transendothelial migration (APLIN *et al*., 1998; SCHUBERT *et al*., 2011).

Recognition as well as contact formation is mediated by several cell adhesion molecules which act in a sequential manner and in concert with regulatory mediators such as the chemokines. In contrast to the rapidly flowing cells in the blood stream, rolling cells are able to sense signals from the endothelium, which stimulates them to adhere more firmly to the endothelial cell surface. Such signals are given by chemokines through receptors (Cxcrs) and G-proteins (DE FANIS *et al*., 2007; ZHU; PAUL, 2008). Decreased expression of Cxcr4 is linked with the decrease of recruitment of inflammatory cells (DALAKAS *et al*., 2005) and the migration of rolling cells (RUBIE *et al*., 2011). Cxcr4 is also involved in migration of macrophages (IMTIYAZ *et al*., 2010) and hematopoietic stem cells (DALAKAS *et al.*, 2005). In the present study, the inhibited expression of Cxcr4 by diclofenac could explain the decreased of migration cells when the migration was challenged by carrageenan.

These migration stimulatory effects caused the activation of a group of adhesion molecules called integrins, which in turn bind to members of the immunoglobulin superfamily on the endothelial cell surface. The major integrin complexes involved in this process are Leukocyte Function-Associated Antigen-1 Lfa1 (Itg-αL and Itg-β2) and Complement receptor 3 (Mac1) (Itg-αM and Itg-β2), which bind to members of the immunoglobulin superfamily such as Intercellular Cell Adhesion Molecule 1 (Icam1) and 2 (Icam2) and Vascular Cell Adhesion Molecule-1 (Vcam1) on the non-lymphoid endothelial cell surfaces. This caused tight adherence of granulocytes to the endothelium (KAIKO *et al*., 2008; ZHU *et al*., 2010). Integrin alpha 1 is linked with heterodimerization, decreased activity and uncapacity to complete the formation of the integrin complex (LEE *et al*., 2013). Cross-linking of integrins with Icams and Vcam1 activates the Erm (ezrin, radixin, moesin) proteins and recruits Cell Surface Antigen Thy1to the cell surface. This interaction enables binding of platelet endothelial cell adhesion molecule-1 (Pecam1) and also facilitates attachment of junctional adhesion proteins like Junctional Adhesion Molecule 2 (Jam2 and 3) with the granulocyte integrins (RAUTAJOKI *et al*., 2008).

In the present work, the expression of radixin decreased, that may have been caused by the incorrect activation of Erm proteins and the consequent binding of Pecam1 and attachment of junctional adhesion proteins (CARLONI *et al*., 2013; YANO *et al*., 2013; ZOU *et al*., 2013).

Following the migration process, the docking of granulocytes to the apical surface of endothelial cell triggers signals through generation of ROS and formation of stress fibers that further results in the activation of Mmps. Activated Mmps and ROS are responsible to degrade the assembly of junctional proteins like Vecam and other Cams, leading to the opening of inter-endothelial cell contacts, allowing granulocytes to transmigrate and reach the underlying tissue (OMENN *et al*., 2005). Among the proteins of the Matrix Metalloproteinase (Mmp) family involved in this migration process, in this study we observed a decrease in expression of Mmp1 and Mmp17. This decrease in Mmp1 and Mmp17 expression in plasma may represent impaired migration due to fewer breakdowns of extracellular matrix (NIKKARI *et al*., 1995; BUTTICE *et al*., 1996; YU *et al*., 2002; OH *et al*., 2013).

Even though we have analyzed some proteins such as Nfκb and Ap1 under the perspective of NO synthesis, those are transcription factors. There are studies which show that the expression of integrin α1, radixin and the MMP are induced during an inflammatory process (LUND; GIACHELLI; SCATENA, 2009; WANG *et al*., 2007; XU *et al*, 2010). Therefore, the reduction of the expression of the proteins involved in the cell migration may also be an indirect effect of the reduction in the expression of these transcription factors.

Therefore, our results clearly showed that the exposure to diclofenac can also reduce another important line of the hosts defense which is the leukocyte migration. No significant gender differences were observed.

4.3 Complement system

The complement system plays an essential role in alerting the host about the presence of potential pathogens. The activation of the complement system contributes significantly to the orchestration and development of immune acquired response (BOSHRA; LI; SUNYER, 2006). The complement system is composed of more than 35 soluble plasma proteins that play key roles in innate and adaptive immunity (GASQUE, 2004). It can be initiated by one pathway or a combination of three pathways: the alternative, lectin and classical. The classical pathway encompasses the formation of a complex between an antigen and an antibody (BOSHRA; LI; SUNYER, 2006). In the alternative pathway, the spontaneous activation of C3 is

amplified upon the covalent binding with various microbial surfaces (XU *et al*., 2001). Lectin activation requires the interaction of lectins such as mannose-binding lectin (MBL) and ficolins, with sugar moieties found on the surface of microbes (FUJITA *et al*., 2004).

The C1q protein is a major constituent of the complement subcomponent (SCHWAEBLE *et al*., 1995). Activation of the classical pathway is triggered by binding of the Fc portion of IgG with the C1q component of the C1 complex (HU *et al*., 2010). Also, C1q is described as an important protein in the regulation of phagocytosis (BOSHRA; LI; SUNYER, 2006). In the present study, both male and female fish exposed to diclofenac had decreased expression of C1q protein, which could predispose these fish to microbiological infection by crippling activation of the classical pathway in the complement system and de-regulating phagocytosis.

One way that the organism distinguishes self from non-self is the deposition of C3 on non-self molecules or structures. A particularly fascinating feature of C3 from fish is the fact that they show differences in their binding efficiencies to complement-activating surfaces (BOSHRA; LI; SUNYER, 2006). It has been hypothesized that this unique structural and functional C3 protein provides a mechanism for recognizing a broader range of microorganisms, thereby allowing fish to expand their innate immune recognition capabilities (SUNYER *et al*., 1998; BOSHRA; LI; SUNYER, 2006). In the present study, female, but not male, fish exposed to diclofenac had decreased plasma expression of the C3 protein, which could also compromise their ability to recognize pathogens.

The CFB protein is a component of the alternative pathway of complement activation. CFB circulates in the blood as a single chain polypeptide. Upon activation of the alternative pathway, it is cleaved by complement factor D, yielding the non-catalytic chain BA and the catalytic subunit BB. Subunit BB is a serine protease which associates with C3B to form the alternative pathway C3 convertase which can be fixed to the target surface. BB is involved specially in the proliferation of preactivated B lymphocytes (CLAIRE *et al*., 2002; ARUMUGAM *et al*., 2006). This illustrates the importance of CFB in the alternative pathway of defense (GONZALEZ *et al*., 2007). In the present work, male and female fish exposed to diclofenac had conflicting CFB protein responses (in male its expression increased and in female it decreased). Studies involving anti-inflammatory drugs and inhibition of the complement system are

rare and not extensive in fish. Previous studies with cloroquine, a well-known anti-malarial and anti-inflammatory agent demonstrated a decrease of CFB in plasma of *Plasmodium falciparum* infected patients (CHAKRABARTY *et al*., 1985). In the present study, the differences observed between males and females are probably associated with differences in hormones and reproductive cycle in the species.

The C7 protein is also a component of the complement system. It participates in the formation of the Membrane Attack Complex (Mac). In C7 deficiency, the ability to react to bacterial infection via the complement system is reduced (PODACK *et al*., 1980; NAKAO *et al*., 2011). In female fish, exposure to diclofenac resulted in decreased C7 protein in plasma.

The lectin pathway requires the interaction of lectins such as mannose-binding lectin (MBL) and ficolins with sugar moieties found on the surface of pathogens (FUJITA *et al*., 2004). Upon binding of the lectins to the microbial sugars, the enzymes associated with these lectins (MBL-associated serine proteases and MASPs) are activated (GAL; AMBRUS, 2001). There are three types of MASP molecules, Masp1, Masp2, and Masp3. Masp1 can activate C3 and C2 (HAJELA *et al*., 2002; MATSUSHITA; ENDO; FUJITA, 2013). Masp1 also has the capacity to increase activation of MASP2 (MATSUSHITA; ENDO; FUJITA, 2013). Masp2 has the ability to cleave C4 and C2 (CHEN; WALLIS, 2004). Masp3 is unable cleave C2, C3 or C4 and its role in the lectin pathway remains a mystery (BOSHRA; LI; SUNYER, 2006).

In the present work, only male fish exposed to diclofenac showed decreased expression of MASP1 protein. However, TAKAHASHI *et al.* (2008) found that a decreased expression of Masp1 resulted in significantly low activity of C4 and C3 deposition and significantly delayed activation of Masp2. Thus, the decrease of MASP1 in male fish plasma is likely to result in reduced resistance to infection. In fact, the reduction of major proteins associated with the complement system demonstrated in female fish exposed to diclofenac reveal that female fish are more susceptible to alterations in the complement system than male fish in this model, which could result in more severe effects in the immunity.

4.4 Class I Major Histocompatibility Complex

Other protein of interest in immune system is the Class I Major Histocompatibility complex (MHC1). The MHC1 protein is critical to the development of the cellular immune response. This protein has a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen (WANG *et al*., 2011). Inhibited expression of MHC1 has been associated with both susceptibility and decreased resistance to diseases, especially infectious disease (GREENE *et al*., 2011). This inhibition can also limit the size of the antigen-specific CD8 T cell response and Natural Killer cells stimulus (ORR *et al*., 2005). This strikingly importance of MHC1 for the immune response has leading to investigate also this protein. Diclofenac exposed greatly reduced the expression of MHC1 in both male and female fishes. Therefore, it is plausible that the whole immune response would be compromised by this exposure since the antigen presentation would be compromised.

5. Conclusion

In the present work, diclofenac exposure resulted in inhibited expression of many proteins involved in NO synthesis, cellular migration, complement system activation and antigen presentation. Some changes in protein expression were gender-specific. However, our results point to the same direction in both male and female fishes and suggest that the exposure to diclofenac at environmentally relevant concentrations can result in immunosuppression.

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4 DISCUSSÃO GERAL

Alguns estudos demonstram que a concentração de produtos farmacêuticos cresce cada vez mais na água, tornando-se uma preocupação emergente como contaminante do ecossistema aquático (ZUCCATO *et al.*, 2006; KHETAN; COLLINS, 2007).

Entre os fármacos encontrados no ecossistema aquático os AINEs como o diclofenaco, ibuprofeno e paracetamol são os mais prevalentes (ZUCCATO *et al.*, 2006; LETZEL; METZNER; LETZEL, 2009).

Por esses motivos, os fármacos escolhidos para esse estudo, no cultivo celular foram o ibuprofeno, o diclofenaco e o paracetamol, visando avaliar o efeito em cultura primária de rim anterior de *Hoplias malabaricus* e no prosseguimento, o diclofenaco em modelo trófico e hídrico. Sendo assim, esta tese destaca-se pelo caráter inovador e busca a obtenção dos primeiros resultados, visando correlacionar seus efeitos à saúde de organismos aquáticos envolvidos neste processo.

Os resultados do presente estudo demonstraram que peixes tanto da espécie *Hoplias malabaricus* quanto *Rhamdia quelen* expostos ao diclofenaco em situações experimentais específicas apresentaram alterações imunológicas importantes, levando a uma imunossupressão considerável com inibição de células e moléculas chaves à defesa do organismo. Nesse contexto, poucos trabalhos relatam sobre a imunologia de peixes, incluindo a imunossupressão, e um número menor ainda descreve essa imunossupressão associada a fármacos em concentrações reais encontradas no ambiente (ALVAREZ-PELLITERO, 2008; SECOMBES, 1994; WHYTE, 2007).

O presente trabalho teve como primeira fase a padronização da cultura primária de rim anterior de *Hoplias malabaricus*, seguido da exposição dessas células aos AINEs ibuprofeno, diclofenaco e paracetamol. No presente trabalho, foi observada uma redução significativa na produção de óxido nítrico basal e após estimulação por LPS em cultivo primário de macrófagos frente à exposição ao diclofenaco, ibuprofeno e paracetamol. Alguns autores já relataram a inibição da produção de óxido nítrico em células de peixes e de roedores expostos à antinflamatórios não-esteroidais (BOULARES *et al*., 2000; COMBS *et al*., 2000; LUNA-ACOSTA *et al.*, 2012; PAROLINI *et al*., 2009; TAKADA *et al*., 2004).

Devido à importância clínica, o volume de utilização e os resultados expressivos de inibição da produção de óxido nítrico, onde a mesma ocorreu especialmente nas menores concentrações testadas, o diclofenaco foi escolhido para prosseguimento dos estudos referentes ao possível mecanismo de ação desta inibição e à continuação das pesquisas relacionadas a essa imunossupressão inicial. Cabe ressaltar ainda que todas as concentrações de diclofenaco a que os organismos foram expostos são concentrações reais encontradas no ambiente, como já demonstrado nos capítulos anteriores.

Na segunda fase, dos estudos com o diclofenaco em *Hoplias malabaricus*, por via trófica, demonstrou-se claramente alterações nos biomarcadores bioquímicos testados como as enzimas referentes ao estresse oxidativo, nos parâmetros hematológicos com uma redução na contagem de leucócitos periféricos, assim como alterações importantes em células mono e polimorfonucleares e nos parâmetros imunológicos com as alterações observadas na migração celular estimulada por carragenina em peixes expostos.

Uma característica essencial na defesa do organismo que foi inibida pela exposição ao diclofenaco foi a contagem global de leucócitos em *Hoplias malabaricus*, também observada com a exposição de ratos ao diclofenaco por AL-SAADY (2011), provavelmente devido a formação de anticorpos dirigidos contra a medula óssea e precursores leucocitários (GUTTING *et al*., 2002). Os leucócitos são células de defesa e participam integralmente da resposta imunológica, além de atuarem especificamente em sua regulação (FERNANDEZ *et al*., 2002; SARAVANAN *et al*., 2012). Sendo assim, com sua redução significativa, o organismo pode ficar mais susceptível a infecções e alterações imunológicas, como a imunossupressão.

Nesses leucócitos globais, a fração linfocitária é responsável pela resposta imune específica, tanto celular como humoral, promovendo a produção de anticorpos e aumento da capacidade citotóxica, além de atuar promovendo o processo de memória imunológica e liberação de fatores regulatórios da função imune, como as linfocinas (FERNANDEZ *et al*., 2002; TIZARD, 2002). Os monócitos, por sua vez, são as células sanguíneas mais importantes na resposta imune, não sendo importantes apenas pela produção de citocinas, mas também de células primárias na apresentação de antígenos em teleósteos (FALCON, 2007). Nesse trabalho, em peixes *Hoplias malabaricus* expostos ao diclofenaco via trófica nas doses de 2,0 e 20,0 μg/Kg, verificou-se uma redução significativa nos linfócitos e monócitos

circulantes, representados pelos mononucleares, sugerindo uma redução expressiva no processo de defesa celular e humoral.

Os neutrófilos, ou polimorfonucleares, que nesse trabalho tiveram uma redução significativa em sua contagem e diferenciação, são as primeiras células envolvidas nos estágios iniciais do processo inflamatório em peixes e desempenham uma importante atividade microbicida (FALCON, 2007; FERNANDEZ *et al*., 2002). Estudos relatam que o diclofenaco é um dos principais fármacos que está associado à neutrofilia em pacientes que o utilizam de forma crônica, evidenciada através da exposição de ratos a esse fármaco (AL-SAADY, 2011; VAN DER KLAUW *et al*., 1998). A provável hipótese desta neutrofilia observada é o fato do diclofenaco induzir anticorpos contra neutrófilos periféricos e até mesmo contra seus precursores na medula óssea (AKAMITZU *et al*., 2001). Assim sendo, os dados reforçam a evidente imunossupressão dos peixes expostos ao diclofenaco.

Outro ponto a ser considerado no presente trabalho foi a inibição da migração celular desafiado por carragenina em peixes *Hoplias malabaricus* expostos ao diclofenaco.

A reação inflamatória, simulada pela carragenina, permite ao organismo a defesa contra a infecção. Um acontecimento chave nesse processo inflamatório é a migração leucocitária a partir do sistema vascular para os sítios expostos (COOK-MILLS; JULGUE, 2005). Todo esse processo se inicia pelo processo de sinalização, de adesão celular, seguido por transmigração. Esse processo é mediado por várias moléculas de adesão que atuam de forma sequencial e de acordo com mediadores regulamentares, como as quimiocinas (LEE *et al.*, 2013; RUBIE *et al.*, 2011; ZHU; PAUL, 2008).

No presente trabalho, tanto a migração desafiada por carragenina, quanto as proteínas envolvidas nesse estímulo e migração, se mostraram inibidas, sugerindo a incapacidade do organismo em reagir frente a um processo inflamatório desencadeado por um agente infeccioso.

Em uma terceira fase, foram realizados ensaios de proteômica na tentativa de se evidenciar os principais mecanismos de ação pelos quais o diclofenaco estava levando os organismos à imunossupressão. Para esses estudos de proteômica, foi utilizada a via hídrica no peixe *Rhamdia quelen*. Os peixes da espécie *Rhamdia quelen* foram utilizados em especial por já apresentarem descritos alguns genes e proteínas em estudos do próprio grupo e também pela facilidade fenotípica na diferenciação

sexual, possibilitando a separação clara e precisa para a exposição ao diclofenaco, retirando toda e qualquer influência hormonal que poderia ocorrer ao se ter sexos distintos. Outro motivo relevante foi a maior disponibilidade de exemplares.

Nos estudos de proteômica, foi observada uma alteração significativa nas proteínas envolvidas na defesa do organismo, representada pela inibição na produção de óxido nítrico e pela inibição de proteínas essenciais à defesa no sistema complemento, tanto na via clássica quanto na via alternativa e da MBL. Vale ressaltar que essas alterações ocorreram tanto perifericamente, encontradas no plasma, quanto diretamente no rim anterior.

Em termos de mecanismo de ação sobre a inibição na produção do óxido nítrico, várias proteínas participantes da cascata de reação foram inibidas, como já relatado em capítulos anteriores.

Outro ponto importante observado no presente trabalho foi a inibição significativa do sistema complemento. Ele reúne 35 proteínas séricas e de membrana, que interagem em uma sequência determinada, gerando funções efetoras de resposta imune, humoral e celular. O conjunto dessas proteínas são a chave de ligação entre o sistema imune inato e adquirido (HU *et al.*, 2010). A ativação do sistema complemento, que após desencadeada, progride de forma sequencial em uma cascata de reação, pode ser iniciada por 3 diferentes formas: (1) pela via clássica envolvendo a ligação do C1 com complexos imunes da circulação (Ag-Ab), (2) pela deposição espontânea do fator do complemento C3 e (3) pela lectina ligadora de manose (MBL) que liga resíduos de manose a outros carboidratos (HU *et al.*, 2010). Além disso, nos peixes, a atividade do sistema complemento, especialmente pela via alternativa, é intensa quando comparada ao dos mamíferos, sugerindo essa via como um mecanismo chave na defesa desses animais (ELLIS, 2001; LIN; SHIAU, 2005). A perda da sequência na cascata de reação leva a interrupção parcial ou total da reação do complemento (CLAIRE; HOLLAND; LAMBRIS, 2002).

Embora os peixes apresentem as proteínas do sistema complemento semelhantes aos mamíferos, algumas diferenças são cruciais. A principal delas é o fato dos peixes possuírem múltiplas isoformas, sugerindo um repertório mais expansivo no reconhecimento do sistema imune (CLAIRE; HOLLAND; LAMBRIS, 2002; NONAKA, 2001). Entendendo-se a função exata do sistema complemento em peixes e como cada proteína se comporta de forma individual, pode-se compreender como se dá o desenvolvimento de novas estratégias para manutenção da integridade

do organismo, especialmente em condições atípicas, como a exposição a poluentes aquáticos (CLAIRE; HOLLAND; LAMBRIS, 2002; BOSHRA; LI; SUNYER, 2006). Em peixes, muitos componentes estão envolvidos com a inicialização das reações do complemento, entre eles o C1q, fator B, C3, C2 e C4, além das MASP1 e MASP2 (HU *et al*., 2010; MATSUSHITA; ENDO; FUJITA, 2013).

No presente trabalho, a inibição da expressão das proteínas relacionadas com a inicialização do sistema complemento, tanto em machos quanto em fêmeas, tanto no plasma quanto no rim anterior, pode atuar impedindo que a cascata de reação se processe, levando a uma falha nos processos de defesa do organismo.

5 CONCLUSÃO E PERSPECTIVAS

A análise dos principais dados do presente estudo levaram às seguintes conclusões:

1. Com a padronização do cultivo de primário de rim anterior de *Hoplias malabaricus*, pôde-se averiguar que o Percoll é o melhor método para separação celular e que o melhor tempo para fazer as exposições foi 24 horas após o plaqueamento;

2. A produção de óxido nítrico basal e estimulado por LPS após exposição com diclofenaco, ibuprofeno e paracetamol reduziu significativamente, indicando uma imunossupressão nas células expostas a esses antinflamatórios

3. Os estudos genotóxicos após exposição das células oriundas do cultivo primário de rim anterior de *Hoplias malabaricus* ao diclofenaco,ibuprofeno e paracetamol demonstraram um potencial de dano elevado nessas células.

4. Após exposição trófica, análises no fígado de *Hoplias malabaricus* demonstraram aumento na atividade da SOD, GSH e da GPx, sugerindo a geração de radicais livres e a presença de estresse oxidativo. A LPO também foi induzida corroborando com os resultados anteriores. Além disso também foi observado a inibição do metabolismo de fase II pela inibição da atividade da GST.

5. *In vivo*, os indivíduos expostos ao diclofenaco via trófica demonstraram uma redução significativa na contagem global dos leucócitos circulantes, com alterações expressivas na diferenciação de mononucleares e polinucleares.

6. A migração celular pós-desafio com carragenina mostrou-se reduzida em indivíduos expostos via trófica ao diclofenaco, demonstrando a capacidade que o diclofenaco tem de reduzir a migração em peixes. Fato esse confirmado através da inibição da expressão de importantes proteínas relacionadas à migração celular.

7. Na avaliação global das proteínas relacionadas ao sistema imunológico, foi observada uma inibição significativa em proteínas-chaves no processo de defesa no plasma e rim anterior de *Rhamdia quelen* expostos ao diclofenaco. Dentre essas proteínas cabe ressaltar a inibição de proteínas responsáveis pela produção de óxido nítrico e envolvidas na defesa através do sistema complemento

8. Os estudos imunológicos realizados nesse trabalho demonstraram claramente uma imunossupressão ocasionada pelos antinflamatórios testados em especial o diclofenaco, em concentrações encontradas no ambiente como micropoluente emergente.

Pelos resultados obtidos ao longo desse trabalho, de fato o que mais fica explícito é a necessidade de uma legislação vigente que tente ao menos limitar a concentração de medicamentos presentes no ambiente. O investimento em estudos que demonstrem os reais efeitos dos fármacos em concentrações ambientalmente relevantes pode ser uma forma de conscientização e mudança do manejo até o descarte adequado dos medicamentos. É necessário, portanto, atenção especial para esse tópico além de constatar a eficácia de ações individuais ou coletivas sobre esse tema.

Talvez uma alternativa muito interessante seja a venda fracionada e exata da quantidade necessária do medicamento, evitando vencimentos e especialmente sobras que tendem a entrar no ambiente da forma mais sorrateira possível.

Cabe lembrar que o passivo ambiental considerado que é gerado pelo medicamento deve ser percebido não somente como um problema para o ambiente, mas para todos os seres vivos.

Enfim, a conscientização da sociedade pela educação ambiental na redução do descarte inadequado, juntamente com um novo olhar sobre a gestão do saneamento ambiental fazem, de fato, um trabalho como esse ganhar destaque e relevância frente às necessidades e desafios que a toxicologia ambiental. Uma pequena gota em um oceano, mas ainda assim uma pequena gota.

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CURITIBA

2014

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EFEITOS TOXICOLÓGICOS DE ANTINFLAMATÓRIOS NÃO ESTEROIDAIS EM PEIXES DE ÁGUA DOCE

Tese apresentada ao Programa de Pós-Graduação em Farmacologia, Setor Ciências Biológicas, Universidade Federal do Paraná.
Orientadora: Drª. Helena Cristina da Silva de Assis
Co-orientador: Dr. Aleksander Zampronio

CURITIBA
2014

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Programa de Pós-Graduação em Farmacologia

PARECER

A Comissão Examinadora da Tese de Doutorado intitulada "EFEITOS TOXICOLÓGICOS DE ANTINFLAMATÓRIOS NÃO ESTEROIDAIS EM PEIXES DE ÁGUA DOCE", de autoria do pós-graduando **JOÃO LUIZ COELHO RIBAS,** sob orientação da Prof.ª Dr.ª Helena Cristina da Silva de Assis e banca composta por: Prof.ª Dr.ª Helena Cristina da Silva de Assis (Presidente – Farmacologia – UFPR), Prof.ª Dr.ª Adriana Frohlich Mercadante (Patologia Básica – UFPR), Prof.ª Dr.ª Glória Emilia Petto de Souza (Física e Química – USP), Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR) e Prof.ª Dr.ª Solange Cristina Garcia (Análises – UFRGS), reuniu-se e de acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, o pós-graduando foi APROVADO. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas pela sua orientadora. Em Curitiba, 26 de agosto de 2014.

Helena C da Silva de Assis
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Às três mulheres da minha vida...

Lúcia, Melissa e Leticia

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“Só porque você dança bem, não significa
que vai ser convidado para o baile.”

Michael Leboeuf

RESUMO

Fármacos antinflamatórios não-esteroidais (AINEs) são amplamente empregados na medicina humana e veterinária e apresentam potencial de contaminar água e sedimentos através de entradas de estações de tratamento de esgoto. No presente estudo, os efeitos de alguns AINEs foram analisados em peixes nativos (*Hoplias malabaricus* e *Rhamdia quelen*). A toxicidade do paracetamol, do diclofenaco e do ibuprofeno foi avaliada em cultura primária da linhagem macrofágica de rim anterior de *H. malabaricus*. Seus efeitos na viabilidade celular, produção de óxido nítrico (NO) induzida por lipopolissacarídeo (LPS) e genotoxicidade foram analisados. Na cultura celular primária, para padronização, a análise por citometria $CD11b^{+}$ mostrou 71,5 % de células progenitoras, 19,5 % de macrófagos e 9,0 % de monócitos. A produção de óxido nítrico induzida por LPS por essas células foi bloqueada após tratamento com dexametasona e L-NMMA. Após 24h de exposição das células ao diclofenaco (0,2-200 ng/mL), paracetamol (0,025-250 ng/mL) e ibuprofeno (10-1000 ng/mL), houve redução na produção basal de NO e inibição da produção de NO induzida por LPS em todas as concentrações testadas. A genotoxicidade ocorreu na maior concentração de diclofenaco, nas concentrações intermediárias de paracetamol e também com ibuprofeno. A toxicidade trófica do diclofenaco em *H. malabaricus* foi avaliada, sendo os peixes alimentados duas vezes por semana com *Astyanax sp*. previamente submetido à inoculação intraperitoneal (IP) com diclofenaco (0; 0,2; 2,0 ou 20,0 µg/Kg), totalizando 12 doses. A metade dos peixes recebeu carragenina IP a 1 mg/Kg e depois de 4 horas, os mesmos foram anestesiados e eutanasiados para estimativa da migração celular. Nos outros peixes (sem carragenina), os parâmetros hematológicos, a produção de NO basal e após estimulação por LPS em rim anterior, o índice hepatossomático (HSI) e a análise hepática das atividades de superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa S-transferase (GST), etoxiresorufina-O-deetilase (EROD) e catalase (CAT) foram determinadas. A glutationa reduzida (GSH) e a lipoperoxidação (LPO) foram também avaliados. Houve aumento na contagem eritrocitária e no hematócrito na menor dose de diclofenaco. A hemoglobina diminuiu na maior dose. A contagem de trombócitos aumentou em todos os grupos expostos ao diclofenaco e a contagem de leucócitos sanguíneos totais diminuiu seguindo a redução de neutrófilos. Os monócitos reduziram na maior dose. O número de macrófagos peritoneais residentes não diferiu entre os grupos, mas a migração celular reduziu após a administração de carragenina, com uma significante diminuição na migração dos polimorfonucleares. A síntese basal de NO das culturas celulares de rim anterior dos animais tratados com diclofenaco foi significantemente menor nas células dos grupos de 2 e 20 µg/Kg. A produção de NO estimulada por LPS decresceu em todos os grupos tratados. No fígado, o diclofenaco causou estresse oxidativo com aumento de LPO e de atividade da GPx. Em contraste, a atividade da GST reduziu. Os efeitos do diclofenaco em componentes do sistema imune também foram avaliados após exposição hídrica de *R. quelen* ao diclofenaco a 0,2, 2,0 e 20,0 µg/L durante 14 dias. Os peixes foram anestesiados, o sangue retirado e após eutanasia o rim anterior foi coletado. As proteínas do plasma e rim anterior envolvidas na produção de NO, migração celular e ativação do sistema complemento foram analisadas por cromatografia líquida acoplada à espectrometria de massas do tipo *tandem*. No plasma foi observada a0 inibição da expressão de receptor toll like 2 (Tlr2), fosfolipase Cγ (Plcγ), quinase quinase quinase 3 (Mekk), 1-fosfatidilinositol 3-quinase (Pi3k), proteína ativadora-1 (Ap-1), fator nuclear de polipeptídeo kappa light (Nf-kb) e proteína NO sintase induzível (iNOS). No rim anterior, a expressão de Tlr2, Plcγ, Mekk,

Pi3k, Ap1 e Nf-kb também foi significantemente inibida. Várias proteínas envolvidas na migração celular foram detectadas no plasma. Nos peixes machos, a expressão da proteína receptora de quimiocina 4 (Cxcr4), Integrina α1 (Itα1), Radixina (Rdx) e Metalopeptidase de matriz (Mmp)-17 foi inibida. Nos peixes fêmeas, a expressão de Cxcr4, Itga1, Rdx, Mmp17 e Mmp1 reduziu. No presente estudo, houve modificação na expressão da proteína componente do complemento 3 (C3), do fator B do complemento (Cfb) e da serina peptidase 1 associada à manana (Masp1), bem como de C1q e do componente do complemento 7 (C7). Adicionalmente, MHC1 no plasma diminuiu significativamente. Em síntese, os NSAIDs estudados influenciaram a produção de NO e causaram danos ao DNA nas células monocíticas oriundas de *H. malabaricus*. Os peixes apresentaram modificações hematológicas e bioquímicas quando submetidos à exposição ao diclofenaco. A inibição da expressão de muitas proteínas envolvidas na síntese de NO, migração celular e ativação do sistema complemento foi observada nos peixes estudados, o que pode comprometer os mecanismos de defesa imune inata destes animais.

Palavras-chave: Fármacos. Cultura celular. Imunotoxicidade. Genotoxicidade. Macrófago. Estresse oxidativo. Migração celular. Parâmetros hematológicos.

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are largely employed in human and veterinary medicine and have the potential to contaminate water and sediments via inputs from sewage treatment plants. Their impacts on humans and ecosystems are emerging issues in environmental health. In the present study, the effects of some NSAIDs were analyzed in native fish species (*Hoplias malabaricus* and *Rhamdia quelen*). The toxicity of acetaminophen, diclofenac and ibuprofen was evaluated on primary culture of monocytic lineage of anterior kidney from *H. malabaricus*. Their effects in cell viability, lipopolysaccharide (LPS)-induced nitric oxid (NO) production and genotoxicity were analyzed. In primary culture cell, cytometry analysis $CD11b^+$ cells showed 71.5 % of stem cells, 19.5 % of macrophages and 9.0 % of monocytes. Cell viability was lower in the Ficoll compared to Percoll separation. LPS-induced NO production by these cells was blocked after treatment with dexamethasone and L-NMMA. After 24 h of cell exposure to diclofenac (0.2-200 ng/mL), acetaminophen (0.025-250 ng/mL) and ibuprofen (10-1000 ng/mL), there was a reduction in basal NO production and an inhibition of LPS-induced NO production at all tested concentrations. Genotoxicity occurred at the highest concentration of diclofenac, at the intermediary concentrations of acetaminophen and also with ibuprofen. The toxicity of diclofenac was also evaluated in *H. malabaricus* after trophic exposure, where fish were fed twice every week with *Astyanax sp*. previously submitted to intraperitoneal inoculation (IP) with diclofenac (0; 0.2; 2.0 or 20.0 µg/Kg), totaling 12 doses. In sequence, half of fish received 1 mg/Kg of carrageenan IP and after 4 hours, they were anesthetized and euthanized for cell migration estimation. In the other fish (without carrageenan), the hematological parameters, NO basal production and after LPS-stimulate in head kidney, hepatosomatic index (HSI) and liver biochemical analysis, such as activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), ethoxyresorufin-O-deethylase (EROD) and catalase (CAT) were measured. Reduced glutathione (GSH) and lipoperoxidation (LPO) were also determined. The results of trophic exposure of *H. malabaricus* showed increases in red blood cells count and in the hematocrit at the low dose of diclofenac. In contrast, the hemoglobin reduced at the highest dose. Thrombocyte count increased in all groups exposed to diclofenac and the total blood leukocyte counts decreased following the neutrophil's reduction. Monocytes decreased at the highest dose. The number of resident peritoneal cells did not differ among the groups, but the cell migration reduced after carrageenan administration, with a significant decrease in the migration of polymorphonuclear cells. The basal NO synthesis of anterior kidney cell cultures from diclofenac-treated animals was significantly lower in the cells from the groups 2 and 20 µg/Kg. The LPS-stimulated NO production reduced in all of the diclofenac-treated groups. Diclofenac also reduced HSI at the 0.2 µg/Kg. In liver, diclofenac caused oxidative stress with increased LPO and GPx activity. In contrast, GST activity decreased. The effects of diclofenac in components of the immune system were also evaluated after hydric exposure of *R. quelen* to diclofenac at 0.2, 2.0 and 20.0 µg/L during 14 days. After the exposure, fish were anesthetized and blood was taken from caudal vein. After this, fish were euthanized and the anterior kidney was collected. Plasma and kidney proteins involved in NO production, cell migration and complement system activation were analyzed using liquid chromatography tandem mass spectrometry in a shotgun proteomic approach. Results obtained after hydric exposure of *R. quelen* to diclofenac for plasma samples showed significant inhibition in the expression of toll like receptor 2 (Tlr2), phospholipase Cγ (Plcγ), kinase kinase

kinase 3 (Mekk), 1-phosphatidylinositol 3-kinase (Pi3k), activator protein-1 (Ap-1), nuclear factor of Kappa light polypeptide (Nf-kb), and the NO synthase inducible protein (iNOS). In the head kidney, the expression of Tlr2, Plcγ, Mekk, PI3K, Ap1 and Nf-kb was also significantly inhibited. Various proteins involved in cell migration were detected in the plasma. In male fish, the expression of Chemokine receptor 4 protein (Cxcr4), Integrin α1 (Itα1), Radixin (Rdx) and Matrix Metallopeptidase (Mmp)-17 was inhibited. In female fish, the expression of Cxcr4, Itga1, Rdx, Mmp17 and Mmp1 decreased. In the present study, the expression of complement component 3 protein (C3), complement factor B (Cfb) and mannan-binding lectin serine peptidase 1 (Masp1) changed as well as C1q and complement component 7 (C7). Additionally, MHC1 in plasma significantly decreased. In summary, the studied NSAIDs influenced NO production and caused DNA damage in monocytic cells from *H. malabaricus*. Fish presented hematological and biochemical changes when submitted to diclofenac exposure. The expression inhibition of many proteins involved in NO synthesis, cell migration and activation of the complement system was observed in the studied fish, which may compromise innate immune defense mechanisms of these animals.

Keywords: Pharmaceuticals. Cell culture. Immunotoxicity. Genotoxicity. Macrophage. Oxidative stress. Cell migration. Hematological parameters.

APRESENTAÇÃO

Esta tese se inicia com uma introdução, seguida de revisão bibliográfica e objetivos. A seguir, a mesma está apresentada na forma de três artigos científicos:

1. Effects of anti-inflammatory drugs in primary kidney cell culture of a freshwater fish
2. Effects of trophic exposure to diclofenac in freshwater fish
3. Immunosuppressive effects of diclofenac in a freshwater fish: a proteomic approach

Na sequência, os tópicos DISCUSSÃO GERAL E CONCLUSÕES E PERPECTIVAS apresentam comentários gerais a respeito dos resultados obtidos nos três artigos bem como as conclusões gerais da tese.

As listas de figuras, siglas, símbolos e abreviaturas e as referências referem-se ao conteúdo apresentado na introdução, revisão bibliográfica e discussão geral da tese, além dos artigos científicos.

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LISTA DE ABREVIATURAS, SÍMBOLOS E SIGLAS

AIEs	-	antinflamatórios esteroidais
AINEs	-	antinflamatórios não esteroidais
Akt	-	serine/threonine-protein kinase
Ap1	-	activator protein-1
C1q	-	complement component 1
C3	-	complement component 3 protein
C7	-	complement component 7
Cfb	-	complement factor b
Cg	-	carrageenan
COX	-	ciclo-oxigenase
Cxcr4	-	chemokine receptor 4 protein
Cxcrs	-	chemokines through receptors
FSC	-	forward scatter
ICAM	-	intercellular cell adhesion molecule
iNOS	-	inducible nitric oxide synthase
Itga1	-	integrin alpha 1
JAM	-	junctional adhesion molecule
LC-MS/MS	-	liquid chromatography tandem mass spectrometry
Lfa1	-	leukocyte function-associated antigen-1
L-NMMA	-	n^{g}-methyl-l-arginine
LPS	-	lipopolysaccharide
m/z	-	relaçãomassa/carga
Mac	-	membrane attack complex
Masp1	-	mannan-binding lectin serine peptidase 1
Mbl	-	mannose-binding lectin
Mekk	-	kinase kinase kinase 3
Mmp1	-	matrix metallopeptidase 1
Mmp17	-	matrix metallopeptidase 17
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nf-kb	-	nuclear factor of kappa light polypeptide
NO	-	nitric oxide

NSAIDs - non-steroidal anti-inflammatory drugs
PBS - phosphate-buffered saline
PECAM - platelet endothelial cell adhesion molecule
Pi3k - enzyme 1-phosphatidylinositol 3-kinase
PLC gamma - phospholipase c gamma
Q-TOF - accurate-mass quadrupole time-of-flight
Rdx - radixin
SSC - side scatter
TCEP - tris(2-carboxyethyl)phosphine
TEAB - triethylammonium bicarbonate buffer
Tlr - toll like receptor
Tlr2 - toll like receptor 2
TNF-α - fator de necrose tumoral α
VCAM - vascular cell adhesion molecule

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1. INTRODUÇÃO

Produtos químicos são depositados no ambiente devido a atividades humanas, resultando em deterioração do ambiente e consequentemente da biodiversidade (MACEDO-SOUZA *et al.*, 2009).

Os produtos químicos farmacêuticos são projetados para ter um modo específico de ação, e muitos deles para ter alguma persistência no organismo. Estas características os tornam um potencial alvo de pesquisas, especialmente por esses medicamentos poderem alcançar o ambiente por diversas vias, levando os animais e os seres humanos a uma exposição direta. Uma visão global e integrada do ciclo dos produtos farmacêuticos inclui não apenas as finalidades de drogas, mas também as suas consequências involuntárias para o meio ambiente e para o homem (FENT; WESTON; CAMINADA, 2006; MORGAN, 2001; RUHOY; DAUGHTON, 2008).

A ocorrência de produtos farmacêuticos no ambiente aquático é hoje em dia um problema bem estabelecido (GINEBREDA *et al.*, 2010), tornando-se uma questão de caráter científico e de preocupação pública, em busca de regulamentação para que as gerações futuras não sofram os males pela presença de produtos farmacêuticos no ambiente.Toneladas de produtos farmacêuticos das mais diferentes classes são depositadas no ambiente, após a sua utilização e excreção através das águas residuais e sistemas de tratamento de esgoto. Esses compostos são encontrados no ambiente aquático em concentrações que podem variar de ng/L a μg/L (CARBALLA; OMIL; LEMA, 2005; CHRISTEN *et al.*, 2010; GROS; PETROVIC; BARCELÓ, 2007; HEBERER; REDDERSEN; MECHLINSKI, 2002; JOSS *et al.*, 2005; KOLPIN *et al.*, 2004; OWEN *et al.,* 2009; SANTOS; APARICIO; ALONSO, 2007; SUÁREZ *et al.*, 2008; VIENO; TUHKANEN; KRONBERG, 2007).

A questão da toxicidade de produtos farmacêuticos e seus metabólitos no meio ambiente é algo que vem ganhando atenção, especialmente nos últimos anos, com real destaque sobre fármacos encontrados em esgotos ou efluentes de estações de tratamento de esgoto (KÜMMERER, 2009a,b), pois todos os produtos farmacêuticos liberados para o ambiente são considerados poluentes, uma vez que exercem efeitos biológicos (KHALAF, 2009).

Com a evolução das técnicas analíticas está sendo possível a identificação de níveis extremamente baixos de fármacos e seus metabólitos no ambiente aquático. Essas detecções remetem a estudos que tentam explicar qual o risco dessas águas à saúde e segurança do homem exposto a esses poluentes (BERCU *et al.*, 2008; GINEBREDA *et al.*, 2010; JONES; LESTER; VOULVOULIS, 2005; KOLPIN *et al.*, 2002). Contudo, a análise da ocorrência e toxicidade de todos os resíduos farmacêuticos é impraticável (KOSTICH; LAZORCHAK, 2008).

Este trabalho propôs estudar possíveis efeitos tóxicos de antinflamatórios encontrados no ambiente aquático e que poderia acarretar alterações bioquímicas, hematológicas e imunológicas. Sendo assim, nós propomos que, os antinflamatórios não esteroidais encontrados no ambiente aquático possam causar alterações na fisiologia normal no peixe, especialmente nas defesas antioxidantes e imunológicas. Especificamente o diclofenaco pode alterar tanto as funções bioquímica e hematológica, quanto a função imunológica. Dessa maneira propomos que a exposição tanto trófica quanto hídrica em espécies distintas de peixes possa apresentar tais resultados.

2. REVISÃO BIBLIOGRÁFICA

A ocorrência de fármacos de uso humano e veterinário vem sendo detectada em águas superficiais, sedimentos e esgotos domésticos no mundo todo. Embora muitos destes fármacos tenham sido submetidos a estudos toxicológicos especialmente utilizando peixes como modelo, informações sobre o seu destino no ambiente e seus riscos e efeitos tóxicos aos organismos aquáticos não são totalmente elucidadas ou compreendidas (AUKIDY; VERLICCHI; VOULVOULIS, 2014; CARMONA; ANDREU; PICÓ, 2014; CORCOLL *et al.*, 2014; FENT; WESTON; CAMINADA, 2006; GALUS *et al.,* 2013; ONESIOS; BOUWER, 2012; ORIAS; PERRODIN, 2013; PAL *et al.*, 2014; SANTOS *et al.*, 2013; STUMPF *et al.*, 1999; TANOUE *et al.*, 2014; YING *et al.* ,2013).

Quimicamente, os compostos ativos contidos em um medicamento são frequentemente formados por moléculas complexas com propriedades especiais, com diferentes funcionalidades e propriedades físico-químicas e biológicas. A maioria desses compostos tem características polares (KÜMMERER, 2009a), e são

frequentemente chamados de “micropoluentes” (COMEAU *et al.*, 2008; KÜMMERER, 2009b; MORLEY, 2009; SCHLÜTER *et al.*, 2007).

Além das substâncias ativas, as formulações farmacêuticas podem incorporar adjuvantes, a exemplo de pigmentos e corantes, e também podem acarretar, em menor grau, impactos sobre o meio ambiente (KOCH *et al.*, 2005).

As características dos produtos farmacêuticos podem ser ácidas, alcalinas ou anfotéricas. Em termos ambientais, as moléculas podem assumir condições catiônicas, aniônicas, anfóteras ou de neutralidade, assumindo assim comportamentos ambientais complexos, o que pode gerar efeitos ambientais diferenciados (KÜMMERER, 2009a).

Poucos dados disponíveis existem sobre a real utilização mundial de produtos farmacêuticos, inclusive sobre o seu consumo e aplicação, que podem variar consideravelmente de um país para outro (GOOSSENS *et al*., 2005, 2007; SCHUSTER; HÄDRICH; KÜMMERER, 2008). Sabe-se, no entanto, que o mercado de produtos farmacêuticos está em franco desenvolvimento, sendo que na União Européia, por exemplo, são catalogadas mais de 3000 substâncias diferentes que são utilizadas na medicina humana. Em relação ao mercado de fármacos, o Brasil é um dos maiores consumidores do mundo, juntamente com Estados Unidos, França e Alemanha (CHRISTEN *et al*., 2010; FENT; WESTON; CAMINADA, 2006; STUMPF *et al*., 1999).

Também se estima, segundo a Organização Mundial de Saúde (OMS), que aproximadamente 80% dos medicamentos produzidos no mundo são consumidos pela população de países ricos, enquanto que no Brasil, a classe mais favorecida economicamente é responsável por 48% do consumo de fármacos. Estima-se também que em países em desenvolvimento como o Brasil, cerca de 30% dos recursos em saúde são destinados à aquisição de medicamentos demonstrando que o medicamento é tido hoje como elemento de primeira ordem (OLIVEIRA; XAVIER; ARAÚJO, 2012).

Como esperado, produtos farmacêuticos estão presentes em maior concentração em efluentes hospitalares em relação ao esgoto municipal (KÜMMERER, 2009a; SCHUSTER; HÄDRICH; KÜMMERER, 2008).

Quanto à eliminação caseira de medicamentos, muitas vezes as sobras são eliminadas no lixo doméstico ou descartadas no ralo, em pias ou banheiros, o que pode também ser uma rota importante de eliminação de fármacos, o que requer

maior atenção, especialmente ambiental (AN *et al.*, 2009; BOUND; VOULVOULIS, 2005; COMEAU *et al.*, 2008; GOTZ; KEIL,2007).

Se os medicamentos forem descartados em pias ou banheiros, estes vão diretamente para as estações de tratamento de esgoto. No entanto, se eles forem descartados no lixo doméstico, os compostos vão acabar em aterro e também podem chegar aos efluentes (AHEL; JELICIC, 2001; METZGER, 2004), sendo uma fonte de contaminação de águas superficiais ou subterrâneas (COMEAU *et al.*, 2008, KÜMMERER, 2009a). As possíveis rotas de fármacos no meio ambiente estão ilustrada na Figura 1.

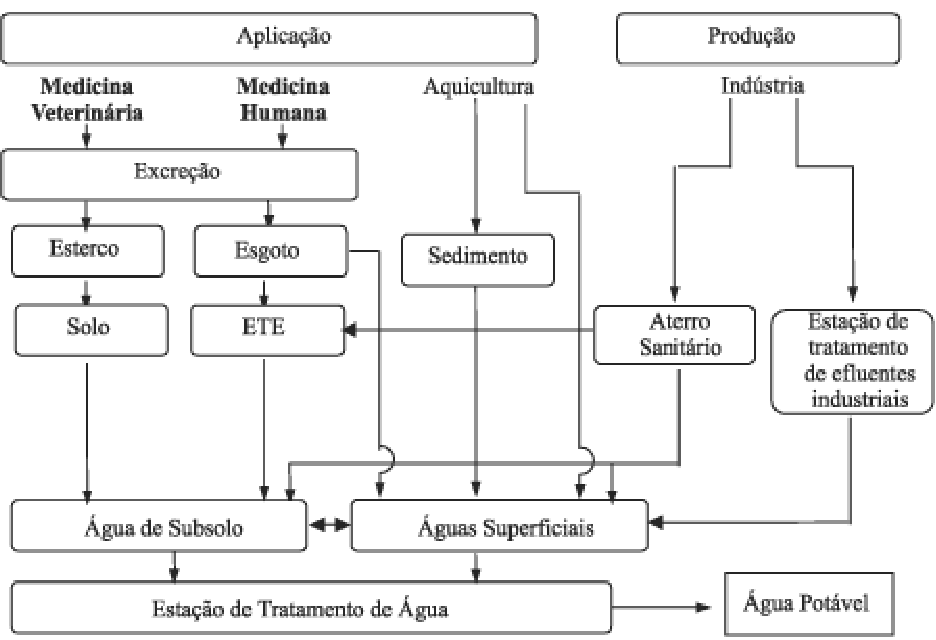

FIGURA 1 - POSSÍVEIS ROTAS DE FÁRMACOS NO MEIO AMBIENTE
FONTE: BILA; DEZOTTI, 2003. ETE – Estação de tratamento de esgoto.

Estudos reportam a presença de mais de 90 tipos de drogas diferentes no ambiente aquático, incluindo os seus metabólitos ativos e inativos, especialmente encontrados em águas superficiais, na União Européia, Estados Unidos, Brasil e Canadá (COMEAU *et al.*, 2008; POMATI *et al.*, 2007).

Devido aos medicamentos e seus metabólitos não serem eliminados durante o tratamento do esgoto (BUERGE *et al.*, 2006; KÜMMERER, 2009a; VERPLANCK *et al.*, 2005), estudos realizados até o momento descrevem a ocorrência desses compostos em efluentes de unidades de esgotos e de estações de tratamento, em

águas superficiais, subterrâneas e até mesmo na água potável, tais como a carbamazepina, o fenobarbital e o diclofenaco (COMEAU *et al.*, 2008; HEBERER, 2002; KALLENBORN *et al.*, 2008; KUMAR; XAGORARAKI, 2010; MORLEY, 2009;STÜLTEN *et al.*, 2008). No entanto, pouco se sabe sobre os efeitos ecotoxicológicos de medicamentos em animais. Especialmente importantes são os organismos aquáticos, que são expostos a águas com resíduos durante toda a sua vida, o que pode afetá-los durante todas as fases de seu desenvolvimento (FENT; WESTON; CAMINADA, 2006; POMATI *et al.*, 2007).

O risco de efeitos adversos em seres humanos através da ingestão de produtos farmacêuticos presentes na água potável parece ser insignificante. Assim, os resíduos de produtos farmacêuticos no ambiente parecem ser mais uma preocupação ambiental do que necessariamente humana (CHRISTENSEN, 1998, KÜMMERER, 2009a). Porém há escassa informação sobre a toxicidade crônica ou o potencial de bioacumulação de produtos farmacêuticos na biota e na cadeia alimentar (CHRISTEN *et al.*, 2010; OAKS *et al.*, 2004).

No entanto, idosos ao administrarem concomitantemente vários medicamentos diferentes já sofrem, frequentemente, os efeitos colaterais destes durante o tratamento. Somado a isso, ao receberem medicação via água potável, podem originar ou mesmo agravar os efeitos colaterais já ocasionados. Infelizmente, dados a este respeito são escassos e os poucos trabalhos nessa área relatam a avaliação de uma substância isoladamente e não de uma mistura, o que é a realidade no ambiente (KÜMMERER, 2009a).

Estudos verificaram que os efeitos negativos dos produtos farmacêuticos contidos no ecossistema aquático podem ser transferidos no interior da cadeia alimentar (CHRISTEN *et al.*, 2010; OAKS *et al.*, 2004). Esses estudos relatam que existe uma correlação direta entre resíduos contendo diclofenaco e insuficiência renal e visceral, o que pode implicar fortemente em mortalidade causada pela ingestão de resíduos de drogas antinflamatórias não esteróides. Outros medicamentos associados à transferência trófica podem ser os antibióticos (SWAN, *et al.*, 2006; TAGGART *et al.*, 2007).

Um fator adicional da toxicidade de um composto farmacêutico no ambiente aquático é a sua persistência. Uma vez liberado no ambiente, o composto é transportado e distribuído para as águas superficiais, sedimentos e biota. As ações e concentrações em cada um destes compartimentos são determinadas por uma

série de fatores e processos, incluindo a concentração da droga e suas propriedades físico-químicas, a separação de sedimentos, a degradação, as características ambientais e as condições climáticas do habitat. A degradação das substâncias pode ocorrer de forma biótica por organismos aeróbios ou anaeróbios, ou de forma abiótica via fotodegradação e/ou hidrólise (BOXALL *et al*., 2004; MORLEY, 2009).

Outro problema relativo a esses compostos é que muitos deles, especialmente os fármacos pouco solúveis, têm um potencial muito grande de bioacumulação. Isso representa um risco ainda maior para a saúde dos organismos aquáticos e da população humana em geral (CHRISTEN *et al.*, 2010; CRANE; WATTS; BOUCARD, 2006; DELÉPÉE; POULIQUEN; LE BRIS, 2004; LUNESTAD, 1992; MIGLIORE *et al*., 1993).

Apesar da existência de uma ampla variedade de classes de fármacos que são usados na terapêutica humana e veterinária, apenas alguns são considerados de real importância para o meio ambiente e para a saúde pública, por causa de seus volumes de consumo, toxicidade e persistência no ambiente. Entre esses, destacam-se os beta-bloqueadores, os quimioterápicos, os hormônios esteróides, os compostos neurológicos, os antiparasitários, os hipolipemiantes, os antibióticos e especialmente os analgésicos e antinflamatórios (CHRISTEN *et al.*, 2010; FENT; WESTON; CAMINADA, 2006; HERNÁNDEZ *et al*., 2007; MORLEY, 2009).

2.1 ANTINFLAMATÓRIOS

A classe dos antinflamatórios pode ser dividida em dois grupos: os antinflamatórios não esteroidais (AINEs) e os antinflamatórios esteroidais (AIEs).

Os AINEs são medicamentos amplamente utilizados e, consequentemente, são frequentemente detectados em esgotos e águas superficiais (GOMEZ *et al*.,2007; MORLEY, 2009). Muitos AINEs, tais como ibuprofeno, diclofenaco e paracetamol (acetaminofeno), são encontrados em corpos d’água, além do ácido salicílico (derivado do ácido acetilsalicílico) e naproxeno (FENT; WESTON; CAMINADA, 2006).

Os AINEs constituem um grupo de compostos muito heterogêneos com várias estruturas químicas, podendo ser distribuídos em diversas classes, de acordo com o grupo químico a que pertencem. Como mecanismo, os AINEs atuam no bloqueio da síntese de prostaglandinas, através da inibição da ciclo-oxigenase (COX). As

prostaglandinas são obtidas através do metabolismo do ácido araquidônico, que se encontra esterificado nos fosfolipídeos das membranas celulares. Uma vez liberado pela ação das fosfolipases, o ácido araquidônico é metabolizado através de duas vias enzimáticas distintas. A via das ciclo-oxigenases, que desencadeia a síntese de prostaglandinas, prostaciclinas e tromboxanos, e a via das lipo-oxigenases, responsável pela síntese de leucotrienos, lipoxinas e outros compostos. A ciclo-oxigenase é encontrada em duas isoformas, denominadas ciclo-oxigenase-1 (COX-1) e ciclo-oxigenase-2 (COX-2). A COX-1 é expressa constitutivamente, ou seja, está presente nas células em condições fisiológicas, principalmente nos vasos sanguíneos, plaquetas, estômago e rins. A COX-2 pode ser induzida na presença de interleucina-1, interleucina-2 e fator de necrose tumoral α (TNF-α), ésteres de forbol, fatores de crescimento e endotoxinas, e é expressa por células envolvidas no processo inflamatório (BURKE; SMYTH; FITZGERALD, 2006; CARVALHO, 2010; KHALAF, 2009).

Em 2002, uma variante da COX-1 em níveis elevados foi isolada no córtex cerebral e no tecido cardíaco de cão. A variante tem sido chamada, desde então, por várias denominações, tais como ciclo-oxigenase-3 (COX-3), COX-1b ou COX-1v. É possível que essa via possa ser o principal mecanismo central pelo qual drogas como paracetamol e fenacetina exercem seus efeitos analgésicos e antipiréticos, estando, talvez, relacionada imunologicamente a COX-2 (Hinz; Brune, 2012; KAM; SO, 2009; LI *et al.,* 2008; MATSUNAGA *et al.*, 2007).

As prostaglandinas estão envolvidas em diversos processos fisiológicos e patológicos, incluindo, por exemplo, vasodilatação ou vasoconstrição; contração ou relaxamento da musculatura brônquica ou uterina; hipotensão; ovulação; metabolismo ósseo; aumento do fluxo sanguíneo renal; proteção da mucosa gástrica e regulação do fluxo sanguíneo local; inibição da secreção gástrica; crescimento e desenvolvimento nervoso; resposta imunológica; hiperalgesia; regulação da atividade quimiotática celular; resposta endócrina; angiogênese; progressão metastásica, entre outros (CARVALHO, 2010; SMYTH; BURKE; FITZGERALD, 2010).

Os AINEs não são efetivamente removidos da água por tratamento convencional sendo, portanto, detectados em afluentes que abastecem vários municípios na União Européia, Estados Unidos e Canadá. Os AINEs atuam de forma sinérgica, com efeitos aditivos em relação à metabolização e bioacumulação em

organismos aquáticos, sendo essa bioacumulação tecidual especialmente produzida por diclofenaco e ibuprofeno (BRUN *et al*., 2006; COMEAU *et al.*, 2008; SANDERSON *et al*., 2003).

Os AINEs apresentam como mecanismo de ação o bloqueio ou retardo do processo inflamatório, reduzindo a permeabilidade do endotélio capilar, inibindo a marginalização e migração leucocitária, reduzindo a cascata que leva a produção de certas prostaglandinas e leucotrienos, através da diminuição de oferta de ácido araquidônico. Essa inibição explica grande parte da ação antinflamatória dos glicocorticóides, devido à importância do ácido araquidônico na produção dos mediadores humorais da inflamação. Os antinflamatórios esteroidais, além de reduzirem a inflamação, suprimem reações alérgicas e a atividade do sistema imune (CHARMAN; WILLIAMS, 2003; MACEDO; OLIVEIRA, 2010; MORLEY, 2009; SCHUERHOLZ *et al*., 2007; TOMLINSON *et al*., 1997).

Os AINEs, tais como os glicocorticóides também são amplamente aplicados na medicina veterinária para restaurar a força muscular, e como promotores de crescimento, para aumento do tamanho muscular em animais.Assim, certa quantidade de glicocorticóides, excretados principalmente na urina de mamíferos, é liberada para o ambiente aquático através de efluentes de estações de tratamento de esgoto ou do escoamento, podendo assim tornar-se potencial contaminante em ambientes aquáticos (CHANG *et al.*, 2007).

Os AINEs empregados no presente estudo toxicológico foram o ibuprofeno, diclofenaco e paracetamol.

2.1.1 Ibuprofeno

.

O ibuprofeno (Figura 2) é frequentemente detectado no ambiente aquático. Assim, os efluentes de águas residuais municipais constituem importante fonte deste fármaco, especialmente em córregos e rios. Devido à sua ocorrência generalizada em ambientes aquáticos, seu potencial de impacto ecológico tem sido uma crescente preocupação (BRUN *et al*., 2006; GOMEZ *et al*., 2007; CHRISTENSEN *et al*., 2009; HAN *et al.*, 2010; KASPRZYK-HORDERN; DINSDALE; GUWY, 2008; KIM *et al*., 2007).

FIGURA 2 - ESTRUTURA QUÍMICA DO IBUPROFENO
FONTE: GROSSER; SMYTH; FITZGERALD (2012).

O ibuprofeno classificado como "perigoso para o ambiente aquático" (CARLSSON *et al*., 2006) é encontrado no ambiente em uma faixa que varia de 0,9 a 27,25 µg/L (WHO, 2012) mas geralmente ele é encontrado em concentração médias de 1,0 µg/L (BILA; DEZOTTI, 2003).

A maioria das avaliações da ecotoxicidade de ibuprofeno realizadas não foi suficiente para compreender o potencial de efeitos crônicos que esse composto pode produzir nos organismos aquáticos nem seu mecanismo de toxicidade (HAN *et al.*, 2010).

Estudos revelam que a exposição ao ibuprofeno no meio aquático leva a um aumento na atividade da aromatase em uma concentração dose-dependente, levando a alterações na reprodução (FLIPPIN; HUGGETT; FORAN, 2007; HECKMANN *et al*., 2007; HAN *et al.*, 2010). Outros trabalhos relatam que a exposição ao ibuprofeno e outros AINEs pode modular a biossíntese estrogênica, inibindo a sua produção em peixes (BRUEGGEMEIER; HACKETT; DIAZ-CRUZ, 2005; HAN *et al.*, 2010; TERRY, 2004).

2.1.2 Diclofenaco

O diclofenaco (Figura 3) é amplamente utilizado devido ao seu efeito antinflamatório, analgésico e às suas propriedades antitérmicas. Estas propriedades terapêuticas são baseadas na inibição da ciclo-oxigenase e inibição subsequente da síntese de prostaglandinas. No entanto, as prostaglandinas não desempenham apenas um papel na mediação da dor, mas estão envolvidas na permeabilidade vascular e função renal. Alguns dos efeitos adversos associados à terapêutica com diclofenaco, como nefropatia, se devem à inibição da síntese das prostaglandinas. No entanto, outros efeitos, incluindo a formação de aductos de proteínas e dano oxidativo também foram sugeridos como causadores de sintomas adversos, por exemplo, ulceração gastrointestinal, nefropatia e hepatotoxicidade idiossincrática

(HARGUS *et al*., 1995; HOEGER *et al.,* 2005; SALI, 2005; SANCHEZ *et al*., 2002; SCHWAIGER *et al.*, 2004).

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O

FIGURA 3 - ESTRUTURA QUÍMICA DO DICLOFENACO
FONTE: GROSSER; SMYTH; FITZGERALD (2012).

Entre os produtos farmacêuticos utilizados em humanos, o diclofenaco é uma das substâncias mais encontradas no ambiente aquático. Ele é encontrado em concentrações médias que variam de 0,2 a 2,3 µg/L (BILA; DEZOTTI, 2003; WHO, 2012). Após o uso terapêutico em humanos, apenas 15% do diclofenaco é excretado de forma inalterada, sendo a maior parte eliminada após biotransformação e excreção via renal. Como consequência, o diclofenaco ou seus metabólitos atingem o ambiente aquático (SCHWAIGER *et al.*, 2004; STÜLTEN *et al.*, 2008).

A exposição ao diclofenaco tem sido apontada como causa de alterações estruturais e de necrose em células específicas dentro das brânquias em trutas, ou seja, as células pilares e as células de cloreto (STÜLTEN *et al.*, 2008; TRIEBSKORN *et al*., 2004). Essas células estão envolvidas tanto nos processos respiratórios como na regulação iônica e osmótica (EADES; WARING, 2009; FREIRE; ONKEN; MCNAMARA, 2008).

Outros estudos indicam que a exposição de truta arco-íris durante 4 semanas a diclofenaco em concentrações variáveis produziu alterações histopatológicas nos rins e nas brânquias, além deste se biocumular e reduzir a síntese de prostaglandina E2 (SCHWAIGER *et al.*, 2004; STÜLTEN *et al.*, 2008).

2.1.3 Paracetamol

Paracetamol (acetaminofeno) (Figura 4) é um analgésico e antipirético considerado seguro, sendo comprado livremente na maioria dos países sendo, por esse motivo, considerado a droga mais amplamente utilizada no mundo. Apesar da venda livre, esse fármaco não pode ser considerado uma droga absolutamente segura, pois pode causar necrose hepática, nefrotoxicidade, lesões extra-hepáticas e até mesmo a morte em seres humanos e animais experimentais, quando administrado em *overdose* (AN *et al.*, 2009; OLALEYE; ROCHA, 2008; RAY *et al.*, 1996).

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FIGURA 4 - ESTRUTURA QUÍMICA DO PARACETAMOL
FONTE: GROSSER; SMYTH; FITZGERALD (2012).

O mecanismo de ação do paracetamol ainda não é completamente elucidado, mas estudos demonstram que ele tem a capacidade de inibir a produção de prostaglandina em nível do sistema nervoso central e tecidos periféricos. Seus efeitos adversos ocorrem principalmente devido à formação de metabólitos hepatotóxicos, principalmente imina *n*-acetil-*p*-benzoquinona, sintetizada quando a disponibilidade de glutationa nas células do fígado é diminuída (FENT; WESTON; CAMINADA, 2006; HINZ, BRUNE, 2012).

O paracetamol é o fármaco mais utilizado ao redor do mundo para alívio da dor. Em efluentes, o paracetamol chegou a ser determinado a uma concentração máxima de 6,0 µg/L (JONES; VOULVOULIS; LESTER, 2001), porém ele foi encontrado nos corpos hídricos em média de 0,25 µg/L (FENT; WESTON; CAMINADA, 2006).

Estudos *in vitro* mostraram que o paracetamol, quando comparado ao ibuprofeno e ao diclofenaco, tem um maior potencial de dano celular, especialmente por sua facilidade de entrada na célula e mudança do pH, o que pode ser um dos responsáveis pelos referidos danos no DNA (PAROLINI *et al.*, 2009).

O paracetamol é excretado principalmente na forma conjugada. Durante o tratamento das águas residuais, a reação de conjugação é degradada, levando a liberação do composto original (KASPRZYK-HORDERN; DINSDALE; GUWY, 2008).

2.2 BIOMARCADORES

Biomarcadores podem ser definidos como qualquer resposta biológica que pode ser representada e mensurada por alterações moleculares, celulares, fisiológicas e até comportamentais no indivíduo ou parte dele. Estas podem ser relacionadas e detectadas devido à exposição a um agente tóxico ou seus efeitos no organismo (PEAKALL, 1994; 1999).

O uso de biomarcadores bioquímicos, genéticos, hematológicos e imunológicos em programas de monitoramento oferece vantagens, pois normalmente estes são os primeiros a apresentar alterações, mostrando boa sensibilidade e especificidade relativa, podendo ser considerados até mesmo sistemas de aviso precoce, indicando a contaminação do ambiente antes que danos mais graves ocorram aos organismos e, possivelmente, ao ecossistema em que estes se encontram inseridos (MCCARTHY; SHUGART, 1990; NUNES *et al.*, 2008).

Os biomarcadores bioquímicos são utilizados, por exemplo, para detectar o estresse oxidativo que se dá quando a geração de radicais livres provocados por substâncias poluentes, ou xenobióticos, é maior que a capacidade antioxidante da célula, promovendo assim a oxidação dos constituintes celulares, tais como os lipídios das membranas, as proteínas e o DNA (AVCI; KAMAZ; DURAKA, 2005). Entre os biomarcadores mais comumente utilizados para se avaliar o estresse oxidativo (Figura 5) estão a atividade enzimática da catalase (CAT), da superóxido dismutase (SOD), e da glutationa S-transferase (GST) que atua na conjugação de substâncias eletrofílicas (KEEN; HABIG; JAKOBY, 1976).

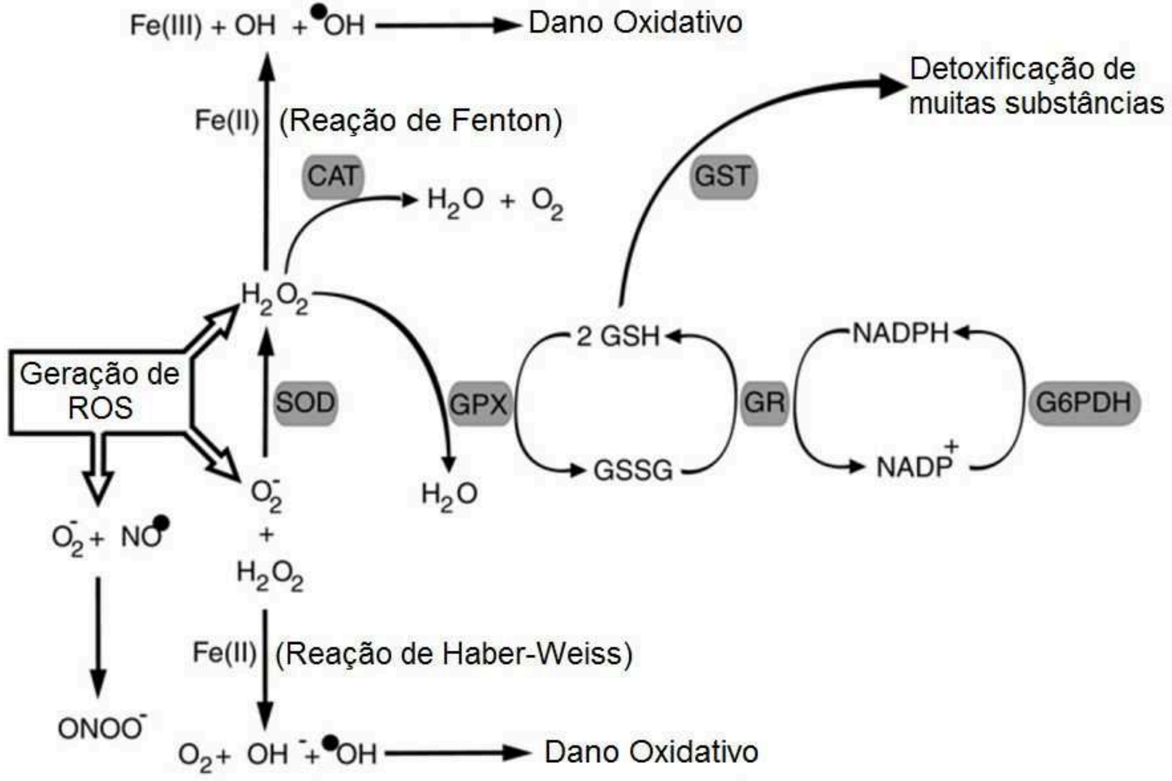

FIGURA 5 - RESPOSTA AO ESTRESSE OXIDATIVO
LEGENDA: CAT: catalase; SOD: superóxido dismutase; GST: glutationa S-transferase; GPX: glutationa peroxidase; GSH: glutationa reduzida; GSSG: glutationa oxidada; GSTs: glutationa S-transferase; GR: glutationa redutase; G6PDH: glucose-6-fosfato desidrogenase; O_2^-: superóxido; H_2O_2: peróxido de hidrogênio.
FONTE: ADAPTADO DE RITTIÉ; FISHER, 2002; KARADAG, 2014

As catalases (CAT) são enzimas que catalisam a eliminação de peróxido de hidrogênio (H_2O_2), liberando como produtos água e oxigênio molecular. São também utilizadas como biomarcadores, embora estejam localizadas nos peroxissomos de muitas células e envolvidas no metabolismo de ácidos graxos, apesar de que mudanças na sua atividade possam ser de difícil interpretação (FASHIMI; CAJARAVILLE, 1995; VAN DER OOST; BEYER; VERMEULEN, 2003).

A superóxido dismutase (SOD) desempenha um papel fundamental na defesa contra os efeitos de agentes tóxicos no organismo. Em eucariontes, ela está presente no citoplasma, núcleo e peroxissomos, além da mitocôndria. Modificações oxidativas em suas isoformas são geradas pela reação com espécies de oxigênio ou de aldeídos derivados da peroxidação lipídica. Essas isoformas da SOD podem ser usadas como bioindicadores de estresse oxidativo provocado por poluentes (LÓPEZ-CRUZ; ZENTENO-SAVÍN; GALVÁN-MAGAÑA, 2010; PASCUAL *et al.,* 2003; PEDRAJAS *et al.,* 1998; VEGA-LÓPEZ *et al.*, 2007). A principal função da SOD é

catalisar a dismutação do radical superóxido (O_2^-) levando à formação do peróxido de hidrogênio (H_2O_2) que por sua vez é degradado pela catalase ou pela glutationa peroxidase (HALLIWELL; GUTTERIDGE, 2007).

A glutationa peroxidase (GPx) é comumente encontrada nas mitocôndrias, sendo utilizados para reduzir diversos tipos de peróxidos, empregando como co-fator a glutationa (GSH), gerando como produto a glutationa oxidada (Gssg) (HAYES *et al*., 1997; HALLIWELL; GUTTERIDGE, 2000). Devido a essa característica, ela é utilizada como importante ferramenta de indicação de estresse oxidativo (VAN DER OOST; BEYER; VERMEULEN, 2003).

Outra glutationa que faz parte dos sistemas oxidantes não enzimáticos que reduz hidroperóxidos é a glutationa reduzida (GSH), sendo responsável por sequestrar espécies reativas de oxigênio e proteger as membranas do estresse oxidativo (ORUC; SEVGILER; UNER, 2004). Ela desempenha papel fundamental na proteção celular contra danos oxidativos causadas por oxidantes, atuando essencialmente como sequestradora de radicais, na homeostase tiólica, manutenção do balanço redox e defesa contra agentes eletrolíticos, como os xenobióticos (PEIXOTO *et al*., 2013; SEVCIKOVA *et al*., 2011).

As glutationa S-transferases (GSTs) são uma superfamília de enzimas diméricas, multifuncionais e primariamente solúveis que catalisam a conjugação de compostos eletrofílicos (ou metabólitos de fase I) com a glutationa reduzida (GSH). Essa reação faz parte do metabolismo de fase II, que envolve a conjugação de um composto xenobiótico ou seu metabólito a um ligante endógeno. As enzimas dessa fase têm um importante papel na homeostase, detoxificação e eliminação de diversas substâncias exógenas, e os níveis de cofatores de fase II podem ser afetados após exposição a poluentes ambientais. Além de seu papel no transporte intracelular e biossíntese de leucotrienos e prostaglandinas, o papel crítico das GSTs é a defesa do DNA e lipídios contra o dano oxidativo e produtos peroxidativos. O processo de lipoperoxidação (LPO), ou oxidação de lipídios tem grande potencial como biomarcador de estresse oxidativo (LIVINGSTONE, 2001; NUNES *et al*., 2008; VAN DER OOST; BEYER; VERMEULEN, 2003).

Alguns biomarcadores podem ainda ser utilizados para verificar a oxidação dos lipídios, como a lipoperoxidação (LPO), e a oxidação de proteínas, tal como a carbonilação de proteínas (PCO) (QUINLAN; GUTTERIDGE, 2000).

A LPO tem como característica causar essencialmente a integridade da membrana celular, podendo resultar em um ambiente favorável ao ataque do DNA, além de potencialmente poder alterar todo o processo de transporte, transdução de sinais mediada por receptores e o gradiente iônico e metabólico (HIGUCHI, 2003).

A PCO tem atraído grande atenção devido a sua natureza irreversível e irreparável. Essa característica é devido ao escape da degradação e formação de agregados de alto peso molecular, que se acumulam e levam ao aumento da produção de ROS, a redução da capacidade de remoção de proteínas oxidadas ou aumento da susceptibilidade de proteínas para o ataque oxidativo (NYSTRÖM, 2005).

Os biomarcadores genéticos, diferentemente dos biomarcadores bioquímicos avaliam uma série de mudanças no material genético, induzido nesse contexto, por substâncias tóxicas encontradas no ambiente, caracterizadas por alterações estruturais do DNA e, consequentemente, o processamento e expressão desse dano como produtos de genes mutantes. A detecção e quantificação desses eventos podem ser empregadas como biomarcadores, como o teste do micronúcleo e o ensaio cometa (VAN DER OOST; BEYER; VERMEULEN, 2003).

A exposição de organismos a substâncias genotóxicas pode induzir uma série de eventos que resultam em mudanças em seu material genético,

O princípio do teste do micronúcleo está baseado no fato de que, durante a anáfase, as cromátides e fragmentos cromossômicos acêntricos não são transportados pelas fibras do fuso para pólos opostos, ao contrário dos fragmentos com centrômero. Após a telófase os cromossomos sem dano são incluídos no núcleo de cada uma das células filhas. Elementos que não foram transportados pelo fuso também podem ser englobados pelos núcleos recém formados. No entanto, alguns destes elementos, normalmente muito pequenos, não são incluídos nos núcleos recém formados e permanecem no citoplasma, constituindo as estruturas caracterizadas como micronúcleos (ALBERTINI *et al*., 2000; FERRARI, 1991; SCHMID, 1975; UDROIU, 2006).

Desta maneira, os efeitos de substâncias que provoquem quebras cromossômicas ou ainda afetem os componentes do fuso ou da região centromérica podem ser detectados a partir da presença de micronúcleos (ALBERTINI *et al*., 2000; HEDDLE *et al*., 1991).

O ensaio cometa é uma técnica capaz de detectar dano ao DNA em células individualizadas (SPEIT; HARTMANN, 1999). O DNA contido em células de organismos eucariotos possui alguns centímetros de comprimento. Para que o DNA seja acomodado no interior do núcleo, que possui entre 5 μm e 10 μm de largura, este DNA tem que ser fortemente condensado. Danos impostos à molécula de DNA provocam um relaxamento desta condensação e ocasionalmente quebras na estrutura molecular (ROJAS; LOPEZ; VALVERDE, 1999).

Os locais do DNA que são susceptíveis ao processo de alquilação são mais sensíveis à degradação. Estes pontos, onde a depurinação está aumentada, transformam-se em pontos de quebras da fita de DNA sendo, portanto, visíveis através do ensaio cometa (HAHN; HOCK, 1999).

Tão importante quanto os biomarcadores bioquímicos e genéticos, os biomarcadores hematológicos conjuntamente agregados aos imunológicos na interface da determinação leucocitária, se destacam na avaliação dos efeitos de um determinado poluente sobre o organismo,

Muitos parâmetros hematológicos em peixes também podem ser empregados como biomarcadores, os quais podem ser sensíveis a certos poluentes (VAN DER OOST; BEYER; VERMEULEN, 2003).

Estudos hematológicos das diferentes espécies de peixe são de interesse ecológico e fisiológico, uma vez que auxiliam na compreensão da relação entre as características sanguíneas, a filogenia, a atividade física, o habitat e a adaptabilidade dos peixes no ambiente. Os valores hematológicos podem ser influenciados não apenas pelo crescimento, mas também pelas condições ecofisiológicas (TAVARES-DIAS; MORAES, 2004). O sangue dos peixes teleósteos é formado basicamente por eritrócitos, trombócitos e leucócitos.

Os eritrócitos são as células mais numerosas que contem essencialmente a hemoglobina, pigmento respiratório que tem por função transportar oxigênio (RANZANI-PAIVA; SILVA-SOUZA, 2004).

Os trombócitos diferentemente das plaquetas nos mamíferos são células completas que participam ativamente tanto da homeostasia quanto na participação no mecanismo de defesa orgânica, demonstrado pela sua presença nos processos de coagulação, inflamação e atividade fagocitária nos processos de infecções (TAVARES DIAS; MORAES, 2004; MARTINS *et al.*, 2004).

Os leucócitos são células que atuam ativamente na defesa do organismo, participando da resposta imunológica com seus principais componentes: linfócitos, monócitos, neutrófilos, eosinófilos e basófilos (FERNANDEZ *et al*., 2002). Os linfócitos são responsáveis pela resposta imune humoral e celular específica, atuando tanto na produção de anticorpos quanto em sua atividade citotóxica (TIZARD, 2002). Os neutrófilos por sua vez, são as primeiras células envolvidas nos estágios iniciais de um processo de inflamação em peixes, com capacidade de fagocitar e gerar radicais livres (FERNANDEZ *et al.*, 2002; FALCON, 2007). Outra célula de importância no contexto dos biomarcadores hematológicos são os monócitos. Os monócitos são considerados as células sanguíneas mais importantes no papel que desempenham na resposta imune, como na produção de citocinas e apresentação de antígenos em teleósteos (VELLEJO *et al*., 1992; SHOEMAKER *et al*., 1997; FALCON, 2007). Os monócitos podem ser encontrados isolados no sangue, no rim anterior e na cavidade peritoneal (SHOEMAKER *et al*., 1997; SIWICKI *et al*., 2009). Dentre as principais características dessas células cabe ressaltar a capacidade de secretarem radicais livres e destruir diferentes tipos de patógenos (FALCON, 2007).

Além de situações adversas serem capazes de diminuir a atividade do sistema imunológico de peixes, a presença de certos poluentes na água tem efeitos supressores sobre a atividade dos fagócitos dos peixes (BOLS *et al.,* 2001; SECOMBES; FLETCHER, 1992).

A caracterização dos principais mecanismos e vias do sistema imune especialmente relacionados a imunotoxicologia, vem preenchendo importantes lacunas no conhecimento a respeito dos numerosos mecanismos imunes relacionados aos peixes (ALVAREZ-PELLITERO, 2008; CHAGAS *et al*., 2009).

O óxido nítrico é uma molécula de sinalização gasosa que regula diversos processos fisiológicos, incluindo a função imune, exibindo atividade citostática ou citotóxica contra diversos microorganismos. A produção de óxido nítrico por macrófagos de peixes pode, assim, ser utilizada como parâmetro funcional dessas células (SARMENTO *et al.*, 2004).

Os peixes são organismos complexos em relação a organização do seu sistema imunológico. Assim como os mamíferos, apresentam dois tipos básicos de sistema de reação imunológica: a imunidade mediada por células e a imunidade humoral (MAGNADOTTIR, 2006). A imunidade celular específica está basicamente

relacionada com os linfócitos T e sua capacidade de reconhecer antígenos com capacidade de ligação à epítopos de certas células. Os linfócitos B, por sua vez, desenvolvem seu papel na produção de anticorpos específicos e células de memória (FALCON, 2007; SECOMBES, 1994). A imunidade humoral está relacionada a produção de imunoglobulina e fatores solúveis como produção e liberação de citocinas (MEDZHITOV, 2007). Além das citocinas, outros mediadores solúveis estão presentes nas reações imunes dos peixes, como as proteínas envolvidas nas reações do sistema complemento. O sistema complemento pode ser ativado pela superfície do patógeno por meio de sua via alternativa, reação inata e inespecífica, por meio da formação de complexo antígeno anticorpo através da via clássica ou ainda pela ligação do complexo protéico de lectina ligada a manose em uma reação antígeno anticorpo, através da ativação da via da lectina (CLAIRE; HOLLAND; LAMBRIS, 2002). As três vias de ativação foram identificadas e descritas em peixes, exceto nos não mandibulados, que não apresentam a forma clássica de ativação (NONAKA, 2001).

A principal diferença do sistema complemento (FIGURA 6) de peixes e mamíferos está no fato de que peixes apresentam uma grande variedade das proteínas expressas para o sistema complemento, com característica de possuir múltiplas isoformas, contribuindo provavelmente para aumentar significativamente sua capacidade de reação e combinação (CLAIRE; HOLLAND; LAMBRIS, 2002).

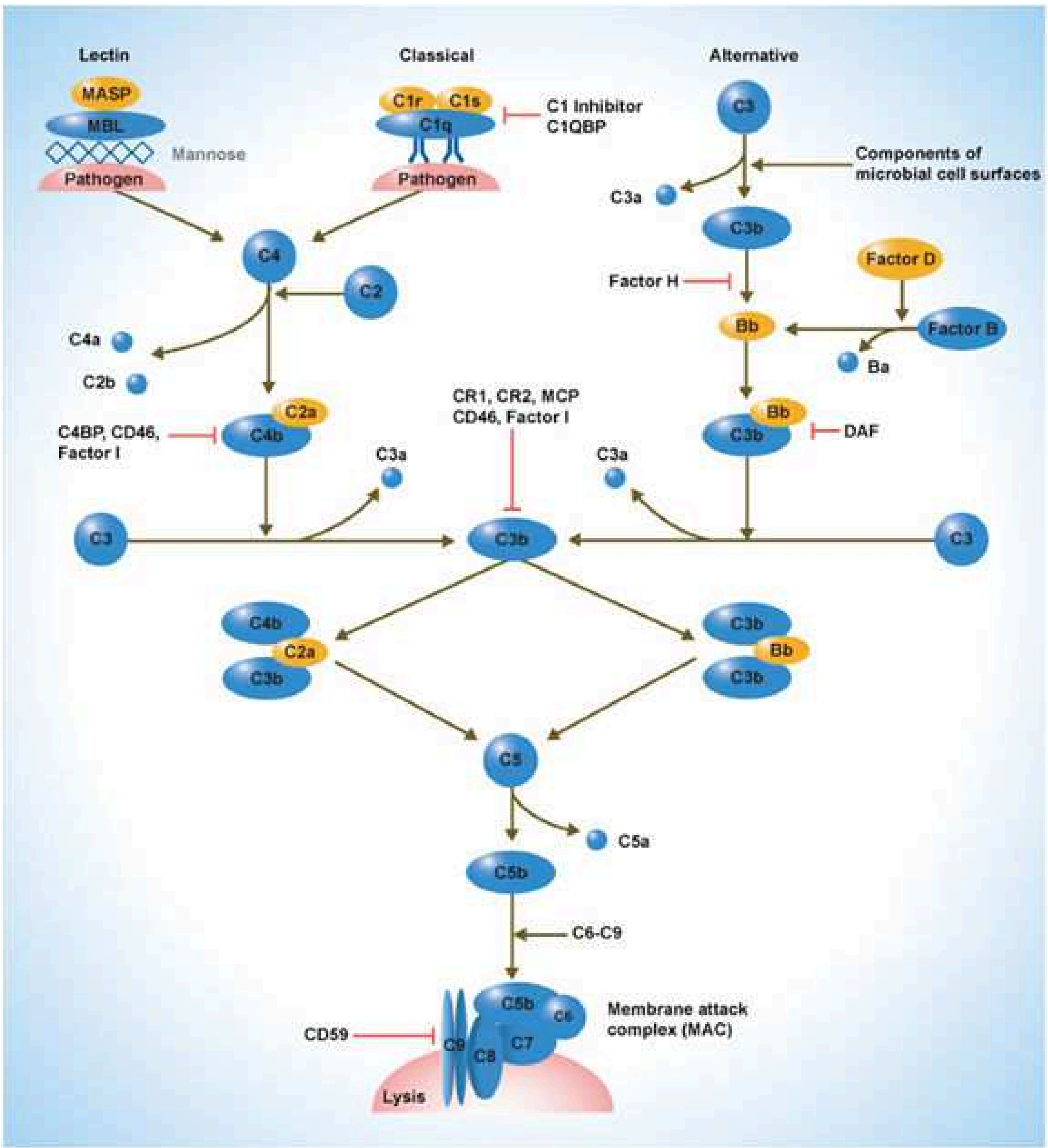

FIGURA 6 - VISÃO GERAL DO SISTEMA COMPLEMENTO
FONTE: http://www.sinobiological.com/Complement-Activation-Pathways-a-1511.html

Outro ponto chave no processo de defesa e proteção do organismo é a migração celular (FIGURA 7), mais especificamente a migração leucocitária. Os macrófagos, por exemplo, migram comumente através dos tecidos, com a principal função de remover células apoptóticas ou necróticas e proteínas desnaturadas. Essa migração depende de uma série de eventos iniciando pela adesão celular e finalizando pela transmigração (COOK-MILLS; DEEM, 2005). Em geral, a migração de leucócitos ocorre por um processo de multi-passos em um processo envolvendo recrutamento,

rolagem, ativação, adesão ao endotélio, diapedese e finalmente migração transendotelial (SCHUBERT *et al*., 2011).

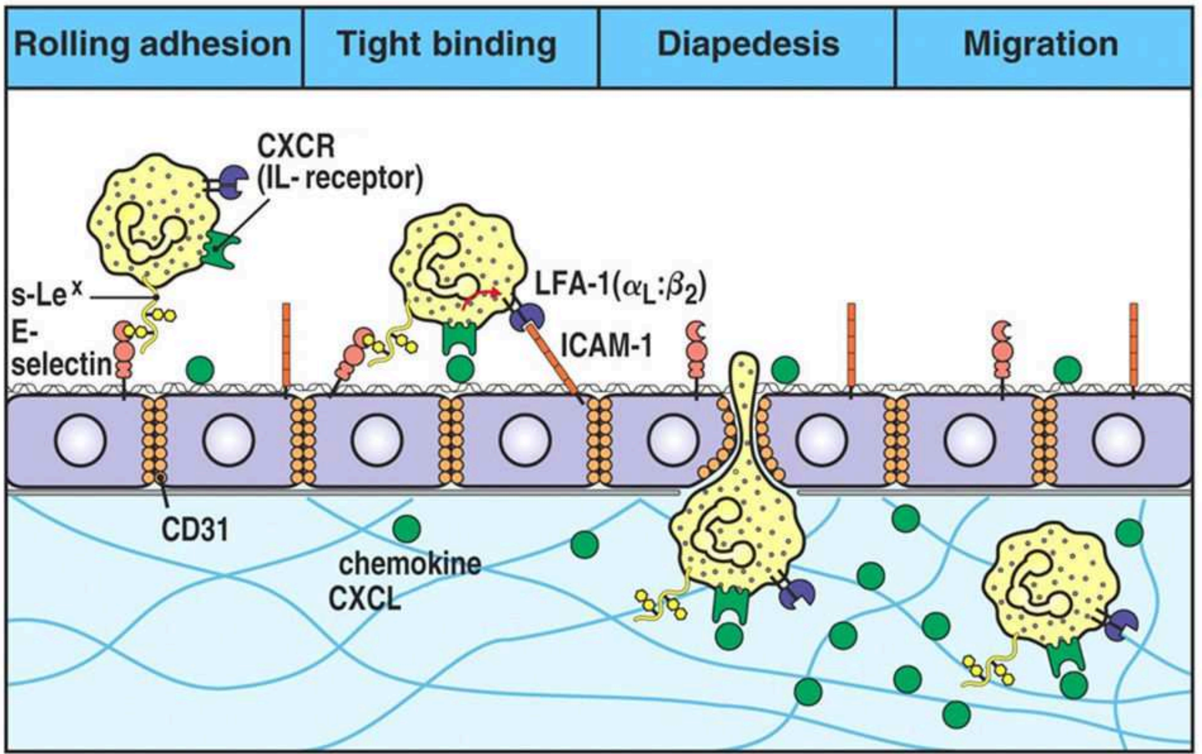

FIGURA 7 - VISÃO GERAL DO PROCESSO DE MIGRAÇÃO CELULAR
FONTE: ADAPTADO DE http://www.bio.davidson.edu/courses/immunology/students/spring2006/latting/home%20copy.html

Os macrófagos residentes são, portanto considerados como sistema de alarme, responsáveis pela primeira sinalização que visa iniciar o processo de migração de neutrófilos (ZHU; PAUL, 2008). O grande problema é que com a supressão do sistema imunológico aumenta a susceptibilidade do peixe à patógenos, um aspecto importante quando consideramos a presença de xenobiótico no ecossistema aquático que pode levar a essa reação (DALAKAS *et al.*, 2005; WEDEMEYER *et al*., 1990).

Uma forma de se avaliar o processo migratório e consequentemente a modulação imunológica em peixes expostos a poluentes ambientais é através da aplicação de elementos flogísticos na cavidade peritoneal (bexiga natatória), simulando a resposta leucocitária à inflamação produzida na cavidade peritoneal (AFONSO *et al*., 1999; KAIKO *et al*., 2008; ZHU *et al*., 2010). Dentre os elementos flogísticos mais utilizados para promover a migração leucocitária em peixes, estão o lipopolissacarídeo (LPS) e a carragenina (MARTINS *et al*. 2001; 2004; 2008; MATUSHI; MARIANO, 1996).

2.3 PROTEÔMICA

O termo proteômica foi inicialmente proposto em 1995 e foi basicamente definido como sendo a caracterização de larga escala do conjunto de proteínas expressas em uma célula ou tecido e o estudo de suas funções (TANVETYANON; CREELAN; CHIAPPORI, 2014; WILKINS *et al*., 1997).

A abordagem proteômica tem permitido estudos da expressão de proteínas em diferentes tecidos e fluidos dos organismos. Agregado a isso os recentes progressos em metodologias nessa área possibilita novas oportunidades de análises e obtenção de informações relevantes sobre os processos fisiológicos e patológicos. Cabe salientar que as proteínas de um organismo podem se modificar dependendo da condição e do estímulo a que esse organismo está exposto. Sendo assim a proteômica reflete a expressão de proteínas que influenciam mais diretamente todo o sistema, sendo por isso uma metodologia de grande importância e aplicação no contexto ambiental (BARBOSA, 2012; SILVA; CORREA; REIS, 2007).

As técnicas proteômicas estão inseridas em diversas áreas da biologia, bioquímica e toxicologia, incluindo a toxicologia ambiental. Nesse contexto, a proteômica busca biomarcadores e alvos de poluentes que demonstrem em termos moleculares o que de fato está ocorrendo com o organismo e elucide os possíveis mecanismos moleculares da ação desses poluentes nos organismos aquáticos (BELLGARD, 2013; KUHNER *et al*., 2009; SANTOS *et al*., 2004). No entanto, a falta de informação genética anterior na maioria das espécies de peixes tem sido um grande problema para uma aplicação mais geral e irrestrita das diferentes tecnologias proteômicas disponíveis (FORNÉ; ABIÁN, CERDÁ, 2010).

O mesmo tipo celular pode apresentar diferentes proteínas e rotas metabólicas em relação, por exemplo, a ação de drogas e/ou poluentes ambientais. Por esse motivo, os dados gerados por uma análise proteômica permitem alcançar objetivos diferentes tais como esclarecer as principais proteínas envolvidas em rotas metabólicas relacionados aos diferentes processos celulares, identificar novos alvos biológicos, caracterizar moléculas bioativas e caracterizar as respostas celulares a determinadas drogas, doenças ou mudanças ambientais (SILVA; CORREA; REIS, 2007).

Apesar de sua relevância, a abordagem proteômica em peixes ainda mostra-se tímida, com um baixo número de espécies pesquisadas. Essas espécies muitas vezes estão restritas a algumas de interesse em aquicultura e modelos experimentais laboratorialmente já estabelecidas, como o *Danio rerio* (zebrafish). Mesmo com essa restrição, a proteômica em peixes tem emergido como uma ferramenta importante para o estudo dos sistemas biológicos e para abordar diferentes questões relacionadas com a biologia do peixe e suas variações quando expostos a alguma substância específica. A possibilidade de combinar os dados de proteômica com os resultados de outros biomarcadores fazem dessa metodologia um grande avanço na abordagem ambiental (AMATRUDA *et al*., 2008; FORNÉ; ABIÁN, CERDÁ, 2010; LOVE *et al*., 2004).

2.4 ANIMAIS DE ESTUDO

Hoplias malabaricus (Bloch) ou traíra (Figura 8) é um peixe de água doce, carnívoro, que devido ao seu comportamento predador ocupa um alto nível trófico na cadeia alimentar aquática, sendo, por isso, um valioso modelo biológico, podendo sofrer bioacumulação. A traíra não tem comportamento migratório, sendo encontrada em toda América do Sul. Esta espécie é um modelo biológico interessante para fins experimentais, devido ao seu comportamento e capacidade de se adaptar às condições experimentais e à sua posição na cadeia alimentar (ALVES COSTA *et al*., 2007; FILIPAK NETO *et al.*, 2007; 2008; MOL *et al*., 2001; OLIVEIRA RIBEIRO *et al*., 2006; OLIVERO-VERBEL, 2006; RABITTO *et al*., 2005; SILVA DE ASSIS *et al*., 2013).

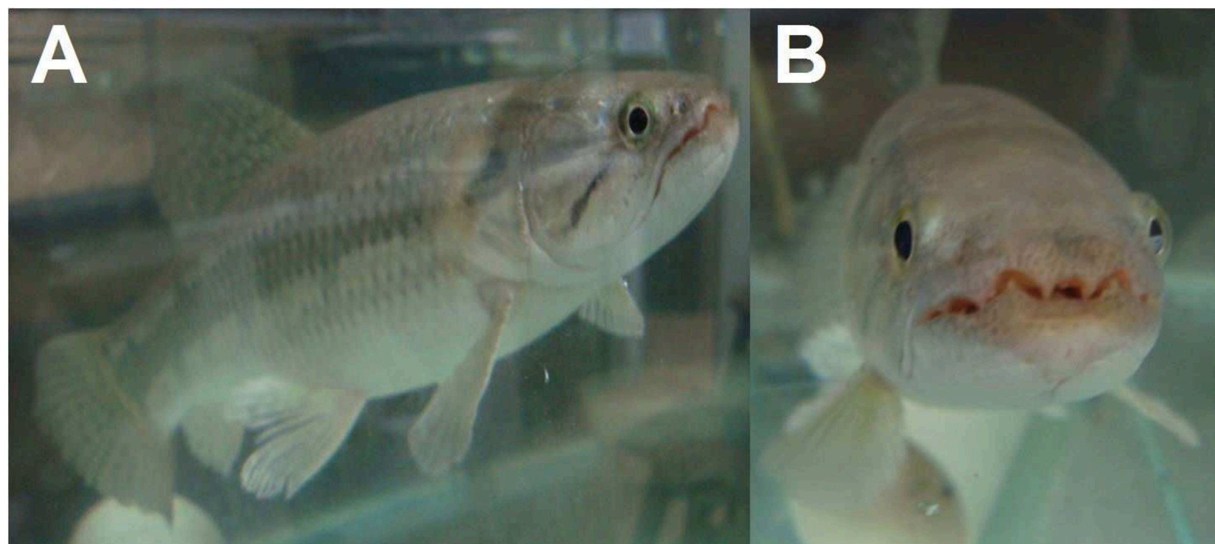

FIGURA 8 - EXEMPLAR DE *H. malabaricus*, CONHECIDO POPULARMENTE COMO TRAÍRA. (A) VISTA LATERAL; (B) VISTA FRONTAL.
FONTE: O AUTOR.

Além disso, *H. malabaricus* tem uma das maiores tolerâncias de privação de alimento, sobrevivendo por períodos de até 180 dias sem redução da taxa metabólica (consumo de oxigênio) (ALVES COSTA *et al*., 2007; RIOS *et al*., 2005).

Rhamdia quelen, ou jundiá (Figura 9), é um peixe que tem distribuição neotropical, sendo encontrado desde o centro da Argentina até o sul do México, e cujo cultivo está aumentando no sul do Brasil. O jundiá tem como característica viver em lagos e rios, e preferir os ambientes de águas mais calmas, com fundo de areia e lama (GOMES *et al.,* 2000). É uma espécie de grande aceitação no mercado consumidor, especialmente por sua carne agradável ao paladar e ausência de espinhos intramusculares. Devido a sua fácil domesticação e adaptação às condições de cultivo, vem atraindo a atenção de pesquisadores, com o qual a espécie tem sido utilizada como modelo de estudos ecotoxicológicos (BARCELLOS *et al*., 2001; BENADUCE *et al*., 2008; BIBIANO MELO *et al*., 2006; COLDEBELLA *et al*., 2011; HERNÁNDEZ *et al*., 2012; PAMPLONA *et al*., 2011; PRETTO *et al*., 2010; 2011), e produtores, por suas características que vêm permitindo sua inclusão na lista de espécies de peixes criadas comercialmente no Brasil (CARNEIRO; MIKOS, 2005).

FIGURA 9 - EXEMPLARES DE *R. quelen*, CONHECIDO POPULARMENTE COMO JUNDIÁ.
FONTES: http://www.scotcat.com/heptapteridae/rhamdia_quelen4.htm; http://planetacuario.com/showthread.php/17693-Rhamdia-quelen

Ambas as exposições, a trófica com o Hoplias malabaricus e a hídrica com o Randia quelen foram escolhidas porque representam avaliações diferentes, mas complementares à avaliação dos riscos ecológicos. A hídrica demonstrando os possíveis efeitos na exposição direta e a trófica que sob condições controladas é reprodutiva e interpreta como age um ou mais agentes ao longo da cadeia alimentar. Ambas com especial poder de demostrar cada um a seu modo possível bioacumulação e biomagnificação (SIMON *et al.*, 2013).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os efeitos toxicológicos de antinflamatórios não esteiroidais por via trófica e hídrica em espécies de peixe nativo através de diferentes biomarcadores de contaminação em doses/concentrações ambientalmente relevantes.

3.2 OBJETIVOS ESPECÍFICOS

- Padronizar o cultivo primário de rim anterior de *Hoplias malabaricus*
- Avaliar a produção de óxido nítrico e danos genotóxicos em cultivo primário de rim anterior de *Hoplias malabaricus* expostos ao diclofenaco, ibuprofeno e paracetamol
- Avaliar o estresse oxidativo em fígado de *Hoplias malabaricus* após exposição trófica ao diclofenaco
- Avaliar o potencial de migração celular do diclofenaco após desafio com a carragenina
- Avaliar proteomicamente proteínas do sistema imunológico relacionados à produção de óxido nítrico, migração celular e sistema complemento, em machos e fêmeas de *Rhamdia quelen* expostos via hídrica ao diclofenaco

Todos os estudos foram aprovados pela Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná sob os números 453 (*Hoplias malabaricus*) e 652 (*Rhamdia quelen*). Os procedimentos também foram realizados de acordo com os princípios éticos estabelecidos pelo Colégio Brasileiro de Experimentação Animal (COBEA) e as exigências estabelecidas no *Guide for the Careand Use of Experimental Animals (Canadian Councilon Animal Care)*.

CAPÍTULO I

PADRONIZAÇÃO DO CULTIVO PRIMÁRIO DE MACRÓFAGOS DO RIM ANTERIOR DE *Hoplias malabaricus* E SUA UTILIZAÇÃO EM ENSAIOS TOXICOLÓGICOS

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Effects of anti-inflammatory drugs in primary kidney cell culture of a freshwater fish

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Abstract

The toxicity of widely used non-steroidal anti-inflammatory drugs (NSAIDs) was evaluated on primary culture of monocytic lineage of *Hoplias malabaricus* anterior kidney. The effects of diclofenac, acetaminophen and ibuprofen in cell viability, lipopolysaccharide (LPS)-induced NO production and genotoxicity were evaluated. Cytometry analysis CD11b^{+}cells showed 71.5% of stem cells, 19.5% of macrophages and 9% of monocytes. Cell viability was lower in the Ficoll compared to Percoll separation. LPS-induced NO production by these cells was blocked after treatment with dexamethasone and L-NMMA. Exposure of the cells to diclofenac (0.2-200 ng/mL), acetaminophen (0.025-250 ng/mL) ibuprofen (10-1000ng/mL) reduced basal NO production and inhibited LPS-induced NO production at all concentrations after 24 h of exposure. Genotoxicity occurred at the highest concentration of diclofenac and at the intermediary concentrations of acetaminophen. Genotoxicity was also observed by ibuprofen. In summary, the pharmaceuticals influenced NO production and caused DNA damage in monocytic cells suggesting that these drugs can induce immunosuppression and genotoxicity.

Keywords: Pharmaceuticals, cell culture, immunotoxicity, genotoxicity, macrophage.

1. Introduction

Some studies have shown that the concentration of pharmaceutical drugs has increased in water bodies all over the world and has became a growing environmental problem and a major source of pollution. These compounds come especially from urban, industrial and hospital wastewater and have therefore been identified as emerging contaminants in aquatic ecosystems (ZUCCATO *et al.*, 2006). The main pharmaceutical group detected is nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, ibuprofen and acetaminophen (ZUCCATO *et al.*, 2006; KHETAN; COLLINS, 2007). Some studies reports that trace NSAIDs concentrations in environment elicit toxic effects, including immunological changes (KHETAN; COLLINS, 2007). Diclofenac, widely used in the world especially for chronic inflammatory conditions, is one the most prevalent water contaminants. It has been identified as one of the most import pharmaceutically active compounds present in the aquatic environment (LETZEL; METZNER; LETZEL, 2009). Diclofenac is found at concentrations ranging from 0.2 to 2.3 µg/L (BILA; DEZOTTI, 2003; WHO, 2012). Similarly to diclofenac, ibuprofen, based on inherent properties, is classified as "dangerous for the aquatic environment" (CARLSSON *et al.*, 2006). The concentration of ibuprofen found in the environment varies from 0.9 to 27.25 µg/L (WHO, 2012), but it has been found at concentrations around 1.0 µg/L (BILA; DEZOTTI, 2003). Acetaminophen is the most widely used over-the-counter common pain reliever. In sewage effluent, the acetaminophen reported maximum concentration is 6.0 µg/L (JONES; VOULVOULIS; LESTER, 2001). However, it has been found at concentrations around 0.25 µg/L (FENT; WESTON; CAMINADA, 2006).

Among aquatic organisms, fish species have been used for detection of the effects of environmental pollution on aquatic ecosystem. The alterations on this organism can be useful to diagnose functional status of the tissue and cells exposed to the toxicant and thereby it is an indirect way to monitor the aquatic environment quality (SARAVANAN *et al.*, 2012).

Functional monocytic lineage and macrophage cells are essential for the establishment of a properly working immune system. They constitute a major, widely dispersed system of cells that regulate homeostasis in the host and respond to tissue

injury by contributing with essential functions during inflammation and repair (BARREDA; BELOSEVIC, 2001).

Several pharmaceuticals have been investigated in *in vitro* systems such as fish cell lines and especially in primary fish cell cultures. Among the advantages of these procedures, based on fish cells or reporter gene systems are their potential for toxicity screening, besides of course, they are important alternatives to animal testing (FENT; WESTON; CAMINADA, 2006). In the present work, the freshwater fish *Hoplias malabaricus* was chosen as a model to establish a lineage of monocytic primary cultures since this species has a large ecological plasticity, with a wide distribution in Brazilian rivers and reservoirs (SILVA *et al.*, 2011).

The aim of the present study was to develop and test a protocol of primary culture of a lineage of monocytic cells from anterior kidney of *H. malabaricus* and expose these cells to different NSAIDs to investigate possible effects on its viability and functionality (NO production at non-stimulated and stimulated conditions). We also evaluated the genotoxic effects of the NSAIDs in these cells.

2. Material and methods

All procedures and protocols were approved by the Institution’s Ethical Committee for Animal Use and are in accordance with international guidelines for Animal Use.

Hoplias malabaricus (100±20g) were purchased from a commercial farm. Fish were kept at 24±1ºC with filtered water on a simulated natural photoperiod and fed twice a week *ad libitum* with fish (*Astyanax* sp) (30±5g). The fish were acclimated in the lab for at least three weeks prior to use in experiments.

2.1 Primary anterior kidney cultures

The monocytic lineage of *H. malabaricus* anterior kidney was obtained, as previously described (NEUMANN; BARREDA; BELOSEVIC, 1998) with some modifications. Briefly, for each experiment, three fishes were anesthetized with benzocaine 0.02% (v/v) and killed by medullar section. The anterior kidneys were removed, pooled and transferred to L-15 medium (Leibovitz-; supplemented with 20U of heparin and 1% antibiotic penicillin/streptomycin) under sterile conditions. The

tissue was homogenized in the same medium, transferred to a 15mL tube and suspended with a sterile Pasteur pipette for 1 min. For a better suspension this procedure was repeated once using a Pasteur pipette of minor diameter. Cellular suspension (in the proportion 2:1) was transferred to a new 15 mL tube containing Percoll gradient 50/50 or 50:40 (v/v) or 60:40 (v/v) or 60:40:10 (v/v) or Ficoll-Pack and centrifuged at 400 x g for 30 min at 22 ºC. Cells at the Percoll or Ficoll interface were removed, washed twice in serum-free medium and centrifuged at 400 x g for 10 min. Viable cells (1.10^6) were placed in 96 wells plates with L-15 medium supplemented with fetal bovine serum 2% and incubated at 20-22 °C, 1.7% CO_2 for 24h for adherence. After this period, non-adherent cells were washed away with phosphate-buffered saline (PBS). The remaining adhered cells were used for subsequent experiments. Cells were detached using ice-cold PBS. For cytometry analysis, the cells were incubated for 40 min with anti-CD11b antibody. Data acquisition was performed in a BD FACS calibur equipped with an argon ion laser tuned to 488 nm. For all measurements, 100,000 cells were collected from each sample tube using Cell Quest software (Becton Dickinson, San Jose, CA, USA). Data analyses were performed using Cell Quest and WinMDI 2.9 software.

2.2 Monocytic stimulation

To establish the optimal LPS concentration and stimulation time for nitric oxide (NO) production, monocytic lineage adherent cells in 96 well plates (1.10^6 cells/well) were stimulated with *Escherichia coli* serotype 0111:B4 lipopolysaccharide (LPS) at different concentrations (0.1,1.0,10.0,100.0,1,000.0 and 10,000.0 ng/mL) diluted in L-15 medium supplemented with fetal bovine serum 2%,at 20-22 °C, 1.7% of CO_2 for 24, 48, 72 and 96 h. At the end of the experiment, cell viability was determined by modified MTT method (MOSMANN, 1983).

2.3 Monocytic cells treatment and stimulation

In subsequent tests, to characterize NO production in monocytic lineage of this species, adherent cells were treated with dexamethasone 0.3 and 0.03 µg/mL (positive control), L-arginine or D-arginine 2 mM or with the inducible NO synthase inhibitor L-NMMA (N^G-Methyl-L-arginine) 1 mM immediately before the stimulation

with LPS 0.1 and 1.0 ng/mL. Cells were incubated at 20-22°C, 1.7% CO_2 and nitrite concentration measured as described below.

2.4 Exposure of the cells to NSAIDs

Adherent cells were treated with Diclofenac (0.2; 2.0; 20.0; 200.0 and 2,000.0 ng/mL), Ibuprofen (0.1; 1.0; 10.0; 100.0 and 1,000.00 ng/L), Acetaminophen (0.025; 0.25; 2.5; 25.0 and 250ng/L) or L15 medium (control) immediately before the stimulation with LPS (1ng/ml). The effect of the NSAIDs in the same concentrations used above was also evaluated in non-stimulated cells. NO concentration was evaluated after 24h in the supernatant of the cultures and the genotoxicity assay was performed in the resulting control cell monolayer (without LPS).

The tested concentrations were selected based on the concentrations found in the environment, especially in water. The average concentration of diclofenac, ibuprofen and acetaminophen usually found in the environment is approximately 2.0 ng/mL, 1.0 ng/mL (BILA; DEZOTTI, 2003) and 0.25 ng/mL (FENT; WESTON; CAMINADA, 2006), respectively. Concentrations ten times lower and ten, one hundred and one thousand times higher were also tested.

2.5 Nitrite assay

NO production was assayed using a modification of the method described by Neumann et al, 1998. This method is based on the Griess reaction (GREEN *et al.,* 1982) that quantifies the nitrite content, since NO is an unstable molecule and degrades to nitrite and nitrate. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

2.6 Alkaline Comet Assay

To evaluate the genotoxic effects of the NSAIDs at 24 h after the treatment, cells were washed and removed from the plate with ice-cold PBS, harvested, centrifuged and ressuspended in 150 µL of PBS. Only the wells that presented cell

viability above 70% were used for the further tests according to the International Workshop on Genotoxicity Test Procedures (TICE *et al.*, 2000).

The comet assay was performed according to Speit and Hartmann (2005). The cell suspension (13 µl) were diluted in low melting point agarose 0.5% (LMP) and placed on a microscope slide pre-coated with a layer of normal melting point agarose (1.5%). The slides were placed for 24 h at 4 °C in lysis working solution (triton X100 1% v/v, DMSO 10% v/v and lysis stock solution 89% v/v (NaCl 2.5 M, EDTA 0.1 M, Tris 0.01 M, NaOH 0.2 M, N-lauroylsarcosine sodium salt0.034 M, pH 10).

An electrophoresis was run at 300 mA and 1V/cm for 25 min. The slides were washed three times for 5 min each with Tris-HCl (0.4 M), pH 7.5 at 4°C and fixed for 5 min in absolute ethanol and stained with 0.02 µg/ml ethidium bromide. DNA strand breaks were scored using one Leica epifluorescence microscope at a magnification of 400×. DNA damage was determined in each slide (three replicates for treatment) and one hundred nucleoids were scored visually according to tail intensity and given a value of 0, 1, 2, 3 or 4 (from undamaged 0, to maximally damage 4). The total score of 100 nucleoids could range from 0 (all undamaged) to 400 (all maximally damage) (COLLINS; AI-GUO; DUTHIE, 1995).

The comets with small or non-existent head and large, diffuse tails (i.e. so-called hedgehogs) are not considered because they are associated with cell death (FAIRBAIRN *et al.*, 1996; FRENZILLI; NIGRO; LYONS, 2009).

2.7 Viability assessment of cell cultures

Different cell viability between Percoll and Ficoll separation was evaluated after 24h, 48h, 72h and 96h of incubation. The cell viability was also evaluated in the same incubation times of exposure to LPS or NSAIDs (or both) by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MOSMANN, 1983). After cells incubation, were added 100µL of MTT and the plate was incubated again. After 24h the reaction was blocked with an acid solution of 10% SDS. The measure was carried out at 550 nm.

2.8 Drugs and reagents

Penicillin/streptomycin, Ficoll-Pack, LPS, MTT, L-arginine, D-arginine, L-NMMA, dexamethasone, diclofenac, acetaminophen and ibuprofen were purchased from Sigma Chemicals & Co., U.S.A. The L-15 medium and Percoll were purchased from Cultilab, Brazil. Trypan Blue dye was from Gibco and anti-CD11b antibody from BD Biosciences. Other reagents used were of analytical grade.

2.9 Statistical Analysis

Data are presented as mean ± SEM. Kolmogorov-Smirnov normality test was used and comparisons between groups were done by One-Way ANOVA followed by Bonferroni's post-hoc test (cells viability and NO) or Kruskal-Wallis test followed by Dunn's post hoc tests (comet assay). Differences of $p < 0.05$ were considered to be significant. The statistics tests were performed using GraphPad Prism version 5.00 for windows (Graph Pad Software, USA).

3. Results and Discussion

3.1 Primary anterior kidney cultures

Isolated anterior kidney cells of *H. malabaricus* were obtained using Percoll or Ficoll separation, and then were plated on culture plates. After 24 h non-adherent cells were washed away using phosphate-buffered saline (PBS) and the viability and predominant adherent cell type were analyzed.

Different concentrations of Percoll were tested and the quantity of isolated cells changed. The gradient proportion 60:40% showed higher cell number after separation of the anterior kidney cells. It has been shown that the density gradient of Percoll for isolation of macrophage cells is variable. Results in anterior kidney cells isolation were achieved with density combinations of 51% in goldfish (HANINGTON *et al.*, 2009), 51:34% in rainbow trout (STAFFORD *et al.*, 2001) and 41:35% in sea bass (SARMENTO *et al.*, 2004). It seems that the best density to be used depends on the fish species.

The anterior kidney was evaluated at light microscopy and it was observed red cells, monocytic lineage as well as steam cells (Figure 1A). In culture plates, after percoll or ficoll separation, the different populations were not evident (Figure 1B and C) and the cells were stained for monocytic lineage with anti-CD11b. Positive cells were evaluated both in relation to size (SSC – side scatter) and in relation to the cellular complexity (FSC – Forward scatter). The resulting plot is shown in Figure 1D. The lineage was composed of 71.5% of stem cells, 19.5% of macrophages and 9% of monocytes.

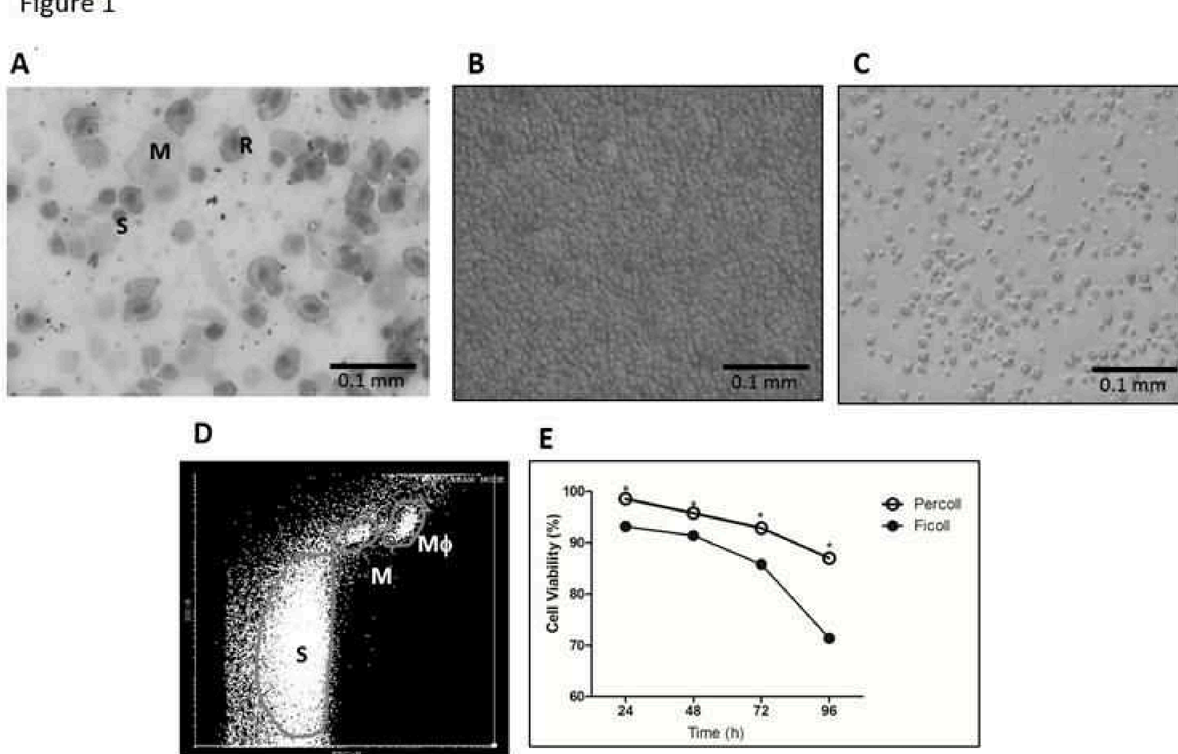

Figure 1 - CULTURE ANTERIOR KIDNEY CELLS OF *Hoplias malabaricus*. Overview of previous anterior kidney cells (A); Placed cells after 24h of incubation in 96 well plates (B); Placed cells after 24h of incubation in 24 well plates (C); Cytometry analysis, using FSC and SSC parameters of cells labeled with $CD11b^+$ antibody (D); Comparison cell viability with percoll and ficoll at different times (E), One-Way ANOVA followed by Bonferroni's post-hoc test $p<0.001$. R-red cells; M-cell monocytic lineage (M – Monocyte; Mϕ – Macrophages); S–stem cells (monocytic lineage).

Similar results were reported before for other species; 12% of macrophage after 24 hours of culture in goldfish (HANINGTON *et al.*, 2009) and rainbow trout (STAFFORD *et al.*, 2001) and 10.4%-58.0% in goldfish (NEUMANN; BARREDA; BELOSEVIC, 1998; NEUMANN; BARREDA; BELOSEVIC, 2000), using a similar protocol.

Cell viability reduced in both separation processes over the time (Figure 1E). However, Percoll separation showed a less pronounced reduction in cell viability when compared to Ficoll separation.

In the present study the cell viability reduced over time. Similar results were described especially after 48 hours. Some studies suggest that this decrease in viability does not seem to be related to nutrient consumption, but with cell senescence (SARMENTO *et al.*, 2004).

3.2 NO production by monocytic cells

Viability and NO production by monocytic lineage from anterior kidney of *H. malabaricus* after stimulation with different concentrations of LPS for 24, 48, 72 and 96h of incubation at 20-22 °C, 1.7% CO_2 are shown in Figure 2.

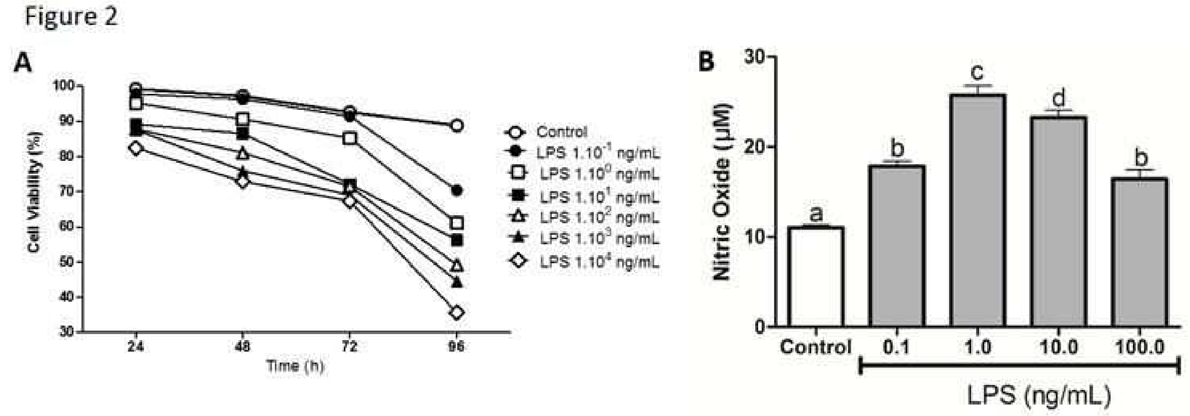

Figure 2 - MONOCYTIC STIMULATION BY LPS. Viability cells in different LPS concentration (A). Nitric oxide production after 24h stimulation by LPS (B); One-Way ANOVA followed by Bonferroni´s post-hoc test $p<0.001$. Different letters represent statistically different values.

Cells viability significantly decreased with incubation time and reductions were evident 48, 72 and 96 h after LPS stimulation. For this reason NO production was analyzed only after 24 hours of incubation (Figure 2B).

NO production blockers and stimulators were tested in order to better characterize the NO production by monocytic/macrophage fish cells after LPS stimulation and its similarities to other species. When the cells were stimulated with LPS at 1 ng/mL NO production significantly increased, which was reverted by the treatment of the cells with dexamethasone at both concentrations 0.3 and 3 µg/mL (Figure 3A). In addition, L-NMMA, an inhibitor of iNOS (inducible nitric oxide synthase)

also abolished NO production induced by LPS (Figure 3B). When monocytic cells were treated with NO precursor L-arginine, NO production increased significantly (Figure 3C) and this increase was more prominent after L-Arg treatment concomitantly with the stimulation of low LPS concentrations (0.1 ng/mL, Figure 3D). In this case, the amount of NO produced was similar to the stimulation of LPS 1 ng/mL (Figure 3D). The effect of L-arginine on NO production was completely blocked with simultaneous exposure to L-NMMA, as expected (Figure 3C). The treatment of the cells with the inactive isomer D-arginine was not significant in the NO production (Figure 3C and 3D).

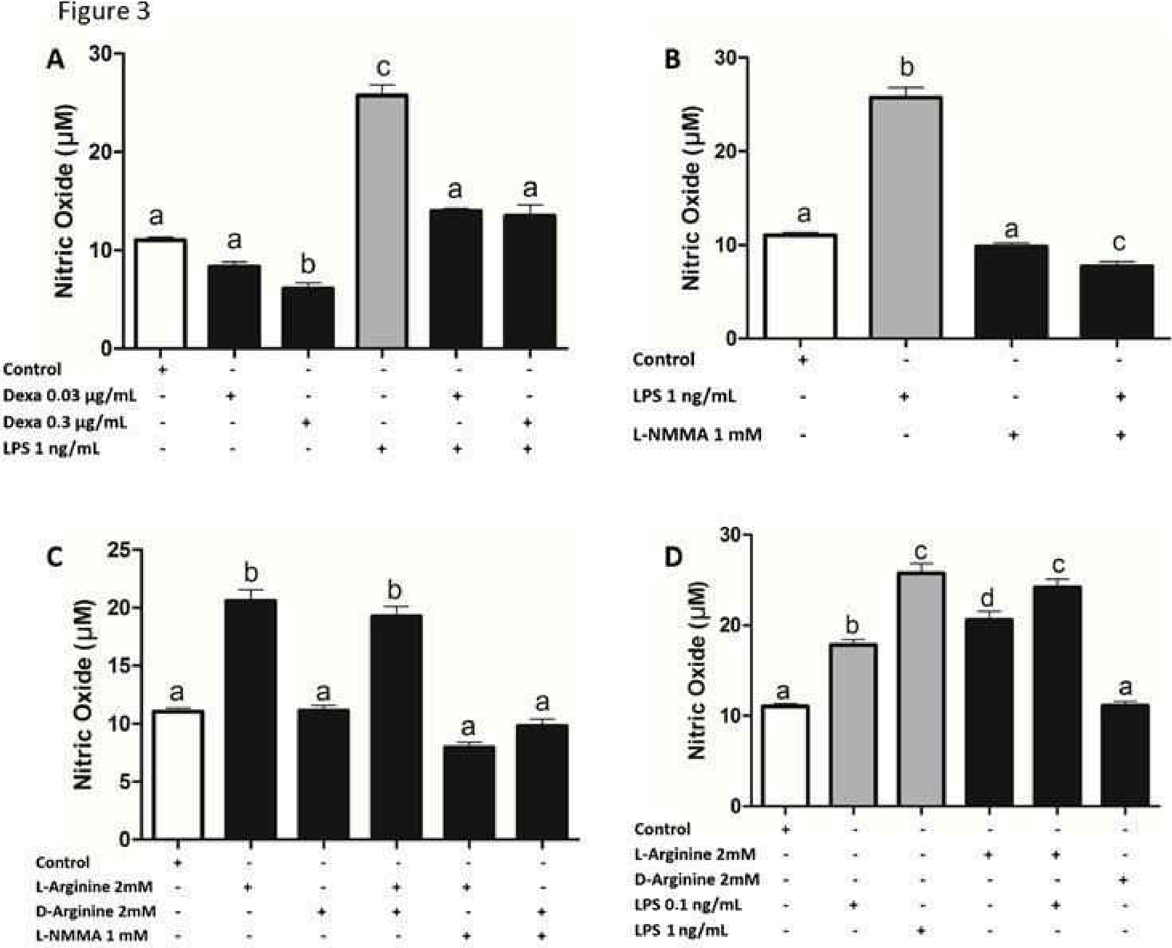

Figure 3 - MONOCYTIC LINEAGE CHALLENGE FROM ANTERIOR KIDNEY CELLS OF *Hoplias malabaricus*. Dexamethasone and LPS challenge (A); LPS and L-NMMA challenge (B); L-arginine, D-arginine and L-NMMA challenge (C); L-arginine, D-arginine and LPS challenge (D). One-Way ANOVA followed by Bonferroni´s post-hoc test $p<0.001$. Different letters represent statistically different values.

LPS has been extensively used for *in vitro* stimulation of fish macrophages. Important molecules associated with the production of the enzyme inducible nitric

oxide synthase (iNOS) (FORLENZA *et al.*, 2011). In our study the lower production of NO at higher LPS concentrations was due to the decrease in cell viability associated with low capacity of monocytic lineage progenitor cells to produce NO. Optimal LPS concentration for NO production by monocytes/macrophages differs among studies. Previous studies showed that optimal LPS concentration required for NO production in monocytes/macrophages isolated form carp and trout was from 0.1 to 50 µg/mL (FIERRO-CASTRO *et al.*, 2012). In a different way, the murine/human macrophage can be stimulated with low LPS concentrations (ng/ml) (REBL; GOLDAMMER; SEYFERT, 2010). The monocytes/macrophages from *H. malabaricus* seem to be very sensitive to LPS stimulation and may represent a good model to study xenobiotics effects.

The activation status of monocytic lineage is important in determining the response to a harmful stimulus. Arginine is an immunomodulator that acts as the unique precursor for NO (LI *et al.*, 2007). Pohlenz *et al.* (2012) showed that the supplementation of L-arginine increased the NO production by monocytic cells stimulated or not by LPS. We confirmed these results using monocytic cells from *H. malabaricus*.

The production of NO in monocytic lineage in response to LPS alone was inhibited by L-NMMA as would have been predicted, but it not occurred with the cells treated with L-NMMA alone. In this case, L-NMMA was not able to decrease spontaneous NO production but only when stimulated by 1 ng/mL of LPS. These results suggested that the production of NO by monocytic/macrophages from *H. malabaricus* is due to *de novo* synthesis of iNOS which transforms L-Arg in NO as usually occurs in other species.

3.3 Effect of NSAIDs on cell viability, NO production and genotoxicity

Diclofenac at 0.2, 2.0, 20.0 and 200.0 ng/mL significantly reduced basal NO production (Figure 4A). Similar results were observed for acetaminophen at 0.025, 0.25, 2.5, 25.0 and 250 ng/mL (Figure 4C), while ibuprofen only reduced basal NO production at the higher concentrations used (10.0, 100.0 and 1000.0 ng/mL) (Figure 4E).

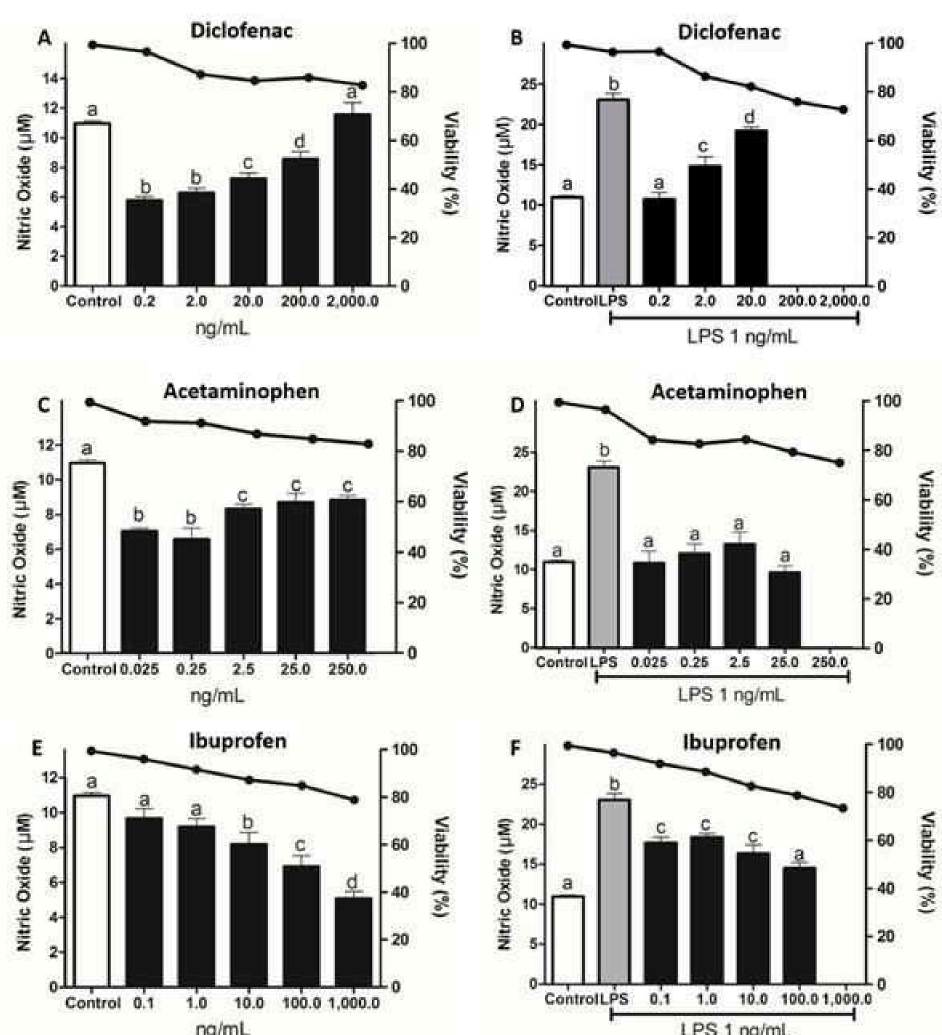

Figure 4 - MONOCYTIC LINEAGE CHALLENGE FROM ANTERIOR KIDNEY CELLS OF *Hoplias malabaricus*. NO production after exposure to diclofenac (A); NO production after exposure to diclofenac with LPS-stimulus (B); NO production after exposure to acetaminophen (C); NO production after exposure to acetaminophen with LPS-stimulus (D); NO production after exposure to ibuprofen (E); NO production after exposure to ibuprofen with LPS-stimulus (F); One-Way ANOVA followed by Bonferroni´s post-hoc test $p<0.001$. Different letters represent statistically different values.

The cells were exposed to NSAIDs and LPS-stimulus, diclofenac, acetaminophen and ibuprofen inhibited LPS-induced NO production in all concentrations tested compared with LPS control (Figure 4B, 4D and 4F). NO production by monocytic cells treated with the highest concentrations of diclofenac (200.0 and 2000.0 ng/mL), acetaminophen (250.0 ng/mL) and ibuprofen (1000.0

ng/mL) and stimulated with LPS was not evaluated, since the cell viability was below 80% (figure 4B, 4D and 4F).

The genotoxicity was observed at the highest concentration of diclofenac (20 ng/mL) (Figure 5A), at the concentrations of acetaminophen (0.25; 2.5 and 25.0 ng/mL) (Figure 5B) and at the ibuprofen concentrations (except 100.0 ng/mL) (Figure 5C).

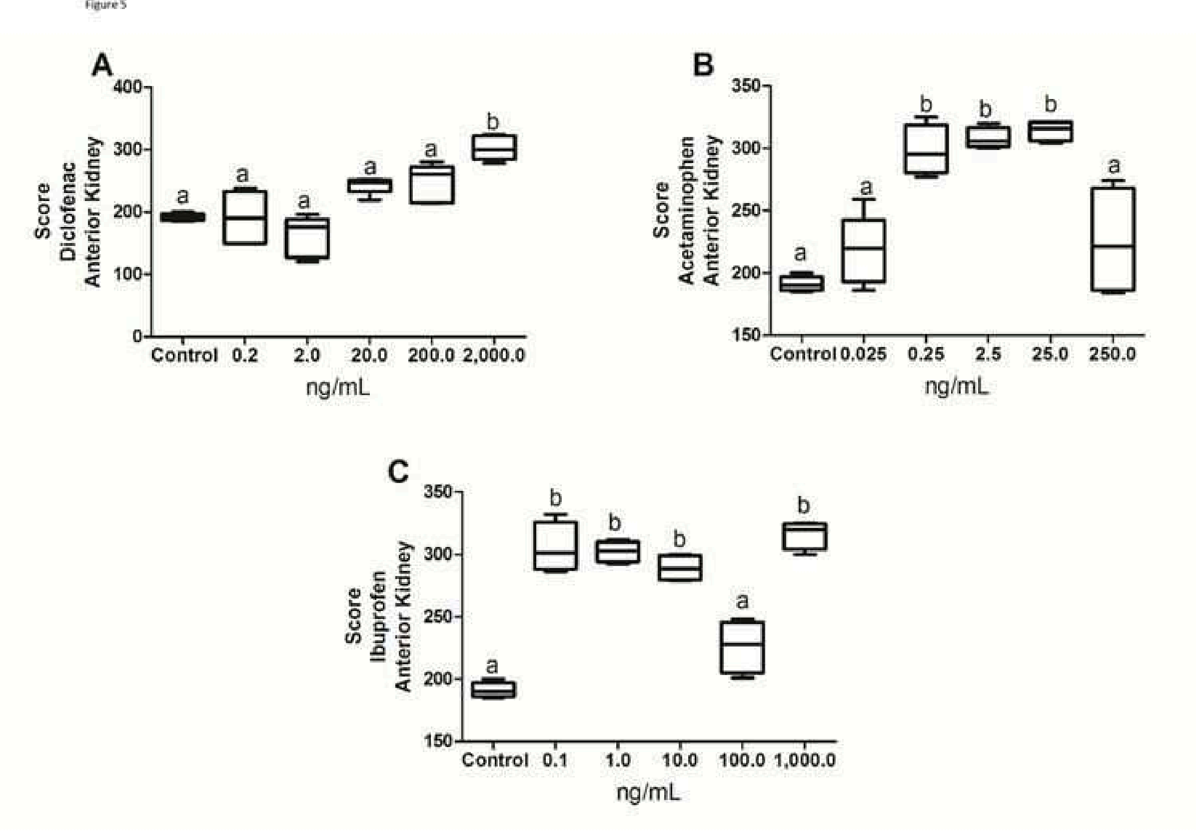

Figure 5 - COMET ASSAY. DNA strand breaks. (A); Score DNA strand breaks after exposure to diclofenac (B); Score DNA strand breaks after exposure to acetaminophen (C); Score DNA strand breaks after exposure to ibuprofen; Kruskal-Wallis test followed by Dunn's post hoc tests $p<0.001$. Different letters represent statistically different values.

The NSAIDs tested in this studies reduced basal NO production. Previous studies, after 24h of incubation the murine macrophage culture with 1 and 100µg/mL of diclofenac, demonstrated an inhibition in the NO production (CIRINO *et al.*, 1996).

In mouse macrophage cells exposed to 10 µmol/mL for 2 and 18h of acetaminophen was also able to decrease the NO production (AL-BELOOSHI *et al.*, 2010). In fish monocytic lineage, acetaminophen inhibited basal NO production.

Ibuprofen also inhibited basal NO production. In other literature studies the inhibition on phagocytosis in haemolymph and catecholase-type phenoloxidade activity in the plasma of the Pacific oyster *Crassos trea gigas* was related with the 5 ng/mL of ibuprofen concentration mixed with herbicides (LUNA-ACOSTA *et al.*, 2012).

In rat glial cell, ibuprofen 178 µg/mL decreased iNOS protein levels in 50%, possibly due to an inhibition of post transcriptional processing of this enzyme, as well as decreased the production of pro-inflammatory cytokines (COMBS *et al.*, 2000).

Furthermore, in the cells from freshwater mussel *Dreissena polymorpha* ibuprofen increased the percentage of haemocytes showing apoptosis and lysosomal membrane destabilization (PAROLINI *et al.*, 2009) suggesting that ibuprofen was toxic for these cells.

In fish monocytic lineage, diclofenac inhibited NO production and probably iNOS induced by LPS. In previous works, Cirino *et al.* (1996) demonstrated that diclofenac was able to inhibit directly iNOS activity, once expressed by LPS-stimulus. Diclofenac (0.38 mM) also decreased activation of NF-kB from KBM5 cells in culture and the levels of iNOS in Raw 264.7 cells, impairing their activation in response to LPS (TAKADA *et al.*, 2004).

Acetaminophen inhibited NO production by LPS-stimulus. In mouse macrophage cells stimulated by 1µg/mL LPS for 12 h prior to treatment with 1 and 10 µmol/mL of acetaminophen for 2 and 18 hours, decreased NO production (concentration dependent) and the expression of the iNOS (AL-BELOOSHI *et al.*, 2010). The acetaminophen (0.5, 3.0 and 10 mM) was able to decrease activation of mouse NF-kB complex in Hepa1-6 cells (BOULARES *et al.*, 2000).

Ibuprofen is also involved in down regulation of iNOS and decreased the expression of iNOS protein activating by LPS-stimulus (HENEKA; KLOCKGETHER; FEINSTEIN, 2000) in granule cells from rat cerebellum. It also decreased the activation of NF-kB in Pc 3, LNCaP and DU145 cells and in nuclei KBM5 cells (TAKADA *et al.*, 2004) previously stimulated.

The production of basal NO or NO LPS-induced by monocytic/macrophages of *H. malabaricus* can be affected by anti-inflammatory drugs, as demonstrated in this work.

The most commonly prescribed NSAIDs, acetaminophen, diclofenac and ibuprofen, were genotoxic to cells of lineage monocytic of *H. malabaricus.*

In lineage DT40 culture cells NSAIDs also induce the DNA damages (LIU *et al.*, 2012) similarly to the results of the present work. MEHINTO, HILL and TYLER (2010) observed a decreasing of cyclooxygenases (COX 1 e COX 2) gene expression in kidney leading to impairment of immune system with diclofenac in trout. Furthermore, affected other enzymes related to metabolism of xenobiotics (CyP) and cell cycle (p53). The genotoxic mechanism of diclofenac is related to oxidative stress.

The hydric fish exposure to 0.18 $ng.mL^{-1}$ of diclofenac (similar to the low concentration used in this study), caused DNA fragmentation, apoptosis and genomic alterations of polymorphic patterns (ROCCO *et al.*, 2010). Diclofenac could induce specific types of DNA lesions. At high concentration diclofenac caused replication blockage leading to chromosomal aberrations as well as translesion DNA synthesis, measured through proliferation of DT40 mutant cells lines (LIU *et al.*, 2012). Induction of the p53 gene and CYP1A gene was observed in fish exposure to diclofenac indicating carcinogenic and/or apoptotic potential effects (HONG *et al.*, 2007).

Studies demonstrated that acetaminophen can covalently bind to DNA and cause chromosomal aberrations in mammalian and zebrafish cells. The genotoxic effects of acetaminophen can be a consequence of cytotoxic events (BLANSET; ZHANG; ROBSON, 2007).

The genotoxicity and carcinogenicity of acetaminophen is widely discussed. For the International Agency for Research on Cancer (IARC) the acetaminophen is non-classifiable as carcinogenic in humans, however this compound might be considered genotoxic and carcinogenic. Studies have demonstrated that acetaminophen can covalently bind to DNA and cause chromosomal aberrations, teratogenesis and inactivation of ribonucleotide reductase with consequent disturbances of DNA repair and replication (BLANSET; ZHANG; ROBSON, 2007; PENG *et al.*, 2010).

The ibuprofen, *in vivo* studies was associated with induction of micronuclei frequency, general DNA damage and punctual genomic alterations (RAGUGNETTI *et al.*, 2011). In Chinese hamster ovary (CHO) cells was reported an increase in the frequencies of micronucleated cells with and without kinetochores indicating aneugenic and clastogenic activity of ibuprofen (DOPPALAPUDI *et al.*, 2012). There is no report of *in vitro* genotoxicity of ibuprofen using fish cells.

Ibuprofen was genotoxic to monocytic cells of *H. malabaricus* including at the environmental reference concentration. The risk to aquatic organisms increases

because ibuprofen is a hydrophobic compound with ability to pass through cell membranes and to accumulate in living organisms (VICQUELIN *et al.*, 2011).

In fish, ibuprofen is associated with induction of micronuclei (RAGUGNETTI *et al.*, 2011); general DNA damage and punctual genomic alterations (ROCCO *et al.*, 2010). In mammals ibuprofen induce sister chromatid exchange (SCE) (PHILIPOSE *et al.*, 1997) and increased micronucleated and binucleated cells with and without kinetochores indicating aneugenic and clastogenic activity (DOPPALAPUDI *et al.*, 2012). There is no report of *in vitro* genotoxicity of ibuprofen using fish cells.

4. Conclusion

In the present study, diclofenac, acetaminophen and ibuprofen reduced NO production and induced DNA damage in the lineage monocytic cells from anterior kidney of *H. malabaricus*. The results suggested potential immunosuppressive action and induction of genotoxicity. The results implied in a better understanding on the toxic effects of these specific pharmaceutical drugs and the methodology can be used as potential immunological biomarkers of freshwater fish toxicity.

Acknowledgments

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CAPÍTULO II

EFEITOS DA EXPOSIÇÃO TRÓFICA DO DICLOFENACO EM *Hoplias malabaricus*

Effects of trophic exposure to diclofenac in freshwater fish

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceutical prescribed in human medicine and have the potential to contaminate water and sediments via inputs from sewage treatment plants. Their impacts on humans and ecosystems are emerging issues in environmental health. The aim of the present work was to evaluate the effects of diclofenac in the fish species Hoplias *malabaricus* after trophic exposure. Fish were fed twice every week with *Astyanax sp*. submitted to intraperitoneal (IP) inoculation with diclofenac (0; 0.2; 2.0 or 20.0 µg/Kg). After 12 doses, half of fish received 1 mg/Kg of carrageenan IP and after 4 hours they were anesthetized and euthanized for cells migration evaluation. In the other fish (without carrageenan) the hematological parameters, nitric oxide (NO) basal production and after LPS-stimulate in head kidney were measured. In the liver hepatosomatic index (HSI) and biochemical analysis, such as activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione S-transferase (GST), Ethoxyresorufin-O-deethylase (EROD) and catalase (CAT) were analyzed. The reduced glutathione (GSH) and lipoperoxidation (LPO) was also evaluated. The results showed an increase in red blood cells count and in the hematocrit at the low dose Therefore, the hemoglobin reduced at the highest dose. The thrombocytes count increased in all groups exposed to diclofenac and the total blood leukocyte counts decreased due to the neutrophils reduction. Monocytes were also decreased at the highest dose. The number of resident peritoneal cells did not differ among the groups, but the cell migration reduced after carrageenan-with a significant decrease in the migration of polymorphonuclear cells. The basal NO synthesis of anterior kidney cell cultures from diclofenac-treated animals was significant lower in the cells from the group 2 and 20 µg/kg. LPS-stimulated NO production was reduced in all the diclofenac-treated groups. Diclofenac also reduced HSI at the 0.2 µg/Kg. In liver, diclofenac caused oxidative stress with increased GPx activity and LPO. The GST activity decreased by diclofenac in liver. The results suggest that a trophic exposure to diclofenac can lead to potential toxic effects, including hematological, immunological and biochemical changes.

Keywords: diclofenac, trophic exposure, oxidative stress, migration cells, hematological parameters, culture cells, *Hoplias malabaricus*

1. Introduction

Pharmaceuticals are considered a new class of pollutants and negative effects on terrestrial and aquatic environments have been attributed recently to their presence in the ecosystems. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently detected pharmaceuticals in treatment plants and surface waters worldwide, due to their volume of consumption and incomplete removal during the wastewater treatment processes (ISLAS-FLORES *et al*., 2013). Diclofenac is a

NSAIDs generally used as an analgesic to reduce pain and to treat inflammatory disorders (ISLAS-FLORES *et al*., 2013). Diclofenac was identified as one of the most important pharmaceutically active compounds present in the water cycle. It was found in groundwater samples and even in treated drinking water (PRASKOVA *et al*., 2014). In the present study antioxidant defense parameters (superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and reduced glutathione) were investigated as biomarkers of cellular oxidative stress. Lipid peroxidation is also associated with oxidative stress resulting in the oxidation of polyunsatured lipids (QUINN *et al*., 2011). Oxidative stress is also considered an important factor affecting reproductive performance (COSTANTINI *et al*., 2011). The biotransformation of some pharmaceuticals in the aquatic environment may modify enzymatic pathways mediated by CYP450 and cause physiological effects and toxicity (BURKINA *et al.*, 2013). The hematological parameters studies of fish are the ecological and physiological interest, since that assist in understanding the relationship between blood characteristics, physical activity, habitat and adaptability of fish in the environment. Hematologic values can be influenced not only by growth but also by the ecophysiological conditions (TAVARES-DIAS; MORAES, 2004).

Several pharmaceuticals have been investigated in *in vitro* systems such as fish cell lines and especially in primary fish cell cultures. Among the advantages of these procedures, based on fish cells or reporter gene systems are their potential for toxicity screening, besides of course, they are important alternatives to animal testing (FENT; WESTON; CAMINADA, 2006).The functional monocytic lineage and macrophage cells constitute a major, widely dispersed system of cells that regulate homeostasis in the host and respond to tissue injury by contributing with essential functions during inflammation and repair. In order to study the immune response in fish cells exposed to diclofenac the analysis of functional monocytic lineage and macrophage cells are essential for the establishment of a properly working immune system. They constitute a major, widely dispersed system of cells that regulate homeostasis in the host and respond to tissue injury by contributing with essential functions during inflammation and repair (BARREDA; BELOSEVIC, 2001).

The experimental model used in this study was *Hoplias malabaricus* (Characiformes, Erythrinidae), a carnivorous fish with large distribution in tropical rivers and lakes and very consumed by human populations (FERREIRA *et al.,* 2003). This

fish has the advantage that it is easily fed under laboratory and has been explored in several biological studies (MONTEIRO *et al.*, 2013; SILVA DE ASSIS *et al*., 2013).

Since diclofenac has been found in aquatic environment, the aim of the present work was to evaluate the biochemical, hematological and immunological effects in the fish *Hoplias malabaricus* after trophic exposure.

2. Material and methods

The present work was approved by the Animal Experimentation Ethics Committee of Federal University of Parana, under number 456 and all protocols were realized in accordance with International Guidelines for Animal Use.

2.1 Chemicals

Diclofenac sodium salt (D6899) was obtained from Sigma Aldrich. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Corporation (USA) and Merck.

2.2 Experimental design

Adult *Hoplias malabaricus* males weighing 171.4 ± 15.7 g (standard length: 25.3± 4.4 cm) were purchased from Santa Candida Commercial Farm, Santa Cruz da Conceição, São Paulo, Brazil. Fish were kept at 24±1°C in glass aquaria (120 L capacity) for 30 days in filtered and dechlorinated tap water on a simulated natural photoperiod (12 hours dark:12 hours light). After acclimation to laboratory conditions, fish were randomly divided into four groups (n=20 fish in each group) and was transferred to test aquarium. During the experiment, each fish were maintained in a 30L aquarium and were fed twice every week with fish (*Astyanax sp*. 30 ± 5g) as pray vehicle prior injected intraperitoneally with diclofenac (0.2; 2.0 or 20.0 µg/Kg simulating a trophic contamination. These doses were based on the concentration of diclofenac (2 µg/L) usually found in the environment (SANTOS *et al*., 2010). After 12 doses, half of fish (n=10) received carrageenan (Cg) 1mg/kg intraperitoneal. The fish were anesthetized and killed by medullar section 4 hours later and the intraperitoneal was washed with PBS. The fluid was removed to determinate the cell migration induced by

Cg. The fish which did not receive Cg were anesthetized in water with benzocaine 1 % (Sigma) and blood was taken from caudal vein for hematology and biochemical analyses. The fish were killed by medullar section weighed and the liver was removed for hepatosomatic index (HIS) calculation and biochemical analysis. The HIS was determined as HSI= (Liver weight/Whole body weight) x 100. The head kidney was used to primary macrophage culture cell.

2.3 Hematological parameters

The erythrocyte count or RBC (Red Blood Cells) was performed with the technique of formaldehyde - citrate, modified by Oliveira-Júnior et al. (2009). Hemoglobin (Hb) contents were determined spectrophotometrically at 540 nm using the cyanomethemoglobin method (COLLIER, 1944; DRABKIN, 1946). The hematocrit (Hct) was determined by the volume occupied by erythrocytes in heparinized microhematocrit (HINE, 1992; NELSON; MORRIS, 1989). The hematimetric indices - MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration) were determined using the following standard formulas described by WINTROBE (1934).

The leukocyte (WBC - White Blood Cells) and the thrombocyte count were realized according to Tavares-Dias and Moraes (2006, 2003). The leukocyte differentiation was realized like described by Tavares-Dias *et al.* (2002, 1999).

2.4 Head kidney primary macrophage culture cell and nitric oxide determination

The monocytic lineage of *H. malabaricus* anterior kidney was obtained, as previously described by Ribas *et al* (2014). Briefly, the head kidney were removed, pooled and transferred to L-15 medium (Leibovitz-; supplemented with 20U of heparin and 1% antibiotic penicillin/streptomycin) under sterile conditions. The tissue was homogenized in the same medium, transferred to a 15mL tube and suspended with a sterile Pasteur pipette for 1 min. For a better suspension this procedure was repeated once using a Pasteur pipette of minor diameter. Cellular suspension (in the proportion 2:1) was transferred to a new

15 mL tube containing Percoll gradient 60:40 (v/v) and centrifuged at 400 x g for 30 min at 22 ºC. Cells at the Percoll interface were removed, washed twice in serum-free medium and centrifuged at 400 x g for 10 min. Viable cells (1.10^6) were placed in 96 wells plates with L-15 medium supplemented with fetal bovine serum 2% and incubated at 20-22 °C, 1.7% CO_2 for 24h for adherence. After this period, the supernatant was removed for Nitric Oxide (NO) determination. Non-adherent cells were washed away with phosphate-buffered saline (PBS). The remaining adhered cells were used for subsequent experiments to determine NO production induced by 1ng/mL of LPS by 24 hour.

NO production was assayed using a modification of the method described by Neumann *et al*. (1998). This method is based on the Griess reaction (GREEN, 1982) that quantifies the nitrite content, since NO is an unstable molecule and degrades to nitrite and nitrate. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

Different cell viability was evaluated in the same incubation times of basal exposure or after stimulus of LPS by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MOSMANN, 1983). After cell incubation, 100µL of MTT were added and the plate was incubated again. After 24h the reaction was blocked with an acid solution of 10% SDS. The measurement was carried out at 550 nm.

2.6 Biochemical analysis

For the biochemical analysis the samples were homogenized in a phosphate buffer (0.1 M) at pH 7.0, and centrifuged at 15,000 *x g* for 30 min, at 4°C.

SOD activity was assayed by measuring its ability to inhibit the reduction of nitrobluetetrazolium (NBT), which was determined by the method described by Crouch *et al.* (1981). The reduction of NBT by O_2^- to blue formazan was measured spectrophotometrically at 560 nm during 30 min. One unit of SOD was defined as the enzymatic activity necessary to inhibit the reduction of NBT to 50% of the blank and was expressed U/mg protein.

CAT activity was assayed by direct measurement of H_2O_2 degradation and absorbance decrease was measured at 240 nm for 1 min at 27°C and the activity was expressed as µmol/ min/mg protein.

GPx activity was measured based on the decrease of absorbance of NADPH at 340 nm, encouraged by the reduction of GSSG, catalyzed by GR, in the presence of NADPH (PAGLIA; VALENTINE, 1967). Absorbance was monitored at 340 nm and the activity was expressed as nmol/min/mg protein.

GSH was measured according Sedlak and Lindsay (1968). Absorbance was determined at415 nm and GSH concentration was calculated by comparison with the standard curve for GSH and the activity was expressed as µg/mg protein.

The analysis of LPO was carried out using the ferrous oxidation–xylenol assay (JIANG *et al.*, 1992). After 30 min of reaction at room temperature, the absorbance was measured at 570 nm and the activity was expressed as µmol hidroperoxides/min/mg protein.

EROD activity was evaluated according to Burke and Mayer (1974). The fluorimeter measurement was at a wavelength of 530 nm (excitation) and 590 nm (emission) for 10 min at 27 °C and the activity was expressed as pmol/min/mg protein.

GST activity was measured using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates (KEEN *et al*., 1976). The absorbance increase was measured at 340 nm for 3 min at intervals of 15 s and the activity was expressed as nmol/min/mg protein.

The protein concentration was determined by using Bradford's method (1976), with bovine serum albumin as the standard at 620 nm.

2.7 Statistical analysis

The data analysis was preceded by the Kolmogorov–Smirnov normality test. Data were analyzed using the One-way Analysis of Variance (ANOVA), followed by the Bonferroni *post hoc* tests. All data were statistically analyzed by the GraphPad Prism 5.00 (GraphPad Software, Inc.). All tests were regarded as statistically significant when $p < 0.05$.

3. Results

3.1 Hematological parameters

In the present work, *H. malabaricus* exposed to diclofenac showed an increase in RBC and Hct determination in the group treated with diclofenac at 0.2 µg/Kg. The Hb determination significantly decreased in the 20.0 µg/Kg exposed group. The hematimetric indices MCV, MCH and MCHC did not change in all groups exposed to diclofenac (Table 1). The leukocyte count decreased significantly in a dose-dependent manner (Figure 1A). Decreased polymorphonuclear leukocytes (0.2, 2.0 and 20.0 µg/Kg) (Figure 1B) and mononuclear leukocytes (2.0 and 20.0 µg/Kg) (Figure 1C) were observed in the present study. In all groups thrombocytes count increased (Anova, followed by Bonferroni post-hoc, $\alpha = 0.05$).

TABLE 1 - *Hoplias malabaricus* HEMATOLOGICAL PARAMETERS AFTER DICLOFENAC EXPOSURE

	Diclofenac Doses			
	Control	0,2 µg/Kg	2 µg/Kg	20 µg/Kg
RBC (10^6/ µL)	1.347 ± 0.06	1.580 ± 0.03*	1.410 ± 0.02	1.410 ± 0.08
Hemoglobin (g/dL)	6.790 ± 0.48	7.070 ± 0.40	6.850 ± 0.40	5.800 ± 0.13*
Hematócrit (%)	23.76 ± 0.51	27.67 ± 0.58*	26.20 ± 0.82	22.66 ± 0.87
MCV (*f*L)	178.2 ± 5.28	175.9 ± 5.88	186.3 ± 6.60	166.6 ± 12.31
MCH (ρg)	51.11 ± 4.24	44.63 ± 2.11	48.74 ± 3.04	42.78 ± 3.16
MCHC (g/dL)	28.68 ± 2.13	25.81 ± 1.81	26.75 ± 2.31	25.76 ± 0.56
Thrombocytes (10^3/ µL)	20.10 ± 1.16	45.16 ± 1.31*	33.34 ± 2.21*	42.40 ± 3.15*

Values are expressed as mean ± standard error of mean. * indicate statistically significant differences ($p<0.05$). ANOVA, Bonferroni, N=10. RBC - Red Blood Cells; MCV - mean corpuscular volume; MCH - mean corpuscular hemoglobin; MCHC - mean corpuscular hemoglobin concentration.

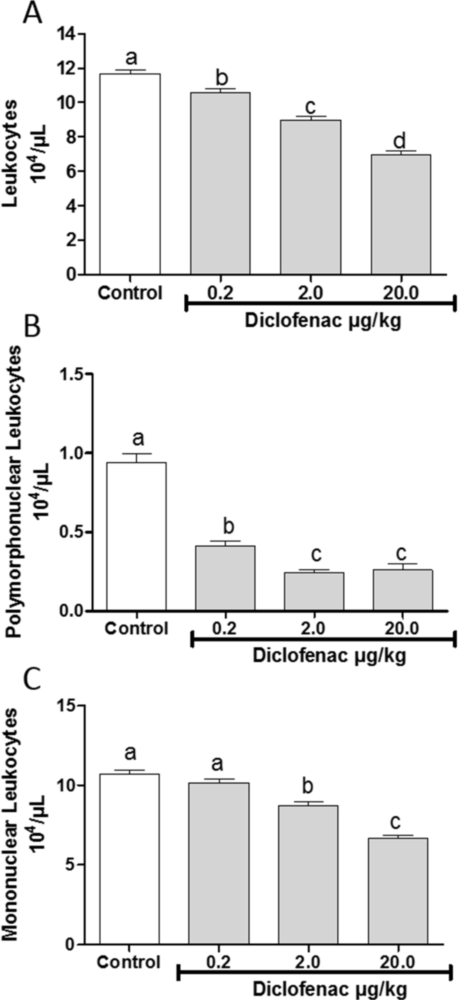

Figure 1 - LEUCOGRAM OF *Hoplias malabaricus* THROPHICALLY EXPOSED TO DICLOFENAC. (A): Leukocyte total number (B): Polymorphonuclear differential leukocytes count (C): Mononuclear differential leukocytes count (Anova, followed by Bonferroni post hoc test ($p<0.0001$). Different letters represent statistically different values.

3.2 Intraperitoneal migration cells induced by carrageenan

In the present study, was not observed difference in the quantity of leukocytes which migrated to peritoneal cavity in *Hoplias malabaricus* exposed to diclofenac. However, after the carrageenan stimulation, the total cell number decreased significantly in all groups, especially in the dose 0.2 μg/Kg (Anova, followed by Bonferroni post-hoc, α = 0.05) (Figure 2A). The differential count of the polymorphonuclear leukocytes increased in the 20.0 μg/Kg diclofenac group, but decreased in the other ones (Anova, followed by Bonferroni post-hoc, α = 0.05) (Figure 2B). The mononuclear cell counts increased in fish exposed to diclofenac and

challenged with carrageenan (Figure 2C) (Anova, followed by Bonferroni post-hoc, α = 0.05).

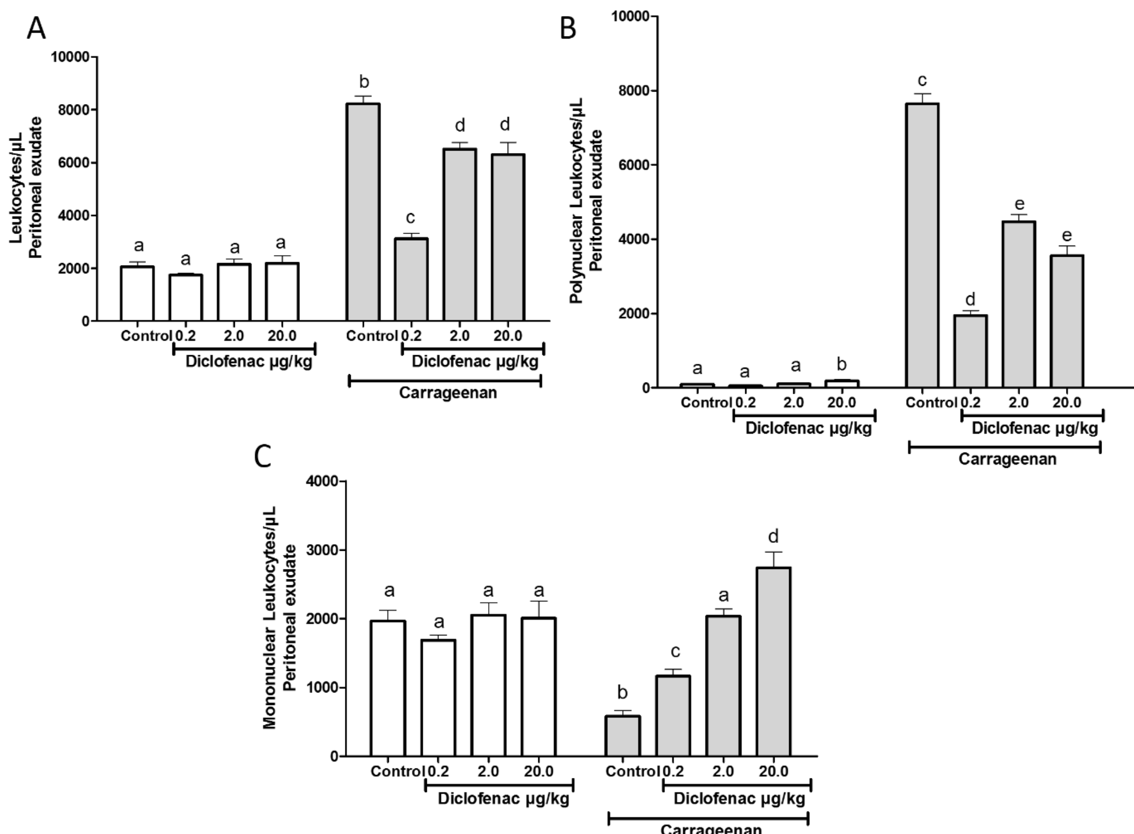

Figure 2 - *Hoplias malabaricus* EXPOSED TO DICLOFENAC AND CHALLENGED WITH CARRAGEENAN. (A) Total cells in fish exposed to diclofenac with and without carrageenan polymorphonuclear differential count in fish exposed to diclofenac with and without carrageenan. (B) Mononuclear differential count in fish exposed to diclofenac with and without carrageenan. (C) One-Way ANOVA followed by Bonferroni's post-hoc test p<0.001. Different letters represent statistically different values.

3.3 Head kidney primary macrophage culture cell and nitric oxide determination

Macrophages cultured from the head kidney of *H. malabaricus* exposed to diclofenac had basal NO production significantly reduced at 2.0 and 20.0 µg/Kg (Figure 3A). After to the LPS-stimulus, the diclofenac inhibited LPS-induced NO production in all the groups compared with LPS control group (Figure 3B) (Anova, followed by Bonferroni post-hoc, α = 0.05).

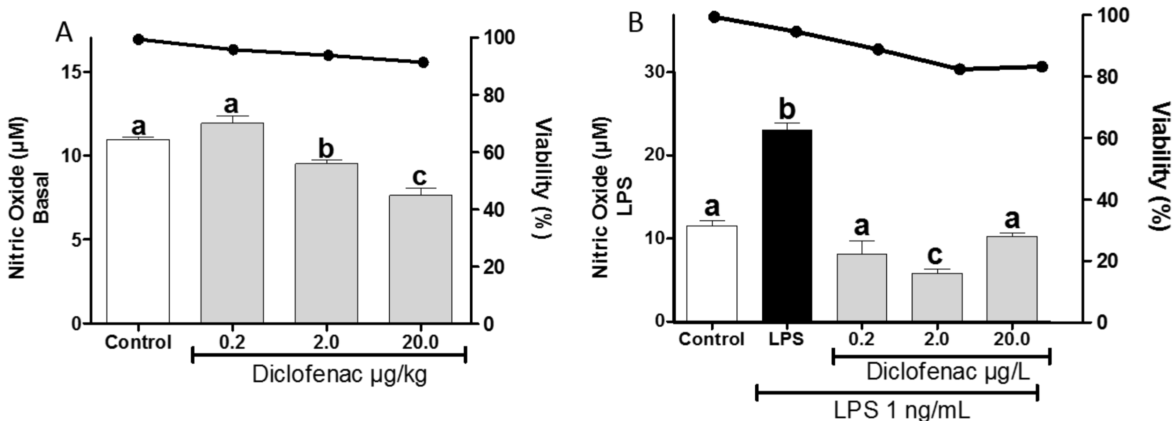

Figure 3 - MACROPHAGE CHALLENGE OF *H. malabaricus* ANTERIOR KIDNEY CELLS. A) Nitric Oxide production after exposure to diclofenac; B) Nitric Oxide production after exposure to diclofenac with LPS-stimulus; One-Way ANOVA followed by Bonferroni´s post-hoc test p<0.001. Different letters represent statistically different values.

3.4 Hepatosomatic Index

Diclofenac reduced the HSI only at the low dose (Figure 4).

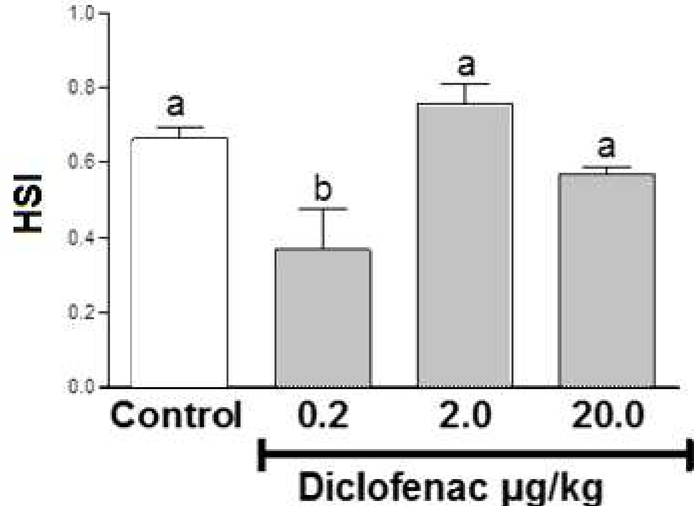

Figure 4 - HEPATOSOMATIC INDEX OF *H. malabaricus* AFTER DICLOFENAC EXPOSURE. One-Way ANOVA followed by Bonferroni's post-hoc test p<0.05. Different letters represent statistically different values.

3.5 Biochemical analysis

In the liver the diclofenac significantly increased the SOD activity, at 2 and 20 µg/Kg doses, the GSH concentration at the 20 µg/Kg and the GPx activity in all doses in relation to the control group, suggesting the generation of free radicals. The LPO was also induced by diclofenac in all exposed groups. Although CAT did not change,

diclofenac reduced the GST activity, demonstrating that inhibited the metabolism of phase II. The EROD activity was not altered by diclofenac (Table 2).

TABLE 2 - BIOCHEMICAL ANALYSIS IN LIVER OF *H. malabaricus* AFTER DICLOFENAC EXPOSURE

	Diclofenac			
	Control	0.2 µg/Kg	2 µg/Kg	20 µg/Kg
SOD (U/mg protein)	68.42 ± 1.50[a]	75.63 ± 4.55[a]	94.70 ± 6.07[b]	114.70 ± 2.57[c]
CAT (µmol/min/mg protein)	84.09 ± 6.22[a]	93.74 ± 9.17[a]	85.38 ± 10.61[a]	99.06 ± 6.45[a]
GPx (nmol/min/mg proteín)	107.50 ± 8.50[a]	161.10 ± 5.58[b]	164.40 ± 6.79[b]	213.40 ± 17.92[c]
EROD (pmol/min/mg proteín)	11.46 ± 2.12[a]	14.83 ±3.76[a]	17.60 ± 3.04[a]	7.56 ± 1.01[a]
GST (nmol/min/mg proteín)	259.00 ± 18.09[a]	41.00 ± 6.09[b]	35.37 ± 2.80[b]	37.90 ± 2.55[b]
GSH (µg/mg protein)	8.78 ± 0.66[a]	8.01 ± 0.86[a]	10.30 ± 1.25[a]	13.07 ± 0.48[b]
LPO (µmol hidroperoxides/mg protein)	7.52 ± 0.57[a]	11.23 ± 0.32[b]	12.15 ± 0.45[b]	8.71 ± 0.60[a]

Values are expressed as mean ± standard error of mean. Different letters indicate statistically significant differences ($p<0.05$). ANOVA, Bonferroni, n=10.

4. Discussion

After a trophic exposure using low doses of diclofenac was observed an increase in level of blood RBC and hematocrit in exposure of 0.2 µg/Kg. Only the hemoglobin reduced at the high dose. There are few studies about the effects of diclofenac on aquatic vertebrates. An increase in the RBC number even at the lowest dose, suggests an increase in the ability of cells to carry oxygen (MARIAPPAN *et al*., 2011), which is corroborated by the increased hematocrit in the same group (0.2 µg/Kg). The decrease of hemoglobin at the highest dose may indicate an anemia process as observed by Saravanan *et al*. (2011) in the exposure of *Cyprinus carpio* to 1, 10 and 100 µg/L of diclofenac during 96 h. This effect also was observed with other toxicants such as heavy metals (LAVANYA *et al*., 2011; SUVETHA *et al*., 2010).

The number of blood leukocytes is a sensitive indicator of stress in fish. Decreased leukocytes counts are indicative of immunosuppression (PIMPÃO *et al*., 2007, SARAVANAN *et al*., 2011). There are several studies that suggest which xenobiotics are capable of inducing immunosuppression in fish. This feature can be detected from decreased leukocyte counts (BARTON, 1991, FISCHER *et al*., 2006, MUÑOZ *et al*., 2014). The polymorphonuclear cells are the most important leukocytes in fish and show great sensitivity to changes in environmental parameters (MUÑOZ *et*

al., 2014). The lymphocytes are the first-line of defense in fish therefore a decrease in the number of mononuclear leukocytes represents a decreased immune response, ultimately predisposing fish to diseases (WEDEMEYER *et al*., 1990; FISCHER *et al*., 2006). In the present study we observed a decreased leukocyte total count and also a decline in the number of polymorphonuclear and mononuclear cells. It is likely that these decreases represent an important change in the fish physiology, contributing to immunosuppression.

Other result that may contribute to immunosuppression is the incapacity of the *H. malabaricus* exposed to diclofenac and challenged with carrageenan for promoting the adequate cell migration especially in an inflammatory process. Inflammatory reactions frequently occur in fish but the mechanisms involved in this process are poorly understood (PIMPÃO *et al*., 2007) include fish exposed to pharmaceutical compounds.The inflammatory reaction enables an organism to defend itself against microbe infection. The migration of leukocytes from the vascular system to sites of pathogenic exposure is a key event in the process of inflammation (WAGNER; ROTH, 2000; COOK-MILLS; DEEM, 2005). Migration of leukocytes is initiated by the process of cell adhesion, followed by transmigration. In general, leukocytes extravasation is a multi-step process that involves the tethering, rolling and activation, firm adhesion to the endothelium, diapedesis and finally transendothelial migration (APLIN *et al*., 1998; SCHUBERT *et al*., 2011). In this work the migration cells decrease in fish exposed to diclofenac, include the polymorphonuclear count demonstrated a possible immunosuppression.

Nitric oxide is an important molecule involved in diverse physiological processes, including vasorelaxation, neuronal communications, inhibition of cell proliferation, and intracellular signaling. Nitric oxide also has potent toxic effects and, as such, is an important component of the arsenal available to animal hosts for effective antimicrobial defenses (RIEGER; BARREDA, 2011); so an imbalance in the NO defense mechanism could predispose the animal to infections.

In the present study, diclofenac decreased NO production in macrophage culture. Other study employing macrophage cell culture also demonstrated an inhibition in the NO production after incubation during 24h with 1µg/mL and 100µg/mL of diclofenac (CIRINO *et al.*, 1996). In the same study, Cirino *et al*. (1996) demonstrated that diclofenac directly inhibited iNOS activity, after induction by LPS-stimulus. Diclofenac (0.38 mM) also decreased activation of Factor Nuclear Kappa B

(NF-kB) in KBM5 cell (leukemic cell line) culture, and levels of iNOS in Raw 264.7 cells, which impaired NO activation in response to LPS (TAKADA *et al.*, 2004). In trout exposed to diclofenac, Mehinto *et al*. (2010) observed a decrease in gene expression of cyclooxygenases (COX 1 and COX 2) in kidney, leading to impairment of immune system.

The production of basal NO or NO LPS-induced by monocytic/macrophages of *H. malabaricus* can be affected by diclofenac, as demonstrated in the present work.

Diclofenac caused a reduction in liver size (HSI) at 0.2 µg/Kg. Exposure of rainbow trout to diclofenac caused a depletion of glycogen in hepatocytes (TRIEBSKORN *et al*., 2004), which could cause reduction of HSI. The hepatocyte growth can be affected by changes in the function of these cells when exposed to NSAIDs (FLIPPIN *et al*., 2007). In exposure for three months at concentrations of 1 to 10,000 µg/L of diclofenac, the fish species *Orizyas latipes*, and Japanese medaka, presented no change in liver somatic index (LEE *et al*., 2011).

Furthermore, diclofenac significantly increased SOD activity at 2 and 20 µg/Kg, GSH concentration at 20 µg/Kg and GPx activity in all doses in relation to the control group, suggesting the generation of free radicals, but not in a dose-dependent manner. There are not many studies of oxidative stress on fish caused by pharmaceuticals. Stepanova *et al.* (2013) observed an increasing of GST of juvenile carp exposed to high concentrations of diclofenac (3 mg/L). The GST activity of fish *Danio rerio* exposed to 40, 120 and 250 mg/L of acetylsalicylic acid also increased (ZIVNA *et al*., 2013). The GST provides cellular protection against toxic effects of a variety of xenobiotics (BARTOSKOVA *et al*., 2014). Dipyrone, other NSAID, diminished the GST activity in *Rhamdia quelen* sub-chronically exposed (PAMPLONA *et al*., 2011).

The superoxide dismutase is the first mechanism of antioxidant defense, converts O_2^- to H_2O_2 (VAN DER OOST *et al.*, 2003). Islas-Flores *et al.* (2013) reported SOD inhibition on brain of *Cyprinus carpio* exposed to diclofenac. In other study, *Cyprinus carpio* exposed to industrial effluent containing NSAIDs had an increase in SOD activity (SAN JUAN-REYES *et al*., 2013).

Glutathione peroxidase is an enzyme which transforms hydroperoxides to hydroxyl compounds using a reduced glutathione as a substrate. As well as diclofenac, the NSAID ibuprofen increased GPx in fish exposed to 0.05; 1; 8 and 25 mg/L (BARTOSKOVA *et al*., 2014).The present study data also showed a decrease in GSH levels in the same concentrations that GPx was diminished. GPx uses GSH as a

cofactor to remove the H_2O_2. Increased of levels of GSH is due to induction of antioxidant system. Several authors previously described the importance of GST, GPx, and GSH in preventing cellular damages (SILVA *et al*., 2011).

Lipid peroxidation has been a major contributor to the loss of cell function under oxidative stress. Diclofenac caused hepatic LPO in all groups exposed. Bivalves exposed to a concentration of 1 to 1000 µg/L diclofenac during 96 h, increased LPO (QUINN *et al.*, 2011) and amphipods *Hyalella azteca* suffered oxidative stress by increasing the LPO and SOD when exposed to artificial sediment containing diclofenac (OVIEDO-GÓMEZ *et al*., 2010).

5. Conclusion

This study highlighted that trophic exposure to diclofenac caused alterations in hematological parameters, migration cells, NO productions in kidney primary culture cell and in liver cause oxidative stress to *H. malabaricus*. These effects may cause negative impacts in aquatic organisms.

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CAPÍTULO III

EFEITO IMUNOSSUPRESSOR DO DICLOFENACO EM PEIXES NEOTROPICAIS

Immunosuppressive effects of diclofenac in a freshwater fish: a proteomic approach

Abstract

Drugs for human and veterinary use, such as anti-inflammatory drugs, have been frequently found in water bodies. In the present study, the effects of diclofenac in some components of the immune system of a tropical fish species were evaluated after hydric exposition. *Rhamdia quelen* fish (male and female) were exposed to 3 concentrations of diclofenac (0.2; 2.0 and 20.0 µg/L) for 14 days. After exposure, the fish were anesthetized, blood was taken from caudal vein and the anterior kidney was collected. Plasma and kidney proteins were analyzed using liquid chromatography tandem mass spectrometry in a shotgun proteomic approach to focusing on proteins involved in nitric oxide production, migration cell and complement system activation. In plasma of *R. quelen*, the expression toll like receptor 2 (Tlr2), phospholipase Cγ (Plcγ), kinase kinase kinase 3 (Mekk), 1-phosphatidylinositol 3-kinase (Pi3k), activator protein-1 (Ap-1), nuclear factor of Kappa light polypeptide (Nf-kb), and the NO synthase inducible protein (iNOS) were significantly inhibited by exposure to diclofenac. In the head kidney, the expression of Tlr2, Plcγ, Mekk, Pi3k, Ap1 and Nf-kb were also significantly inhibited. Various proteins involved in cell migration were detected in the plasma. In male fish, the expression of Chemokine receptor 4 protein (Cxcr4), Integrin α1 (ITα1), Radixin (Rdx) and Matrix Metallopeptidase (Mmp)-17 was inhibited. In female fish, the expression of Cxcr4, Itga1, Rdx, Mmp17 and Mmp1 decreased. In the present study, the expression of complement component 3 protein (C3), complement factor B (Cfb) and mannan-binding lectin serine peptidase 1 (Masp1) changed as well as C1q and complement component 7 (C7). In addition, MHC1 in plasma significantly decreased. In summary, the diclofenac exposure inhibited the expression of many proteins involved in NO synthesis, cell migration and activation of the complement system in fish which may compromise innate immune defense mechanisms in these animals.

Keywords: Emerging contaminants, diclofenac, migration cell, Nitric Oxide production, Complement system, immunosuppression

1. Introduction

The contamination of freshwater with a wide range of pollutants has become a matter of concern over the last few decades (ABOUD, 2010). Numerous environmental chemicals like pharmaceuticals drugs have been identified as emerging and persistent

toxicants. These chemicals are known by their potential to modulate immune system components with adverse consequences to individual’s resistance (SEGNER, 2011). The suppression of immune system by these pollutants is not fully understood (ABOUD, 2010).

Studies have shown that the amount of pharmaceutical drugs found in water bodies has increased all the world, becoming a growing environmental problem and a major source of pollution (OVIEDO-GÓMEZ, 2010; KOLPIN, 2004) especially from urban, industrial and hospital wastewater (PAROLINI, 2009). These compounds have been identified as emerging contaminants in aquatic ecosystems (KIM *et al.*, 2007; ZUCCATO, 2006). The most frequently detected pharmaceuticals groups detected are the nonsteroidal anti-inflammatory drugs (NSAIDs), a class of pain relievers which blocks cyclooxygenase (COX) enzymes. The COX enzyme family catalyzes the synthesis of prostaglandins from arachidonic acid (KHETAN; COLLINS, 2007; VAN HECKEN *et al*., 2000). Some studies report that traces of NSAIDs in environment may elicit toxic effects, including immunological changes (KHETAN; COLLINS, 2007).

Diclofenac is a widely used NSAID all over the world, especially in chronic inflammatory conditions and is one of the most prevalent in aquatic environment (MYCEK, 2004). It has been identified as one of the most important pharmaceutically active compounds present in the aquatic environment (LETZEL; METZNER; LETZEL, 2009). Low levels of diclofenac (µg/L) have been detected in the influents and effluents of municipal sewage treatment plants and also in surface waters (ng/L - µg/L) (LETZEL; METZNER; LETZEL, 2009). Global concentrations of diclofenac in the water bodies range from 0.2 to 2.3 µg/L (BILA; DEZOTTI, 2003; HONG *et al*., 2007; WHO, 2012).

Despite these high levels of contamination, few studies have attempted to characterize the health risks associated with exposure to pharmaceutical products in water. Kumar and Xagoraraki (2010) showed a quantitative pharmaceutical risk assessment after accidental contamination of stream water by fish consumption and direct ingestion of finished drinking water in children and adults (KUMAR; XAGORARAKI, 2010). Results of their studies have identified none potential risk of adverse effects for human populations. However, fish are particularly sensitive to water contamination and pollutants including drugs for human and veterinary use, which may impair many physiological and immunological parameters (OAKES; VAN DER KRAAG, 2003; SILVA DE ASSIS *et al*., 2013a). In addition, it should be considered

that the fundamental immune molecules and mechanisms are similar in fish and mammals (RAUTA, 2012) and therefore, even though some adverse effects are not observed in humans, long-time exposure can also affect our species in the future.

In fish, nonspecific immunity is a fundamental defense mechanism (URIBE, 2011). The innate response has been considered an essential component in combating pathogens due to limitations of the adaptative immune system (WHYTE, 2007; URIBE, 2011). However, the intensity of this immunologic reaction has been shown to vary between different species and environmental conditions (WHYTE, 2007). Fish health depends on the interrelationship of some major components of the fish and the environment in which they live (KUM; SEKKIN, 2011). The environment may be the most critical component of the fish health especially because the environment and the pollutants may influence the fish’s immune physiology and the ability to maintain natural and acquired resistance and immunity (PLUMB; HANSON, 2011).

Previous studies of our group have shown that fish exposed to diclofenac exhibited a reduction in NO production (RIBAS *et al*., 2014), circulating leukocytes and abnormal cell migration after challenge with carrageenan (data not published).

Thus, the aim of the present study was evaluate the effects of diclofenac exposure on the immune system of *Rhamdia quelen*, in proteins related to NO production, cell migration and complement system activation using a proteomic approach.

2. Material and methods

All procedures and protocols were approved by the Institution’s Ethical Committee for Animal Use and are in accordance with international guidelines for Animal Use.

2.1 Bioassay

In the present study, exposure concentrations were selected because they are representative of concentrations detected in the aquatic environment (typical concentrations of diclofenac are 2.0 μg/L) (BILA; DEZOTTI, 2003; HONG *et al.*, 2007).

Thus, a range of concentrations ten times lower and ten times higher (0.2, 2.0 and 20.0 µg/L) were selected for waterborne test exposures.

Rhamdia quelen fish were selected because exhibits a large ecological plasticity, with a wide distribution in Brazilian rivers and reservoirs (HENSLEY; MOODY, 1975). It is a omnivorous fish, endemic from South America and of economical importance in the Southern Brazil (BARCELLOS *et al*., 2001). The responses of this species after exposure to environmental pollutants has been studied for some authors (BECKER *et al*., 2009; DOS SANTOS MIRON *et al*., 2004; MELA *et al.*, 2013; MIRON *et al.*, 2005).

2.2 *Rhamdia quelen*

Rhamdia quelen weighing 80±10g were purchased from a Krahu Commercial Farm, Pomerode, Santa Catarina, Brazil. The fish were kept at 28±2°C in filtered water on a simulated natural photoperiod of 12-12 hlight:dark cycle (lights on at 0700 AM), and they were fed *ad libitum* with commercial feed once a day. The fish were acclimated to this environment for at least three weeks prior the use in experiments.

After the acclimation period, fish were divided into 4 experimental groups (0.2 µg/L, 2.0 µg/L and 20 µg/L of diclofenac plus one control group of male and female fish). One third of the water volume in each tank was replaced twice daily in order to maintain the diclofenac concentration. Male (n=20) and female (n=20) fish were exposed to each diclofenac concentration. After 14 days of exposure, the fish were anesthetized in water with benzocaine 1% (Sigma) and blood was taken from caudal vein. The fish were then euthanized by medullar section, and the anterior kidney was removed. All the samples were immediately frozen at -80ºC until analysis.

2.3 Proteomic analyses

Samples were prepared according to previously established methods (SIMMONS *et al*., 2012; SILVA DE ASSIS *et al*., 2013b). Head kidney tissue samples were added to microcentrifuge tubes and 10x the volume (v/w) of Triethylammonium bicarbonate buffer (TEAB) was added to each tube along with two 4mm stainless-steel balls. Tissues were homogenized using a ball mill (Retsch MM300) for 1 min at 20 Hz. Tubes were centrifuged for 15 min at 14,000 x *g* at 4 °C and the supernatant was

collected into low-density microcentrifuge tubes. Protein concentrations were estimated by light absorbance at 280 nm (Thermo Scientific Nanodrop 1000). Approximately 1mg of total protein of each kidney homogenate or 15 µL of plasma were transferred to a low-retention microcentrifuge tube after used for subsequent digestion.

All samples were then diluted with TEAB to a total volume of 50 µL, followed by the addition of 2.65 µL of 100 mM Tris(2-carboxyethyl)phosphine (TCEP) in TEAB to each tube, which were then vortex mixed, and left to incubate for 1 h to reduce disulfide bonds. To acetylate cysteine residues, 2.8 µL of 200 mM Iodoacetamide (IAA) in 50 mM Tris-HCl (pH = 7.8) was added to each tube, mixed by vortex, and then left to incubate for another hour. A 50 µL portion of 20% v/v formic acid was added to each tube, mixed thoroughly by vortex, and then left to digest on a heating block for 30 min at 115 °C. The resulting peptide mixtures in each tube were then evaporated to near dryness using centrifugal evaporation (Savant Instruments, Inc., model AES1010-120) for approximately 45 min and then resuspended by vortex in 20 µL of 5% acetonitrile, 95% water, and 0.1% formic acid. Finally, the tubes were centrifuged for 10 min at 15,000 x *g* to remove debris, and the supernatant was transferred from each tube into glass chromatography vials containing 200 µL polypropylene conical vial inserts for subsequent analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Peptides were separated on a Zorbax, 300SB-C18, 1.0 mm × 50 mm 3.5 µm column, with a thermostat controlled column temperature of 40 °C, using the Agilent 1260 Infinity Binary LC system. The Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) was used as the detector in tandem to the Agilent 1260 system. The pump timetable was the following: 0−2 min 2% solvent B, 2−22 min 2−40% solvent B, 22−27 min 40−60% solvent B, 27−32 min 90% solvent B, 32−50 min 5% solvent B. A blank, peptide standard and BSA digest standard injection was performed every 10 samples in order to monitor baseline, carry-over, drift, and sensitivity during the runtime. Reference mass correction was enabled using the dual-ESI reference nebulizer for m/z 121.051 and m/z 922.010.

Both centroid and profile mass spectral data files were collected using Mass Hunter Data Acquisition Software (Version B.02.00). Data files were pooled into groups by treatment, and database search was performed on each group separately. Spectrum Mill Software (Version A.03.03 SR4) was used to extract good quality

spectra and filter noise spectra and poor quality spectra from raw data files, sequence peptide sequences, and then search protein databases. All Spectrum Mill MS/MS search settings were left to default values except; spectra were searched using reversed database scores and dynamic peak thresholding, and a precursor mass tolerance of ± 20 ppm and product mass tolerance of ± 50 ppm. Mixed acetamide (C), oxidized methionine, pyroglutamic acid (NtermQ), deamidated (N), phosphorylated (S, T, and Y), and ubiquitination-GG (K) were specified as variable modifications. Proteins were searched using a subset database that contained only protein sequences of teleost fish species from within the NCBInr and Swissprot databases. Proteins were validated manually and accepted when matched to multiple members of a protein family or it had a summed score greater than five and a minimum of one peptide with a %SPI of greater than 60% (as recommended for Agilent Q-TOF data). Mean spectral intensity values were calculated by the Spectrum Mill Software and are defined as the sum of intensity for all spectra of peptides belonging to the protein, divided by the number of spectra. Peptide intensities were calculated from the sum of the precursor m/z abundance from the MS scans. The mean spectral intensity from all peptides detected for each protein was used as a relative quantitative measure of protein expression.

Valid protein matches were analyzed through the use of Ingenuity Pathways Analysis (IPA, 2013) Data was pooled for each treatment, and was then uploaded into the application using corresponding gene identifiers and expression values (mean peptide spectral intensity values). Each identifier was mapped to its corresponding molecule in Ingenuity’s Knowledge Base. These network eligible molecules were overlaid onto a global molecular network developed from information contained in Ingenuity’s Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. Functional analysis identified the biological functions and/or diseases that were most significant to the data set based upon p-values obtained by the IPA software using the right-tailed fisher exact test and the Ingenuity Knowledge Base.

2.4 Statistical analysis

Data are presented as mean ± standard error mean (SEM) values for each condition. Kolmogorov-Smirnov normality test was used and comparisons among groups were done by Kruskal-Wallis test followed by Dunn's *post hoc* tests. The χ^2 test was performed to identify significant differences in the number of proteins that were significantly associated with a particular biological function compared to the total number of proteins among treatments. Differences of p-value < 0.05 were considered significant. The statistics tests were performed using GraphPad Prism version 5.00 for windows (Graph Pad Software, USA).

3. Results

3.1 Effects on NO production-related proteins

Since our previous results had shown that diclofenac exposure impairs LPS-induced NO production by macrophages from head kidney of fish, shotgun proteomics was employed in the present study to determine which proteins related to NO production could be affected by diclofenac exposure. Many of these proteins, particularly the kinases and the transcription factors are also involved in other processes. In the plasma of male fish exposed to diclofenac, expression of phospholipase Cγ (Plcγ), kinase kinase kinase 3 (Mekk), 1-phosphatidylinositol 3-kinase (Pi3k), and the NO synthase inducible protein (iNOS) were significantly reduced (Figure 1). Unexpectedly, in females, Plcγ and Mekk expression was not changed by the exposure to diclofenac (Figure 1). In contrast to the obtained results for females, the expression of the enzyme serine/threonine-protein kinase (Akt) was significantly increased in the male fish exposed to 20 µg/L of diclofenac (Figure 1) (Kruskal-wallis, followed by Dunn's post-hoc, α = 0.05).

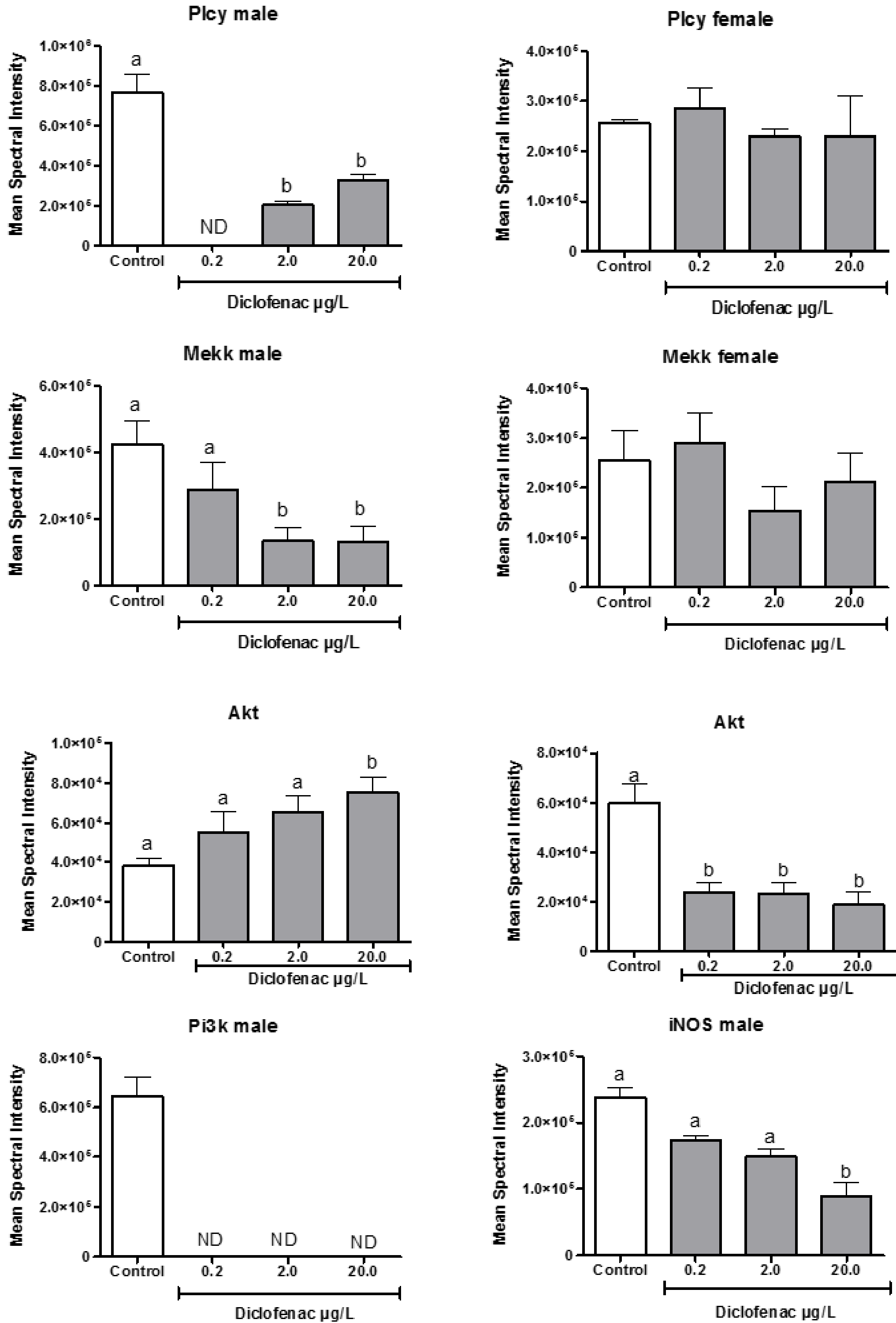

Figure 1 - EXPRESSION OF PROTEINS RELATED TO THE NITRIC OXIDE PRODUCTION IN PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Phospholipase Cγ (Plcγ, male and female), Protein Kinase Kinase Kinase (Mekk, male and female), serine/threonine protein kinase (Akt, male and female), 1-phosphatidylinositol 3-kinase (Pi3k, only in males) and Nitric Oxide Synthase (iNOS, only in males) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

Additionally, in male fish plasma, expression of the transcription factor nuclear factor κB light peptide (Nf-κb) was reduced only in the higher concentration of diclofenac while activator protein-1 (AP1) was roughly reduced in all exposure concentrations tested (Figure 2) (Kruskal-wallis, followed by Dunn's post-hoc, α = 0.05). In contrast with the enzymes showed previously, the reduction observed in the transcription factor Nf-κb in females was more severe than in males (Figure 2).

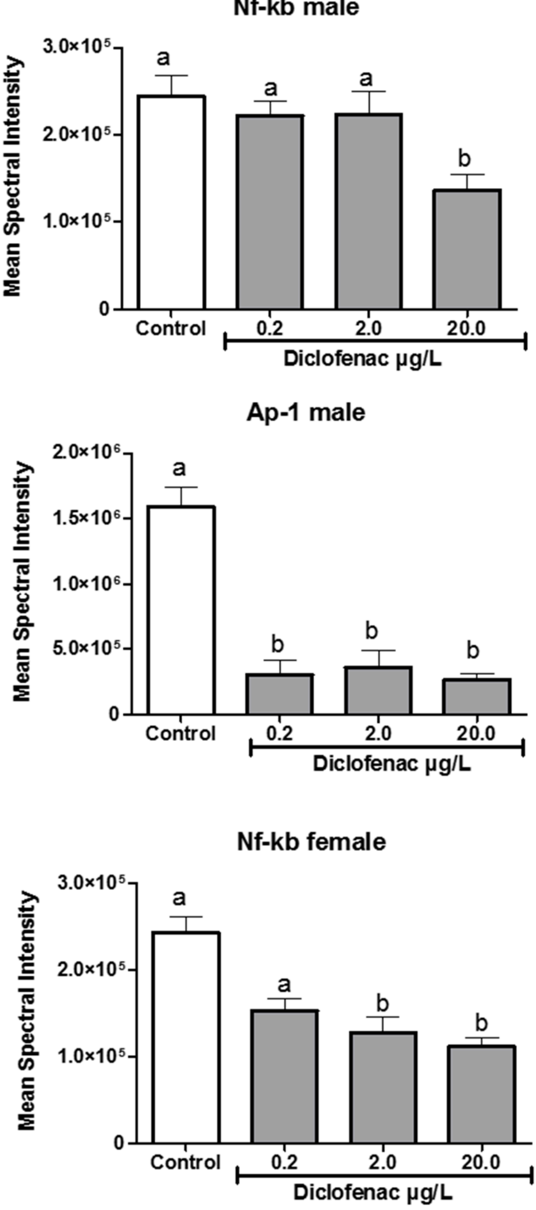

Figure 2 - EXPRESSION OF TRANSCRIPTION FACTORS RELATED TO NITRIC OXIDE PRODUCTION IN THE PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of nuclear factor κB light peptide (Nf-κb, males and females) and Activator protein-1 (males) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

In the head kidney of male *Rhamdia quelen* exposed to diclofenac, the expression of the Plcγ, Mekk, Pi3k were also significantly inhibited for all concentrations tested (Kruskal-wallis, followed by Dunn's post-hoc, α = 0.05) (Figure 3). Similar results were observed for female fishes concerning Plcγ and Pi3k (Figure 3).

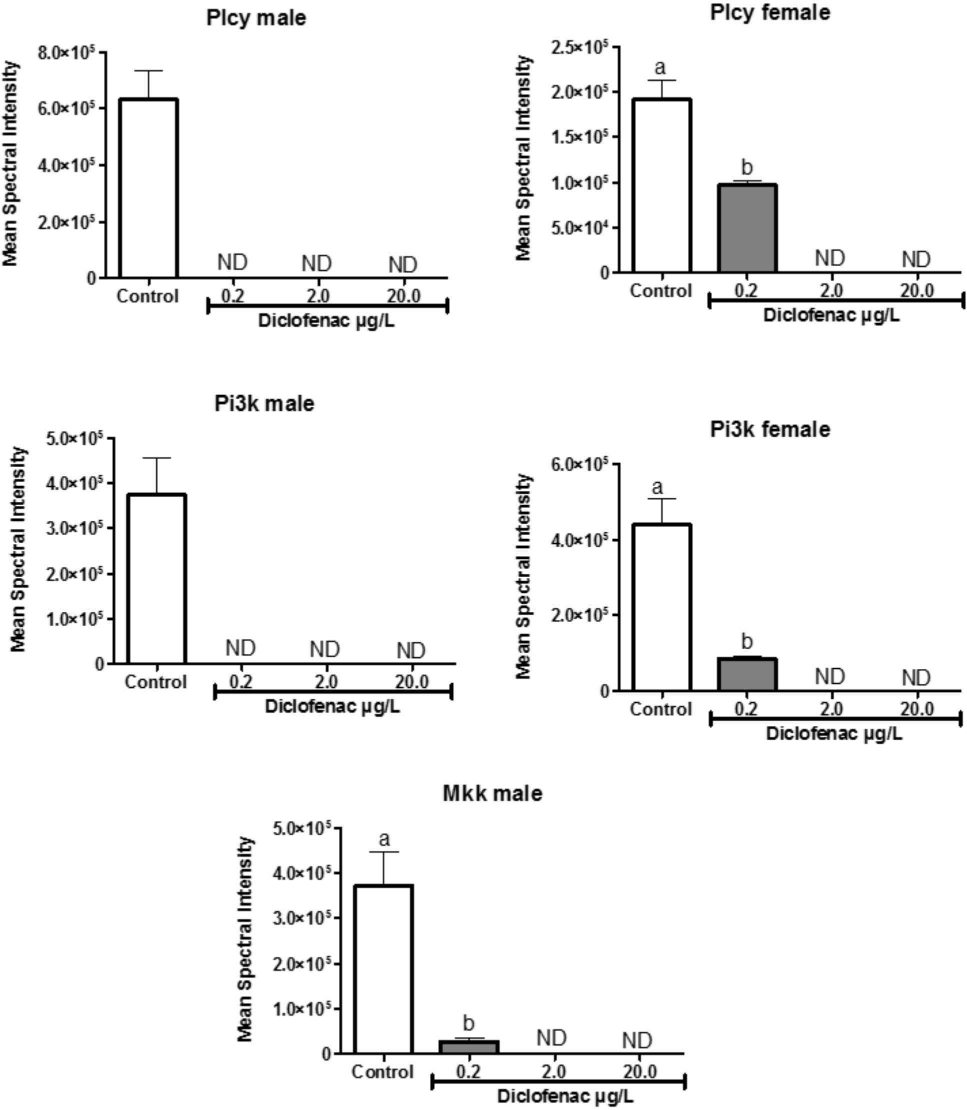

Figure 3 - EXPRESSION OF PROTEINS RELATED TO THE NITRIC OXIDE PRODUCTION IN THE HEAD KIDNEY OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Phospholipase Cγ (Plcγ, male and female), Protein Kinase Kinase Kinase (Mekk, only in male), and 1-phosphatidylinositol 3-kinase (Pi3k, in male and female) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

Similarly to plasma results, the expression of the transcription factor nuclear factor κB light peptide (Nf-κb) reduced at the higher concentration of diclofenac while activator protein-1 (Ap1) was reduced in all exposure concentrations tested (Figure 4). The reduction observed in the transcription factor Nf-κb in females was more intense than in males (Figure 4).

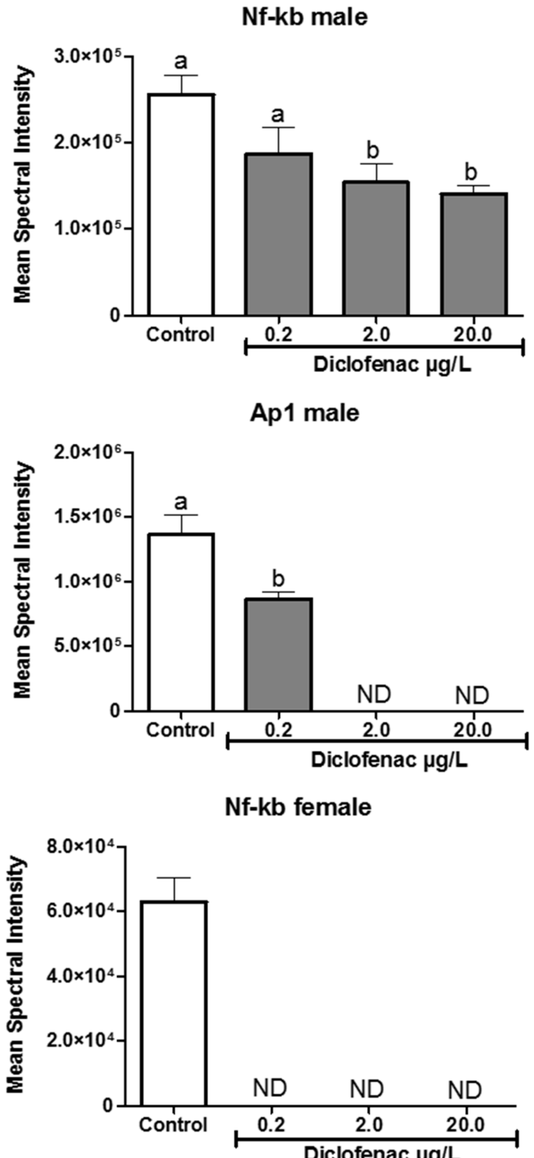

Figure 4 - EXPRESSION OF TRANSCRIPTION FACTORS RELATED TO NITRIC OXIDE PRODUCTION IN THE HEAD KIDNEY OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of nuclear factor κB light peptide (Nf-κb, males and females) and Activator protein-1 (Ap1 - males) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

The activation of Plcγ and Pi3k is macrophages can be done through the activation of toll-like receptors (TLR). Interestingly we found that in female fish plasma, even though Plcγ was not altered as it was in males, Tlr2 expression was reduced in all doses tested (Figure 5). An even more intense reduction of this receptor expression was seen in the head kidney (Figure 5).

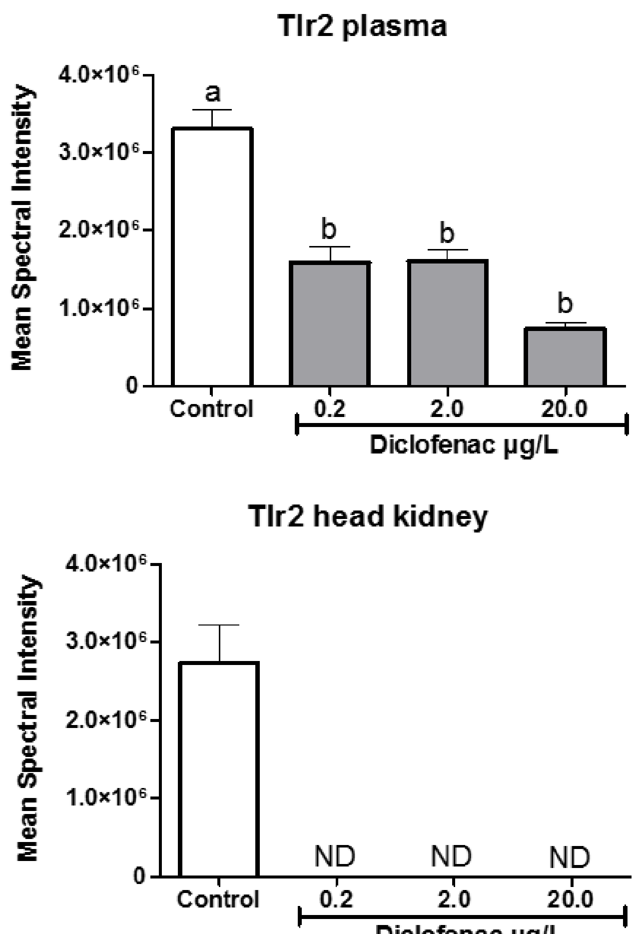

Figure 5 - EXPRESSION OF Tlr2 IN THE PLASMA AND HEAD KIDNEY OF FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Tlr2 is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

A visual pathway containing the proteins involved in NO production that were detected in the present study in male and female fish exposed to diclofenac is presented in Figure 6.

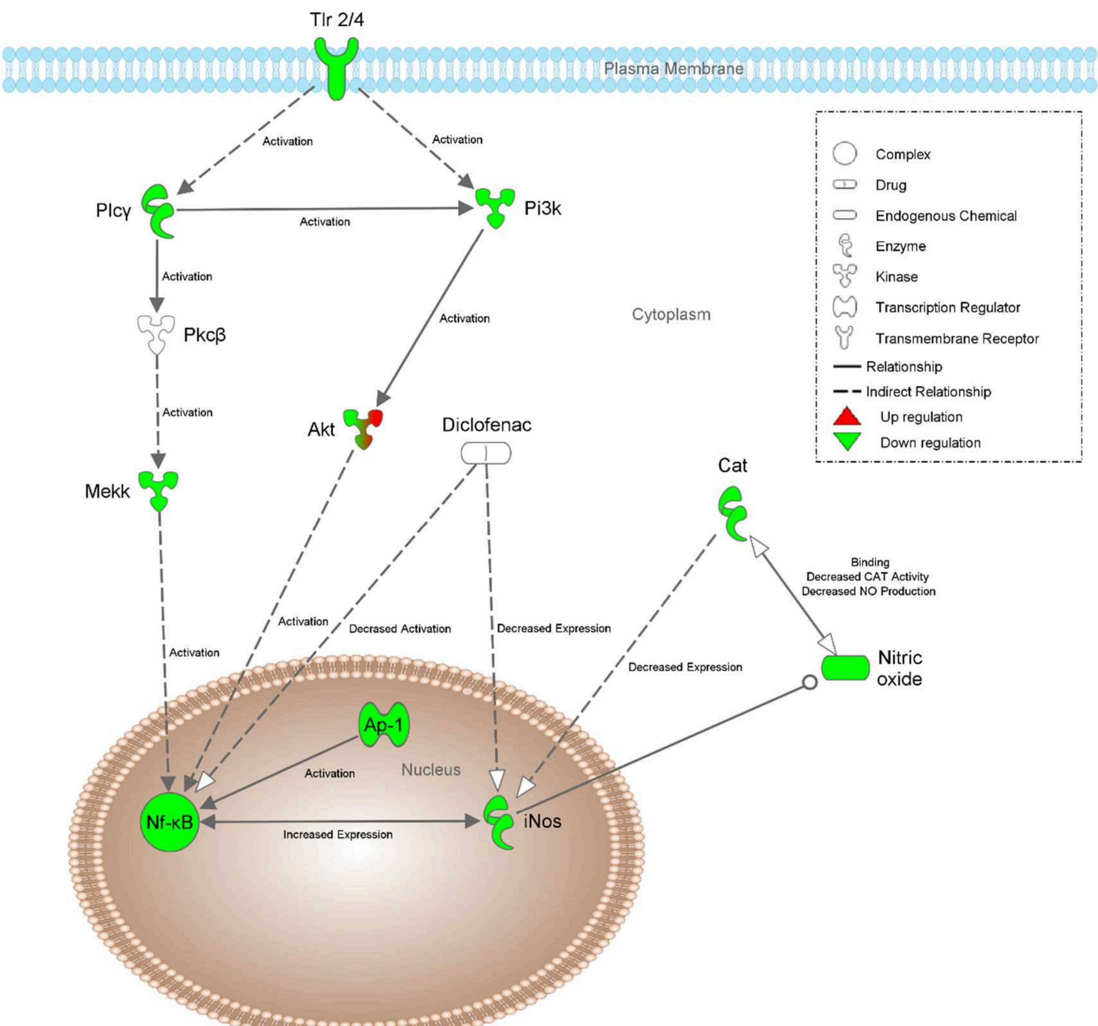

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Figure 6 - VISUAL PATHWAY OF THE PROTEINS INVOLVED IN THE NITRIC OXIDE PRODUCTION FOUND IN MALE AND FEMALE FISH PLASMA THAT ARE KNOWN TO INTERACT WITH OR BE AFFECTED BY DICLOFENAC. Arrows with dotted lines represent indirect associations and arrows with solid lines represent direct relationships. The direction of the arrow indicates which molecule is known to affect the other. Relationship lines that point to the same molecule from which they originate indicate a feedback mechanism. Relationship lines with a flat head instead of an arrow indicate a negative or inhibitory relationship. Legend: Tlr 2/4= Toll Like Receptor 2 and 4; Plcy = phospholipase C gamma; Pkcβ= Protein Kinase C Beta; Mekk = kinase kinase kinase 3; Ikb = Nuclear factor of Kappa light polypeptide inhibitor; Nf-kb = Nuclear factor of Kappa light polypeptide; iNOS = nitric oxide synthase inducible; Pik3 = 1-phosphatidylinositol 3-kinase; Akt = serine/threonine-protein kinase; Cbp=Creb binding protein; Ap-1= activator protein-1.This Figure was generated by the Ingenuity Knowledge Base (IPA, Ingenuity Systems).

3.2 Effects on cellular migration-related proteins

Among the various proteins involved in cellular migration, some proteins were found in plasma of *Rhamdia quelen* in the present study. In male fish exposed to diclofenac, the expression of Chemokine receptor 4 protein (Cxcr4) was inhibited at the higher concentration while the expression of Integrin alpha 1 (Itga1), Radixin (Rdx), and Matrix Metallopeptidase 17 (membrane-inserted, Mmp17) was inhibited was inhibited at all concentrations tested (Kruskal-Wallis test followed by Dunn's post hoc test, α = 0.05) (Figure 7). Matrix Metallopeptidase 1 (interstitial collagenase, Mmp1) was not significantly different in males after exposure to diclofenac (Kruskal-Wallis, followed by Dunn's post-hoc, α = 0.05) (Figure 7). Similar results were obtained in the plasma of female fish (Figure 7).

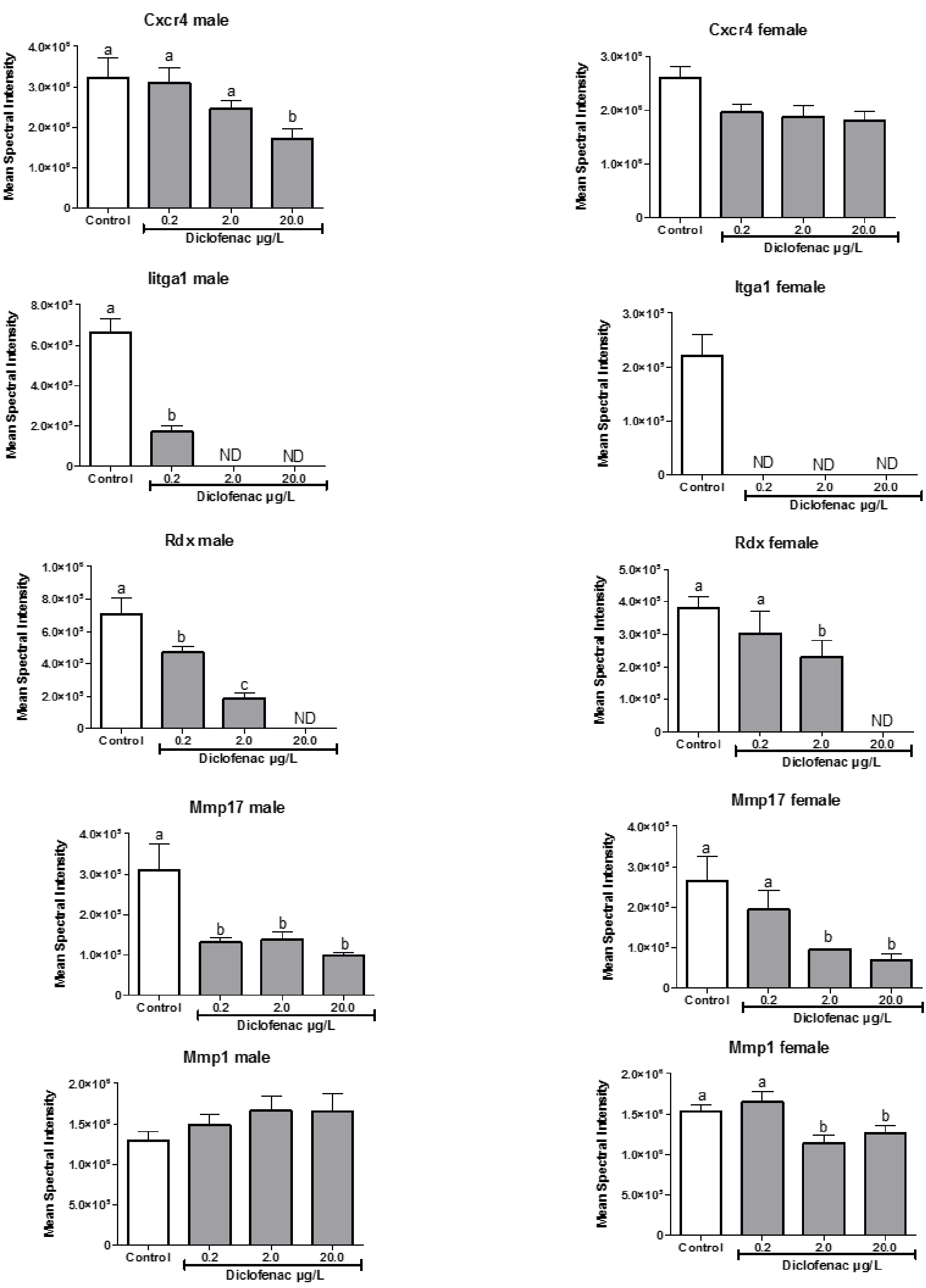

Figure 7 - EXPRESSION OF PROTEINS RELATED TO CELLULAR MIGRATION IN THE PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Chemokine receptor 4 protein (Cxcr4), Integrin alpha 1 (Itga1), Radixin (Rdx), Matrix Metallopeptidase 17 (Mmp17) and Matrix Metallopeptidase 1 (Mmp1) in both male and female fish is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

3.3 Effects on complement system-related proteins

The effects of diclofenac exposure on the complement system-related proteins were more variable and more complex than for the previous ones. In male fish plasma, while the complement component 1 (C1q) and the mannan-binding lectin serine peptidase 1 (Masp1) expression were reduced the expression of complement factor B (Cfb) was increased (Figure 8). The complement component 3 protein (C3) expression was unchanged (Figure 8). For the female fish the expression of C3, C1q and Cfb was reduced (Figure 8).

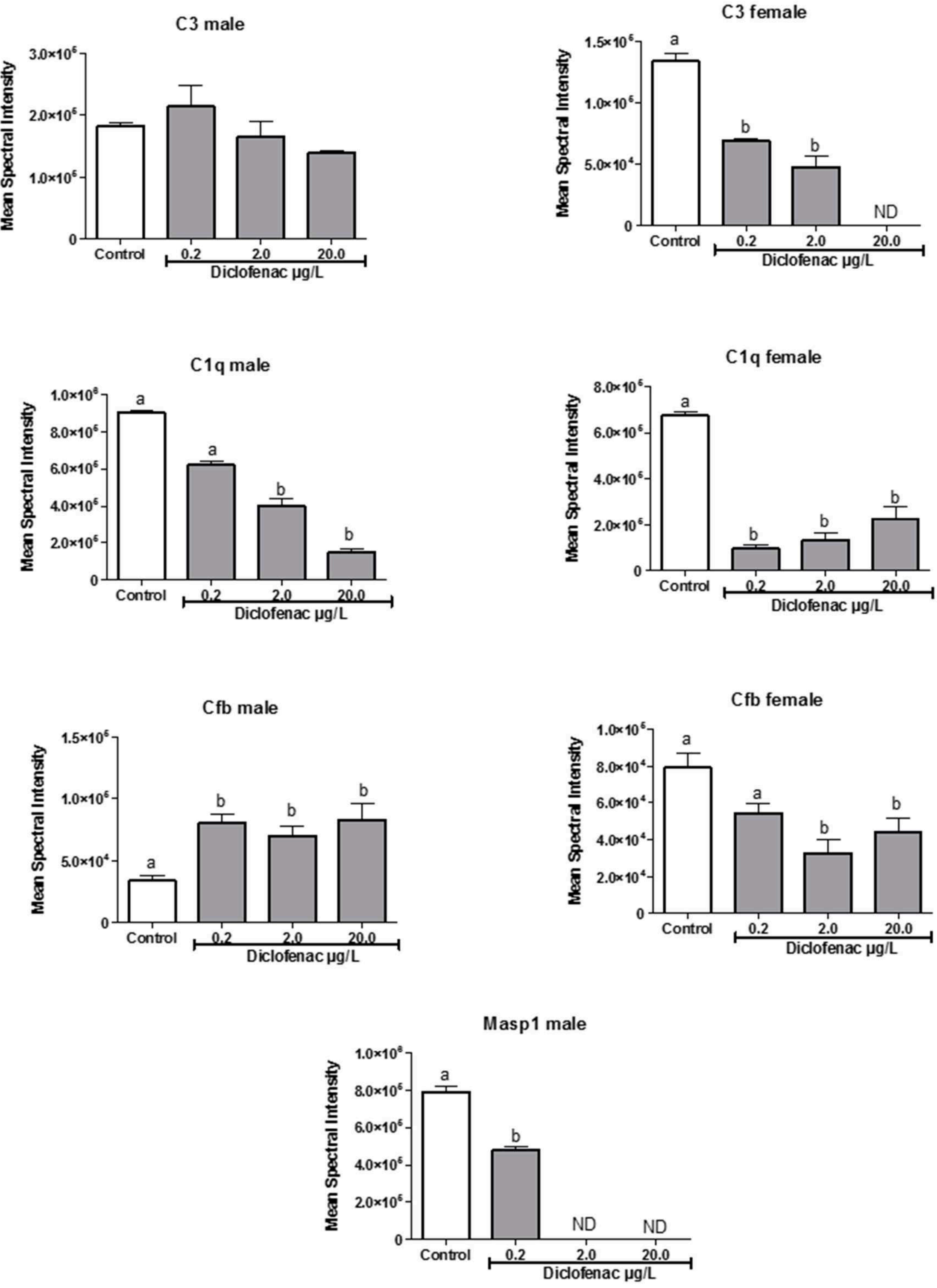

Figure 8 - EXPRESSION OF PROTEINS RELATED TO THE COMPLEMENT SYSTEM IN THE PLASMA OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Complement component 3 protein (C3, male and female), Complement component 1 protein (C1q, male and female), Complement Factor B (male and female) and Mannan-Bilding Lectin Serine Peptidase 1 (Masp1, only in males) is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

3.4 Effects on the Class I Major Histocompatibility complex

In both in male and female fish, the expression of MHC1 in plasma and kidney was significantly reduced in all concentrations of diclofenac tested (Figure 9) (Kruskal-Wallis, followed by Dunn's post-Hoc, α = 0.05).

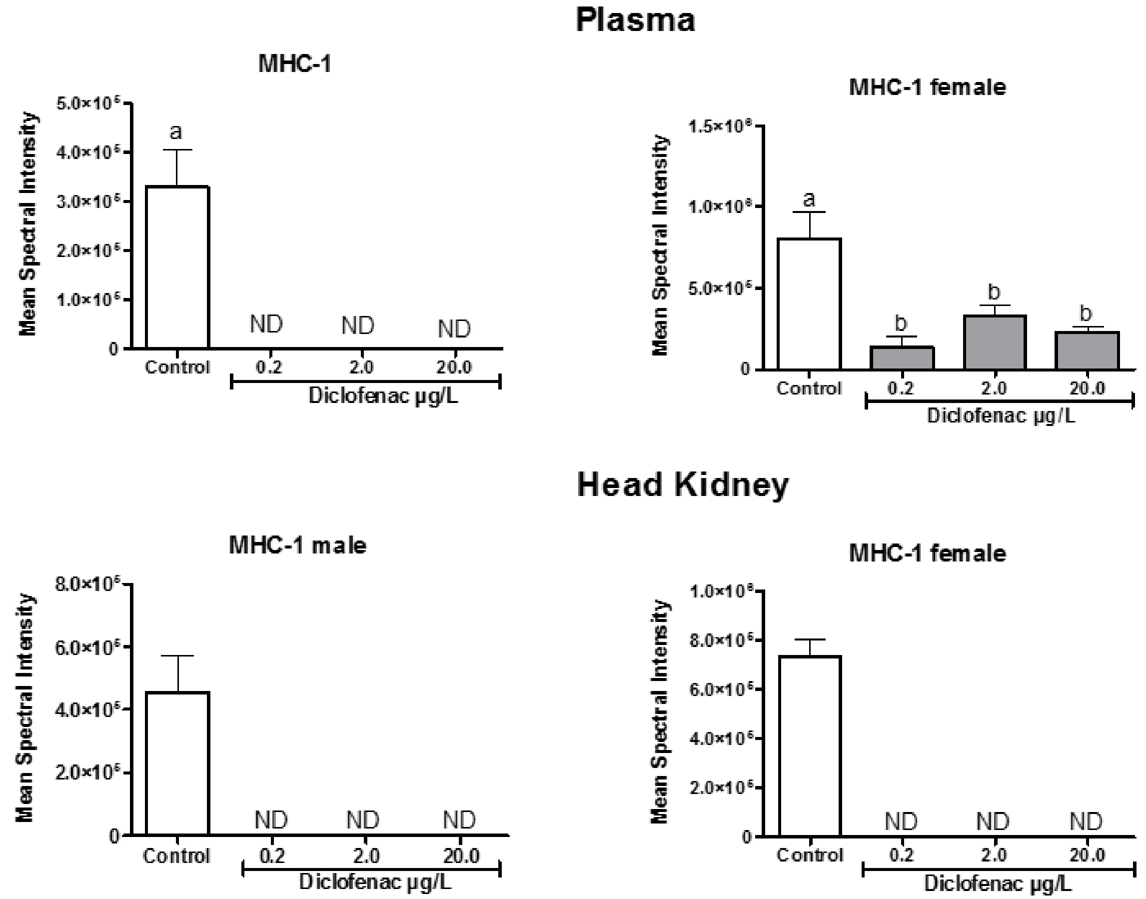

Figure 9 - EXPRESSION OF CLASS I MHC IN THE PLASMA AND KIDNEY OF MALE AND FEMALE FISH EXPOSED TO DICLOFENAC. The mean of spectral intensity of Class I MHC is shown. Kruskal-Wallis test followed by Dunn's post hoc test was used for statistical analysis and different letters represent statistically different values, $p < 0.05$. ND = Not detected.

4. Discussion

4.1 Nitric oxide production

Nitric oxide is an important molecule involved in several physiological processes, including vasorelaxation, neuronal communication, inhibition of cell proliferation, and intracellular signaling. Nitric oxide also has potent toxic effects and,

as such, is an important component of the arsenal available to animal hosts for effective antimicrobial defenses (RIEGER; BARREDA, 2011). For this reason an imbalance in the NO defense mechanism could predispose the animal to infections. In our experiments, we found significant changes in the expression of proteins known to be involved in NO production after diclofenac exposure, especially a decrease in expression of several enzymes which are involved in the final steps of NO production.

The stimulation of Plcγ leads to increased of activity of the protein kinase Cβ, which increases activity of Mekk protein (ZHOU *et al*., 2006). Mekk phosphorylates the Ikk complex, which ultimately increases the phosphorylation of Ikb protein (LI; VERMA, 2002) and, as a consequence, activates Nf-kb (CHAN *et al*., 2001). Increased activity of the Nf-kb protein is thought to increase expression of several proteins including of iNOS protein, and therefore the NO production (MEMET, 2006; ZHOU *et al*., 2008). In an alternative pathway, the phosphorylation of Plcγ causes increased activation of phosphorylated Pi3k complex (CHAO, 2003; CHAO *et al.,* 2006) that increases activation of Akt protein (DARIEVA *et al*., 2004; BOULBES *et al.,* 2011).

In the present study we show that the exposure of male fishes to diclofenac inhibited the expression of Plcγ, even at lower doses and in both plasma and kidney, which could indirectly promote a decrease in Mekk phosphorylation and activity. This decrease could result in decreased Nf-kb activation and consequently reduced iNOS expression. However, diclofenac reduced not only this first component of the NO production mechanism. The expression of the following components such as mkk, Pi3k, Nfκb and Ap-1 were also reduced. Another protein apparently inhibited by diclofenac in the present study was Ap1 (activator protein 1). The expression of Ap1 is known to increase by Pi3k activation (PERON *et al*., 2001; KIM *et al*., 2012). The expression of Akt increased while the expression of Pi3k decreased. Although Pi3k is cited as the main regulator of Akt (CHEN *et al*., 2001), other studies relate that E-cadherin (MUNSHI *et al*., 2002) and Hb-egf protein (MEHTA; BESNER, 2005) can both increase Akt protein expression regardless of Pi3k regulation. In the present study, diclofenac affected Pi3k negatively, but it seems to affect positively other protein intermediaries, leading to an increased expression of Akt. Additionally, the activation of Akt is known to result in the increased activation of Nf-kb complex in nuclei and thus increased expression of iNOS (WRIGHT; WARD, 2000; HSU *et al*., 2007). Even though the Akt expression had augmented at the higher dose of diclofenac, the

addictive effect of all proteins reductions would not be compensated. These results are in accordance with our previous study (RIBAS *et al.*, 2014). In previous studies, diclofenac (0.38 mg/L) decreased activation of Nf-kb in cultured kbm5 cells (TAKADA *et al.*, 2004) and the levels of iNOS in raw 264.7 cells (mouse leukaemic monocyte macrophage cell line), impairing NO production in response to LPS (VILLALONGA *et al.,* 2010).

The changes observed in the plasma of male fishes were similar to that observed in the kidney being more evident in this organ for some protein such as Mkk and Nfκb. These results confirmed the effects of diclofenac in the doses used in this species. Interestingly, some proteins in plasma of female fishes were less affected by the exposure to diclofenac such as Plcγ and Mkk. However, even in plasma reduced levels of Nfκb and Akt were observed. In addition, an important reduction in kidney proteins in female fish was observed in a similar manner that in males. These results suggested that, even though some gender differences may exist, both male and female may have an impaired production of NO after exposure to diclofenac.

The Tlr2 protein, which is located in the plasma membrane of macrophage cells, is known to increase expression of Plc gamma (ZHOU *et al*., 2006) and the expression of Pi3k (LEE; PARK; KIM, 2008). In the present study, diclofenac inhibited the Tlr2 protein in female fish, which likely explains the observed decrease in PI3K expression.

A visual pathway containing the proteins involved in NO production that were detected in the present study in male and female fish exposed to diclofenac is presented in Figure 7.

4.2 Cellular migration

The inflammatory reaction enables an organism to defend itself against microbe infection. The migration of leukocytes from the vascular system to sites of pathogenic exposure is a key event in the inflammatory process (WAGNER; ROTH, 2000; COOK-MILLS; DEEM, 2005). Migration of leukocytes is initiated by the process of cell adhesion, followed by transmigration. In general, leukocytes extravasation is a multi-step process that involves the tethering, rolling and activation, firm adhesion to the endothelium, diapedesis and finally transendothelial migration (APLIN *et al*., 1998; SCHUBERT *et al*., 2011).

Recognition as well as contact formation is mediated by several cell adhesion molecules which act in a sequential manner and in concert with regulatory mediators such as the chemokines. In contrast to the rapidly flowing cells in the blood stream, rolling cells are able to sense signals from the endothelium, which stimulates them to adhere more firmly to the endothelial cell surface. Such signals are given by chemokines through receptors (Cxcrs) and G-proteins (DE FANIS *et al*., 2007; ZHU; PAUL, 2008). Decreased expression of Cxcr4 is linked with the decrease of recruitment of inflammatory cells (DALAKAS *et al*., 2005) and the migration of rolling cells (RUBIE *et al*., 2011). Cxcr4 is also involved in migration of macrophages (IMTIYAZ *et al*., 2010) and hematopoietic stem cells (DALAKAS *et al.*, 2005). In the present study, the inhibited expression of Cxcr4 by diclofenac could explain the decreased of migration cells when the migration was challenged by carrageenan.

These migration stimulatory effects caused the activation of a group of adhesion molecules called integrins, which in turn bind to members of the immunoglobulin superfamily on the endothelial cell surface. The major integrin complexes involved in this process are Leukocyte Function-Associated Antigen-1 Lfa1 (Itg-αL and Itg-β2) and Complement receptor 3 (Mac1) (Itg-αM and Itg-β2), which bind to members of the immunoglobulin superfamily such as Intercellular Cell Adhesion Molecule 1 (Icam1) and 2 (Icam2) and Vascular Cell Adhesion Molecule-1 (Vcam1) on the non-lymphoid endothelial cell surfaces. This caused tight adherence of granulocytes to the endothelium (KAIKO *et al*., 2008; ZHU *et al*., 2010). Integrin alpha 1 is linked with heterodimerization, decreased activity and uncapacity to complete the formation of the integrin complex (LEE *et al*., 2013). Cross-linking of integrins with Icams and Vcam1 activates the Erm (ezrin, radixin, moesin) proteins and recruits Cell Surface Antigen Thy1to the cell surface. This interaction enables binding of platelet endothelial cell adhesion molecule-1 (Pecam1) and also facilitates attachment of junctional adhesion proteins like Junctional Adhesion Molecule 2 (Jam2 and 3) with the granulocyte integrins (RAUTAJOKI *et al*., 2008).

In the present work, the expression of radixin decreased, that may have been caused by the incorrect activation of Erm proteins and the consequent binding of Pecam1 and attachment of junctional adhesion proteins (CARLONI *et al*., 2013; YANO *et al*., 2013; ZOU *et al*., 2013).

Following the migration process, the docking of granulocytes to the apical surface of endothelial cell triggers signals through generation of ROS and formation of stress fibers that further results in the activation of Mmps. Activated Mmps and ROS are responsible to degrade the assembly of junctional proteins like Vecam and other Cams, leading to the opening of inter-endothelial cell contacts, allowing granulocytes to transmigrate and reach the underlying tissue (OMENN *et al*., 2005). Among the proteins of the Matrix Metalloproteinase (Mmp) family involved in this migration process, in this study we observed a decrease in expression of Mmp1 and Mmp17. This decrease in Mmp1 and Mmp17 expression in plasma may represent impaired migration due to fewer breakdowns of extracellular matrix (NIKKARI *et al*., 1995; BUTTICE *et al*., 1996; YU *et al*., 2002; OH *et al*., 2013).

Even though we have analyzed some proteins such as Nfκb and Ap1 under the perspective of NO synthesis, those are transcription factors. There are studies which show that the expression of integrin α1, radixin and the MMP are induced during an inflammatory process (LUND; GIACHELLI; SCATENA, 2009; WANG *et al*., 2007; XU *et al*, 2010). Therefore, the reduction of the expression of the proteins involved in the cell migration may also be an indirect effect of the reduction in the expression of these transcription factors.

Therefore, our results clearly showed that the exposure to diclofenac can also reduce another important line of the hosts defense which is the leukocyte migration. No significant gender differences were observed.

4.3 Complement system

The complement system plays an essential role in alerting the host about the presence of potential pathogens. The activation of the complement system contributes significantly to the orchestration and development of immune acquired response (BOSHRA; LI; SUNYER, 2006). The complement system is composed of more than 35 soluble plasma proteins that play key roles in innate and adaptive immunity (GASQUE, 2004). It can be initiated by one pathway or a combination of three pathways: the alternative, lectin and classical. The classical pathway encompasses the formation of a complex between an antigen and an antibody (BOSHRA; LI; SUNYER, 2006). In the alternative pathway, the spontaneous activation of C3 is

amplified upon the covalent binding with various microbial surfaces (XU *et al*., 2001). Lectin activation requires the interaction of lectins such as mannose-binding lectin (MBL) and ficolins, with sugar moieties found on the surface of microbes (FUJITA *et al*., 2004).

The C1q protein is a major constituent of the complement subcomponent (SCHWAEBLE *et al*., 1995). Activation of the classical pathway is triggered by binding of the Fc portion of IgG with the C1q component of the C1 complex (HU *et al*., 2010). Also, C1q is described as an important protein in the regulation of phagocytosis (BOSHRA; LI; SUNYER, 2006). In the present study, both male and female fish exposed to diclofenac had decreased expression of C1q protein, which could predispose these fish to microbiological infection by crippling activation of the classical pathway in the complement system and de-regulating phagocytosis.

One way that the organism distinguishes self from non-self is the deposition of C3 on non-self molecules or structures. A particularly fascinating feature of C3 from fish is the fact that they show differences in their binding efficiencies to complement-activating surfaces (BOSHRA; LI; SUNYER, 2006). It has been hypothesized that this unique structural and functional C3 protein provides a mechanism for recognizing a broader range of microorganisms, thereby allowing fish to expand their innate immune recognition capabilities (SUNYER *et al*., 1998; BOSHRA; LI; SUNYER, 2006). In the present study, female, but not male, fish exposed to diclofenac had decreased plasma expression of the C3 protein, which could also compromise their ability to recognize pathogens.

The CFB protein is a component of the alternative pathway of complement activation. CFB circulates in the blood as a single chain polypeptide. Upon activation of the alternative pathway, it is cleaved by complement factor D, yielding the non-catalytic chain BA and the catalytic subunit BB. Subunit BB is a serine protease which associates with C3B to form the alternative pathway C3 convertase which can be fixed to the target surface. BB is involved specially in the proliferation of preactivated B lymphocytes (CLAIRE *et al*., 2002; ARUMUGAM *et al*., 2006). This illustrates the importance of CFB in the alternative pathway of defense (GONZALEZ *et al*., 2007). In the present work, male and female fish exposed to diclofenac had conflicting CFB protein responses (in male its expression increased and in female it decreased). Studies involving anti-inflammatory drugs and inhibition of the complement system are

rare and not extensive in fish. Previous studies with cloroquine, a well-known anti-malarial and anti-inflammatory agent demonstrated a decrease of CFB in plasma of *Plasmodium falciparum* infected patients (CHAKRABARTY *et al*., 1985). In the present study, the differences observed between males and females are probably associated with differences in hormones and reproductive cycle in the species.

The C7 protein is also a component of the complement system. It participates in the formation of the Membrane Attack Complex (Mac). In C7 deficiency, the ability to react to bacterial infection via the complement system is reduced (PODACK *et al*., 1980; NAKAO *et al*., 2011). In female fish, exposure to diclofenac resulted in decreased C7 protein in plasma.

The lectin pathway requires the interaction of lectins such as mannose-binding lectin (MBL) and ficolins with sugar moieties found on the surface of pathogens (FUJITA *et al*., 2004). Upon binding of the lectins to the microbial sugars, the enzymes associated with these lectins (MBL-associated serine proteases and MASPs) are activated (GAL; AMBRUS, 2001). There are three types of MASP molecules, Masp1, Masp2, and Masp3. Masp1 can activate C3 and C2 (HAJELA *et al*., 2002; MATSUSHITA; ENDO; FUJITA, 2013). Masp1 also has the capacity to increase activation of MASP2 (MATSUSHITA; ENDO; FUJITA, 2013). Masp2 has the ability to cleave C4 and C2 (CHEN; WALLIS, 2004). Masp3 is unable cleave C2, C3 or C4 and its role in the lectin pathway remains a mystery (BOSHRA; LI; SUNYER, 2006).

In the present work, only male fish exposed to diclofenac showed decreased expression of MASP1 protein. However, TAKAHASHI *et al.* (2008) found that a decreased expression of Masp1 resulted in significantly low activity of C4 and C3 deposition and significantly delayed activation of Masp2. Thus, the decrease of MASP1 in male fish plasma is likely to result in reduced resistance to infection. In fact, the reduction of major proteins associated with the complement system demonstrated in female fish exposed to diclofenac reveal that female fish are more susceptible to alterations in the complement system than male fish in this model, which could result in more severe effects in the immunity.

4.4 Class I Major Histocompatibility Complex

Other protein of interest in immune system is the Class I Major Histocompatibility complex (MHC1). The MHC1 protein is critical to the development of the cellular immune response. This protein has a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen (WANG *et al*., 2011). Inhibited expression of MHC1 has been associated with both susceptibility and decreased resistance to diseases, especially infectious disease (GREENE *et al*., 2011). This inhibition can also limit the size of the antigen-specific CD8 T cell response and Natural Killer cells stimulus (ORR *et al*., 2005). This strikingly importance of MHC1 for the immune response has leading to investigate also this protein. Diclofenac exposed greatly reduced the expression of MHC1 in both male and female fishes. Therefore, it is plausible that the whole immune response would be compromised by this exposure since the antigen presentation would be compromised.

5. Conclusion

In the present work, diclofenac exposure resulted in inhibited expression of many proteins involved in NO synthesis, cellular migration, complement system activation and antigen presentation. Some changes in protein expression were gender-specific. However, our results point to the same direction in both male and female fishes and suggest that the exposure to diclofenac at environmentally relevant concentrations can result in immunosuppression.

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4 DISCUSSÃO GERAL

Alguns estudos demonstram que a concentração de produtos farmacêuticos cresce cada vez mais na água, tornando-se uma preocupação emergente como contaminante do ecossistema aquático (ZUCCATO *et al.*, 2006; KHETAN; COLLINS, 2007).

Entre os fármacos encontrados no ecossistema aquático os AINEs como o diclofenaco, ibuprofeno e paracetamol são os mais prevalentes (ZUCCATO *et al.*, 2006; LETZEL; METZNER; LETZEL, 2009).

Por esses motivos, os fármacos escolhidos para esse estudo, no cultivo celular foram o ibuprofeno, o diclofenaco e o paracetamol, visando avaliar o efeito em cultura primária de rim anterior de *Hoplias malabaricus* e no prosseguimento, o diclofenaco em modelo trófico e hídrico. Sendo assim, esta tese destaca-se pelo caráter inovador e busca a obtenção dos primeiros resultados, visando correlacionar seus efeitos à saúde de organismos aquáticos envolvidos neste processo.

Os resultados do presente estudo demonstraram que peixes tanto da espécie *Hoplias malabaricus* quanto *Rhamdia quelen* expostos ao diclofenaco em situações experimentais específicas apresentaram alterações imunológicas importantes, levando a uma imunossupressão considerável com inibição de células e moléculas chaves à defesa do organismo. Nesse contexto, poucos trabalhos relatam sobre a imunologia de peixes, incluindo a imunossupressão, e um número menor ainda descreve essa imunossupressão associada a fármacos em concentrações reais encontradas no ambiente (ALVAREZ-PELLITERO, 2008; SECOMBES, 1994; WHYTE, 2007).

O presente trabalho teve como primeira fase a padronização da cultura primária de rim anterior de *Hoplias malabaricus*, seguido da exposição dessas células aos AINEs ibuprofeno, diclofenaco e paracetamol. No presente trabalho, foi observada uma redução significativa na produção de óxido nítrico basal e após estimulação por LPS em cultivo primário de macrófagos frente à exposição ao diclofenaco, ibuprofeno e paracetamol. Alguns autores já relataram a inibição da produção de óxido nítrico em células de peixes e de roedores expostos à antinflamatórios não-esteroidais (BOULARES *et al*., 2000; COMBS *et al*., 2000; LUNA-ACOSTA *et al*., 2012; PAROLINI *et al*., 2009; TAKADA *et al*., 2004).

Devido à importância clínica, o volume de utilização e os resultados expressivos de inibição da produção de óxido nítrico, onde a mesma ocorreu especialmente nas menores concentrações testadas, o diclofenaco foi escolhido para prosseguimento dos estudos referentes ao possível mecanismo de ação desta inibição e à continuação das pesquisas relacionadas a essa imunossupressão inicial. Cabe ressaltar ainda que todas as concentrações de diclofenaco a que os organismos foram expostos são concentrações reais encontradas no ambiente, como já demonstrado nos capítulos anteriores.

Na segunda fase, dos estudos com o diclofenaco em *Hoplias malabaricus*, por via trófica, demonstrou-se claramente alterações nos biomarcadores bioquímicos testados como as enzimas referentes ao estresse oxidativo, nos parâmetros hematológicos com uma redução na contagem de leucócitos periféricos, assim como alterações importantes em células mono e polimorfonucleares e nos parâmetros imunológicos com as alterações observadas na migração celular estimulada por carragenina em peixes expostos.

Uma característica essencial na defesa do organismo que foi inibida pela exposição ao diclofenaco foi a contagem global de leucócitos em *Hoplias malabaricus*, também observada com a exposição de ratos ao diclofenaco por AL-SAADY (2011), provavelmente devido a formação de anticorpos dirigidos contra a medula óssea e precursores leucocitários (GUTTING *et al.*, 2002). Os leucócitos são células de defesa e participam integralmente da resposta imunológica, além de atuarem especificamente em sua regulação (FERNANDEZ *et al.*, 2002; SARAVANAN *et al.*, 2012). Sendo assim, com sua redução significativa, o organismo pode ficar mais susceptível a infecções e alterações imunológicas, como a imunossupressão.

Nesses leucócitos globais, a fração linfocitária é responsável pela resposta imune específica, tanto celular como humoral, promovendo a produção de anticorpos e aumento da capacidade citotóxica, além de atuar promovendo o processo de memória imunológica e liberação de fatores regulatórios da função imune, como as linfocinas (FERNANDEZ *et al.*, 2002; TIZARD, 2002). Os monócitos, por sua vez, são as células sanguíneas mais importantes na resposta imune, não sendo importantes apenas pela produção de citocinas, mas também de células primárias na apresentação de antígenos em teleósteos (FALCON, 2007). Nesse trabalho, em peixes *Hoplias malabaricus* expostos ao diclofenaco via trófica nas doses de 2,0 e 20,0 µg/Kg, verificou-se uma redução significativa nos linfócitos e monócitos

circulantes, representados pelos mononucleares, sugerindo uma redução expressiva no processo de defesa celular e humoral.

Os neutrófilos, ou polimorfonucleares, que nesse trabalho tiveram uma redução significativa em sua contagem e diferenciação, são as primeiras células envolvidas nos estágios iniciais do processo inflamatório em peixes e desempenham uma importante atividade microbicida (FALCON, 2007; FERNANDEZ *et al*., 2002). Estudos relatam que o diclofenaco é um dos principais fármacos que está associado à neutrofilia em pacientes que o utilizam de forma crônica, evidenciada através da exposição de ratos a esse fármaco (AL-SAADY, 2011; VAN DER KLAUW *et al*., 1998). A provável hipótese desta neutrofilia observada é o fato do diclofenaco induzir anticorpos contra neutrófilos periféricos e até mesmo contra seus precursores na medula óssea (AKAMITZU *et al*., 2001). Assim sendo, os dados reforçam a evidente imunossupressão dos peixes expostos ao diclofenaco.

Outro ponto a ser considerado no presente trabalho foi a inibição da migração celular desafiado por carragenina em peixes *Hoplias malabaricus* expostos ao diclofenaco.

A reação inflamatória, simulada pela carragenina, permite ao organismo a defesa contra a infecção. Um acontecimento chave nesse processo inflamatório é a migração leucocitária a partir do sistema vascular para os sítios expostos (COOK-MILLS; JULGUE, 2005). Todo esse processo se inicia pelo processo de sinalização, de adesão celular, seguido por transmigração. Esse processo é mediado por várias moléculas de adesão que atuam de forma sequencial e de acordo com mediadores regulamentares, como as quimiocinas (LEE *et al.*, 2013; RUBIE *et al.*, 2011; ZHU; PAUL, 2008).

No presente trabalho, tanto a migração desafiada por carragenina, quanto as proteínas envolvidas nesse estímulo e migração, se mostraram inibidas, sugerindo a incapacidade do organismo em reagir frente a um processo inflamatório desencadeado por um agente infeccioso.

Em uma terceira fase, foram realizados ensaios de proteômica na tentativa de se evidenciar os principais mecanismos de ação pelos quais o diclofenaco estava levando os organismos à imunossupressão. Para esses estudos de proteômica, foi utilizada a via hídrica no peixe *Rhamdia quelen*. Os peixes da espécie *Rhamdia quelen* foram utilizados em especial por já apresentarem descritos alguns genes e proteínas em estudos do próprio grupo e também pela facilidade fenotípica na diferenciação

sexual, possibilitando a separação clara e precisa para a exposição ao diclofenaco, retirando toda e qualquer influência hormonal que poderia ocorrer ao se ter sexos distintos. Outro motivo relevante foi a maior disponibilidade de exemplares.

Nos estudos de proteômica, foi observada uma alteração significativa nas proteínas envolvidas na defesa do organismo, representada pela inibição na produção de óxido nítrico e pela inibição de proteínas essenciais à defesa no sistema complemento, tanto na via clássica quanto na via alternativa e da MBL. Vale ressaltar que essas alterações ocorreram tanto perifericamente, encontradas no plasma, quanto diretamente no rim anterior.

Em termos de mecanismo de ação sobre a inibição na produção do óxido nítrico, várias proteínas participantes da cascata de reação foram inibidas, como já relatado em capítulos anteriores.

Outro ponto importante observado no presente trabalho foi a inibição significativa do sistema complemento. Ele reúne 35 proteínas séricas e de membrana, que interagem em uma sequência determinada, gerando funções efetoras de resposta imune, humoral e celular. O conjunto dessas proteínas são a chave de ligação entre o sistema imune inato e adquirido (HU *et al*., 2010). A ativação do sistema complemento, que após desencadeada, progride de forma sequencial em uma cascata de reação, pode ser iniciada por 3 diferentes formas: (1) pela via clássica envolvendo a ligação do C1 com complexos imunes da circulação (Ag-Ab), (2) pela deposição espontânea do fator do complemento C3 e (3) pela lectina ligadora de manose (MBL) que liga resíduos de manose a outros carboidratos (HU *et al*., 2010). Além disso, nos peixes, a atividade do sistema complemento, especialmente pela via alternativa, é intensa quando comparada ao dos mamíferos, sugerindo essa via como um mecanismo chave na defesa desses animais (ELLIS, 2001; LIN; SHIAU, 2005). A perda da sequência na cascata de reação leva a interrupção parcial ou total da reação do complemento (CLAIRE; HOLLAND; LAMBRIS, 2002).

Embora os peixes apresentem as proteínas do sistema complemento semelhantes aos mamíferos, algumas diferenças são cruciais. A principal delas é o fato dos peixes possuírem múltiplas isoformas, sugerindo um repertório mais expansivo no reconhecimento do sistema imune (CLAIRE; HOLLAND; LAMBRIS, 2002; NONAKA, 2001). Entendendo-se a função exata do sistema complemento em peixes e como cada proteína se comporta de forma individual, pode-se compreender como se dá o desenvolvimento de novas estratégias para manutenção da integridade

do organismo, especialmente em condições atípicas, como a exposição a poluentes aquáticos (CLAIRE; HOLLAND; LAMBRIS, 2002; BOSHRA; LI; SUNYER, 2006). Em peixes, muitos componentes estão envolvidos com a inicialização das reações do complemento, entre eles o C1q, fator B, C3, C2 e C4, além das MASP1 e MASP2 (HU *et al*., 2010; MATSUSHITA; ENDO; FUJITA, 2013).

No presente trabalho, a inibição da expressão das proteínas relacionadas com a inicialização do sistema complemento, tanto em machos quanto em fêmeas, tanto no plasma quanto no rim anterior, pode atuar impedindo que a cascata de reação se processe, levando a uma falha nos processos de defesa do organismo.

5 CONCLUSÃO E PERSPECTIVAS

A análise dos principais dados do presente estudo levaram às seguintes conclusões:

1. Com a padronização do cultivo de primário de rim anterior de *Hoplias malabaricus*, pôde-se averiguar que o Percoll é o melhor método para separação celular e que o melhor tempo para fazer as exposições foi 24 horas após o plaqueamento;

2. A produção de óxido nítrico basal e estimulado por LPS após exposição com diclofenaco, ibuprofeno e paracetamol reduziu significativamente, indicando uma imunossupressão nas células expostas a esses antinflamatórios

3. Os estudos genotóxicos após exposição das células oriundas do cultivo primário de rim anterior de *Hoplias malabaricus* ao diclofenaco,ibuprofeno e paracetamol demonstraram um potencial de dano elevado nessas células.

4. Após exposição trófica, análises no fígado de *Hoplias malabaricus* demonstraram aumento na atividade da SOD, GSH e da GPx, sugerindo a geração de radicais livres e a presença de estresse oxidativo. A LPO também foi induzida corroborando com os resultados anteriores. Além disso também foi observado a inibição do metabolismo de fase II pela inibição da atividade da GST.

5. *In vivo*, os indivíduos expostos ao diclofenaco via trófica demonstraram uma redução significativa na contagem global dos leucócitos circulantes, com alterações expressivas na diferenciação de mononucleares e polinucleares.

6. A migração celular pós-desafio com carragenina mostrou-se reduzida em indivíduos expostos via trófica ao diclofenaco, demonstrando a capacidade que o diclofenaco tem de reduzir a migração em peixes. Fato esse confirmado através da inibição da expressão de importantes proteínas relacionadas à migração celular.

7. Na avaliação global das proteínas relacionadas ao sistema imunológico, foi observada uma inibição significativa em proteínas-chaves no processo de defesa no plasma e rim anterior de *Rhamdia quelen* expostos ao diclofenaco. Dentre essas proteínas cabe ressaltar a inibição de proteínas responsáveis pela produção de óxido nítrico e envolvidas na defesa através do sistema complemento

8. Os estudos imunológicos realizados nesse trabalho demonstraram claramente uma imunossupressão ocasionada pelos antinflamatórios testados em especial o diclofenaco, em concentrações encontradas no ambiente como micropoluente emergente.

Pelos resultados obtidos ao longo desse trabalho, de fato o que mais fica explícito é a necessidade de uma legislação vigente que tente ao menos limitar a concentração de medicamentos presentes no ambiente. O investimento em estudos que demonstrem os reais efeitos dos fármacos em concentrações ambientalmente relevantes pode ser uma forma de conscientização e mudança do manejo até o descarte adequado dos medicamentos. É necessário, portanto, atenção especial para esse tópico além de constatar a eficácia de ações individuais ou coletivas sobre esse tema.

Talvez uma alternativa muito interessante seja a venda fracionada e exata da quantidade necessária do medicamento, evitando vencimentos e especialmente sobras que tendem a entrar no ambiente da forma mais sorrateira possível.

Cabe lembrar que o passivo ambiental considerado que é gerado pelo medicamento deve ser percebido não somente como um problema para o ambiente, mas para todos os seres vivos.

Enfim, a conscientização da sociedade pela educação ambiental na redução do descarte inadequado, juntamente com um novo olhar sobre a gestão do saneamento ambiental fazem, de fato, um trabalho como esse ganhar destaque e relevância frente às necessidades e desafios que a toxicologia ambiental. Uma pequena gota em um oceano, mas ainda assim uma pequena gota.

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