UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

CARACTERIZAÇÃO DE UM MODELO DE INFLAMAÇÃO EM LARVAS DE ZEBRAFISH: O PAPEL DO SISTEMA PURINÉRGICO

CARLOS EDUARDO LEITE

Porto Alegre
2013

CIP - Catalogação na Publicação

Leite, Carlos Eduardo
Caracterização de um modelo de inflamação em larvas de zebrafish: o papel do sistema purinérgico / Carlos Eduardo Leite. -- 2013.
114 f.

Orientadora: Ana Maria Oliveira Battastini.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Medicina: Ciências Médicas, Porto Alegre, BR-RS, 2013.

1. Inflamação. 2. Sistema purinérgico. 3. Larvas de zebrafish. 4. Toxicologia. I. Oliveira Battastini, Ana Maria, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

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Tese apresentada ao Programa de Pós-Graduação em Medicina: Ciências Médicas, UFRGS, como requisito para obtenção do título de Doutor.

Porto Alegre
2013

Dedico esta tese ao meu pai José Carlos, minha mãe Zilamar e a minha avó Francisca Ana, os primeiros mestres da minha vida.

Agradecimentos

À Universidade Federal do Rio Grande Sul (UFRGS) e ao Programa de Pós-Graduação em Medicina: Ciências Médicas (PPGCM) pela oportunidade de realização do doutorado. Agradeço também ao coordenador do curso Professor Wolnei Caumo e aos funcionários Vera Ribeiro e Rodrigo Peçanha pela atenção e auxílio.

À Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) e ao Instituto de Toxicologia e Farmacologia da PUCRS (INTOX) pela colaboração e disponibilização de laboratórios e equipamentos de pesquisa.

À Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) pelo apoio financeiro necessário para a realização do projeto.

À minha orientadora Ana Maria Battastini pela oportunidade, orientação e atenção que me foram dados.

À Professora Maria Martha Campos, com quem tenho o prazer de trabalhar, pela oportunidade, estímulo, auxílio e pelo exemplo de dedicação a ciência.

Às Professoras Fernanda Bueno Morrone e Carla Denise Bonan pela oportunidade, confiança e, principalmente, pelos exemplos de dedicação e profissionalismo.

À toda equipe do INTOX pelo auxílio e amizade.

Aos alunos de iniciação científica Lucas, Fernanda e a Simone pelo auxílio fundamental na realização deste trabalho.

À minha amiga Ariane, que sempre esteve me guiando e ajudando a trilhar este caminho.

À amiga Fernanda pelo incentivo e companheirismo.

Aos meus irmãos de coração Juliano e Rodrigo por ajudarem no meu crescimento pessoal e pela amizade de sempre.

Aos meus tios João, Pedro e Joceli e, as minhas primas Liana e Luize por sempre estarem perto de mim e pelo amor que sempre me foi dedicado.

Aos meus tios Zildomar, Zolmir e Zelmar pela amizade e por estarem sempre comigo.

Ao meu irmão Alexandre pela amizade que temos desde criança.

Aos meus pais José Carlos e Zilamar pela educação, amor e incentivo em todos os momentos da minha vida.

Todo aquele que se dedica ao estudo da ciência chega a convencer-se de que nas leis do Universo se manifesta um Espírito sumamente superior ao do homem.

(Albert Einstein 1879 - 1955)

Resumo

Introdução: Entre os modelos utilizados para o estudo da inflamação está o que utiliza o cobre como agente inflamatório. O sistema purinérgico está envolvido na inflamação e em alguns tecidos pode ser afetado pela ação do cobre. Não obstante, nada se sabe sobre o comportamento do sistema purinérgico frente à inflamação induzida por este metal em larvas de zebrafish. **Objetivo:** Estabelecer e caracterizar um modelo de inflamação induzida pelo cobre em larvas de zebrafish e elucidar o envolvimento do sistema purinérgico. **Métodos:** Desenvolvemos uma metodologia para a quantificação de óxido nítrico (NO) por cromatografia líquida para a avaliação inicial dos efeitos do cobre sobre o estresse oxidativo. Posteriormente, realizamos curvas de mortalidade para o cobre e o lipopolissacarídeo (LPS) de *Escherichia coli* (*E.coli*). A metodologia foi utilizada para avaliar os níveis de NO após a indução da inflamação pelo cobre e a comparação com o outro agente inflamatório, bem como para quantificar os níveis basais desse íon em larvas de diferentes dias pós-fertilização. Na seguinte etapa, inserimos outros marcadores de estresse oxidativo e testamos as concentrações de 1, 10 e 25 µM de cobre durante um período de vinte e quatro horas. A concentração de 10 µM foi utilizada para indução da inflamação e a caracterização do sistema purinérgico. Os parâmetros avaliados foram estresse oxidativo, migração de neutrófilos, prostalandina E_2 (PGE_2), expressão gênica de mediadores inflamatórios, hidrólise extracelular de ATP e atividade das ecto-nucleotidases e da adenosina deaminase (ADA). As expressões gênicas das enzimas e dos receptores também foram avaliadas, bem como a absorção de cobre durante o período de exposição. **Resultados:** Por conseguinte, a metodologia utilizada para a determinação de NO foi validada com êxito e considerada adequada para a determinação do analito em alterações fisiológicas e patológicas em larvas de zebrafish. As curvas de mortalidade mostraram que as concentrações subletais foram de 10 µM para o cobre e 150 µg/ml para o LPS. As concentrações de NO em larvas de diferentes dias pós-fertilização apresentaram níveis crescentes de acordo com o estágio de desenvolvimento, ao passo que a exposição ao cobre e ao LPS induziram ao aumento do NO em comparação ao grupo controle nos tempos testados. Posteriormente, foi constatado que após a exposição ao cobre, ocorreu uma série de eventos inflamatórios que envolveram a PGE_2 e a migração de neutrófilos para o foco inflamatório. A absorção do cobre foi tempo-dependente e coincidiu com o aumento do estresse oxidativo. As atividades da ecto-5´-nucleotidase (Ecto-5´-NT) e da ADA apresentaram redução, sendo que a nível molecular também foram constatadas alterações nas expressões gênicas destas enzimas. Os receptores de adenosina apresentaram aumento das suas expressões gênicas. **Conclusão:** O acúmulo do cobre absorvido durante o período de 24 horas induziu a inflamação e o estresse oxidativo. Dentro do sistema purinérgico o mais envolvido foi o adenosinérgico, que parece estar envolvido principalmente na resolução da inflamação. Esses resultados trazem novos avanços na caracterização da resposta inflamatória induzida pelo cobre em larvas de zebrafish, fornecendo novos conhecimentos e ferramentas para o estudo de mecanismos de ação e para a triagem de efeitos de novas terapias anti-inflamatórias.

PALAVRAS-CHAVE
Zebrafish; inflamação; cobre; sistema purinérgico; adenosina.

Abstract

Introduction: Inflammation induced by copper exposure has been recently characterized in zebrafish. The purinergic system is closely involved in inflammation, although there is no available study correlating this system and the inflammatory effects of copper. **Objective:** The present study was aimed to characterize a model of inflammation induced by copper in zebrafish larvae, with special attempts to elucidate the involvement of the purinergic system in this condition. **Methods:** Firstly, we developed a methodology for the measurement of nitric oxide (NO) by liquid chromatography, for the initial evaluation of the effects of copper on oxidative stress. Subsequently, we carried out mortality curves for exposition to copper and lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*). NO was quantified in both inflammatory models, and in larvae at different days post-fertilization. Additional markers of oxidative stress were quantified, and the concentrations of 1, 10 and 25 µM of copper were evaluated during 24 h of treatment. The concentration of 10 µM of copper was used for induction of inflammation and to characterize the relevance of purinergic system in this context. The parameters assessed were: oxidative stress, neutrophil migration, prostaglandin E_2 (PGE_2), expression of inflammatory cytokines, extracellular ATP hydrolysis and ecto-nucleotidases and adenosine deaminase (ADA) activities. The gene expression of enzymes and receptors related to the purinergic system were evaluated. The absorption of copper during the exposure period was also measured. **Results:** The methodology for the determination of NO was successfully validated and considered suitable to assess the physiological and the pathological changes of NO levels in zebrafish larvae. The mortality curves showed that sub-lethal concentrations were 10 µM and 150 µg/ml for copper and LPS, respectively. The concentrations of NO in zebrafish larvae at different days post-fertilization showed increased levels according to the stage of development. The exposure to copper and LPS induced a significant increase in NO levels. After copper exposure, there was an increase of PGE_2 levels and migration of neutrophils to the inflammatory focus. The absorption of copper was time-dependent and coincides with increased oxidative stress. The activities of ecto-5'-nucleotidase (Ecto-5'-NT) and ADA decreased, and at the molecular level, it was possible to detect changes in expression of genes related to these enzymes. Relevantly, the mRNA expression of adenosine receptors was found augmented. **Conclusion:** The increase in copper concentrations in zebrafish larvae correlated well with the evolution of inflammation and oxidative stress in the zebrafish larvae. Within the purinergic system, the adenosinergic mechanisms appear to be mainly involved in the resolution of inflammation. Our results provide new advances on the characterization of inflammatory response induced by copper in zebrafish larvae, providing new knowledge and tools for the study of mechanisms of action and screening effects of new anti-inflammatory therapies.

KEYWORDS

Zebrafish; inflammation; copper; purinergic system; adenosine.

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Lista de Abreviaturas

ADA	Adenosina deaminase
AK	Adenosina quinase
ATP, ADP e AMP	Adenosina trifosfato, difosfato e monofosfato
COX-2	Ciclooxigenase 2
E. coli	*Escherichia coli*
Ecto-5'-NT	Ecto-5'-nucleotidase
E-NTPDases	Ecto-nucleosideo-trifosfo-difosfoidrolases
ERO	Espécies reativas do oxigênio
FDA	*Food and Drug Administration*: órgão governamental americano responsável pelo controle de alimentos e medicamentos
IFN-γ	Interferon- γ
IL	Interleucina
iNOS	Óxido nítrico sintase induzível
LPS	Lipopolissacarídeo bacteriano
MAPK	Proteínas quinases ativadas por mitógenos
NF-κB	Fator de transcrição nuclear κB
P1	Receptor purinérgico para nucleosídeos
P2	Receptor purinérgico para nucleotídeos
P2X	Receptor purinérgico para nucleotídeos ionotrópico
P2Y	Receptor purinérgico para nucleotídeos metabotrópico
PKC	Proteína quinase C
TNF-α	Fator de necrose tumoral-α
UTP e UDP	Uridina trifosfato e difosfato
ZFIN	Base de dados internacional sobre o zebrafish

Sumário

1. INTRODUÇÃO

A descoberta de novos medicamentos envolve processos complexos de análises bioquímicas e celulares, necessitando de avaliações pré-clínicas em modelos animais para chegar às fases clínicas de desenvolvimento. Modelos utilizando mamíferos para avaliação da farmacocinética e farmacodinâmica são caros, trabalhosos e consomem uma grande quantidade das substâncias a serem testadas. Há também um considerável aumento na conscientização e na limitação do uso de animais em pesquisas científicas. Uma alternativa útil, de grande valor científico e de menor custo que outros animais é o peixe zebrafish (*Danio rerio*), conhecido no Brasil como Paulistinha. Ele está sendo utilizado em vários estágios dos processos de descoberta de novos fármacos em substituição a roedores, cães e porcos (Zon and Peterson, 2005).

Nos últimos anos, o uso desse peixe tem sido adotado em estudos de comportamento animal, imunologia e toxicologia, estabelecendo a sua importância como modelo para doenças humanas e como ferramenta para triagem (*screening*) de novas drogas (Sullivan and Kim, 2008). As características do zebrafish fazem dele um modelo útil na descoberta de novos alvos farmacológicos, podendo dessa forma contribuir nas várias etapas do processo de desenvolvimento de fármacos (Zon and Peterson, 2005).

Esse teleósteo possui um sistema imune muito semelhante ao dos mamíferos, incluindo a presença de linfócitos T e B, células apresentadoras de antígenos, células fagocíticas que produzem espécies reativas do oxigênio (ERO) e elementos da imunidade humoral, incluindo o complemento. As similaridades entre o sistema imune de peixes e mamíferos, aliados a um genoma totalmente sequenciado e a um aumento na produção de zebrafish transgênicos e *knockouts*, têm estimulado diversos pesquisadores a estabelecer modelos de inflamação induzidos por agentes infecciosos e substâncias químicas, como os induzidos por *Escherichia coli* (*E. coli*) (Vojtech *et al*., 2009; Loynes *et al*., 2010) e cobre (d'Alencon *et al*., 2010; Leite *et al*., 2012), respectivamente.

O cobre é um metal essencial, mas que pode induzir danos oxidativos quando a exposição a ele é alta e crônica em exposição acidental, ocupacional ou ambiental. Além disso, a exposição crônica ao cobre está associada a desordens de metabolismo, como a encontrada na doença de Wilson e doenças neurodegenerativas, como o Alzheimer (Gaetke and Chow 2003; Brenner, 2013). Modelos de inflamação utilizando o cobre surgiram com o principal objetivo de dar suporte à triagem de potenciais drogas anti-inflamatórias. Desde

então, estudos têm buscado caracterizar os diferentes sistemas envolvidos na inflamação induzida por este metal.

Rosemberg *et al*. (2007) demonstraram que a toxicidade induzida pelo cobre no sistema nervoso central de zebrafish adultos está relacionada com a inibição das atividades das ecto-nucleosidio-trifosfo-difosfoidrolases (E-NTPDases) e a ecto-5'-nucleotidase (ecto-5'-NT), enzimas responsáveis pela controle dos níveis de nucleotídeos e nucleosídeos por hidrólise sequencial da adenosina 5´-trifosfato (ATP) a adenosina.

Os nucleotídeos extracelulares atuam como moléculas sinalizadoras endógenas de danos teciduais, exercendo efeitos sobre a resposta inflamatória e imune (Lazaroswski *et al*., 1997; Burnstock, 2006). O ATP é importante na secreção de citocinas e no recrutamento e diferenciação de células imunes, enquanto a adenosina desencadeia ações contrárias àquelas do ATP, mediando uma resposta imunossupressora para proteger os tecidos saudáveis dos ataques promovidos pelas células de defesa (Bours *et al*., 2006).

Durante o processo inflamatório, ATP e adenosina são liberados no sítio de inflamação como resultado do dano celular. Além disto, os nucleotídeos podem ser transportados ativamente ao meio extracelular por células ativadas. Plaquetas e células endoteliais, por exemplo, secretam ATP, ADP e adenosina sob condições de estresse fisiológico (Burnstock, 2006; Luttikhuizen *et al*., 2004). O ATP está envolvido no desenvolvimento da inflamação através de uma combinação de ações, que compreendem a liberação de histamina dos mastócitos (induzindo a produção de prostaglandinas) e a produção e liberação de citocinas das células imunes. Ao contrário do ATP, a adenosina tem uma ação, principalmente, anti-inflamatória (Burnstock, 2006).

A caracterização de diferentes sistemas dentro de um modelo animal é de suma importância para o desenvolvimento de novos estudos, além de fornecer suporte científico e ferramentas para a triagem de novos fármacos. Considerando que o zebrafish tem se posicionado como um modelo pré-clínico de bastante relevância cientifica; que o sistema purinérgico atua ativamente nos processos inflamatórios; que pouco se sabe sobre o comportamento deste sistema na inflamação induzida nas larvas deste teleósteo, o presente trabalho visou, principalmente, à busca pela elucidação do papel do sistema purinérgico na inflamação induzida pelo cobre.

Esta dissertação originou dois artigos:

O primeiro apresenta o desenvolvimento e a validação de uma metodologia para a determinação de NO em larvas de zebrafish. O método foi validado com êxito e os resultados

comparados com uma metodologia já reconhecida para a determinação desse analito em fluidos biológicos. Além disso, foi possível evidenciar que a liberação de NO aumenta de acordo com o período de desenvolvimento do animal e que diferentes estímulos como a inflamação induzida pelo cobre e pelo LPS de *E. coli*, induzem respostas diferentes. Este artigo intitulado ***Analytical method for determination of nitric oxide in zebrafish larvae: toxicological and pharmacological applications*** foi publicado na revista *Analytical Biochemistry* (2012).

O segundo artigo apresenta a extensa caracterização da inflamação induzida pelo cobre, a determinação dos níveis absorvidos do metal por larvas de zebrafish ao longo do período de exposição, a relação temporal entre os inícios da inflamação e do estresse oxidativo, a migração de células para os tecidos lesados, a expressão de interleucinas e, principalmente, o comportamento do sistema purinérgico frente à exposição ao cobre. O trabalho intitulado ***Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae*** foi publicado no periódico *Toxicology and Applied Pharmacology* (2013).

2. REVISÃO DA LITERATURA

2.1 Zebrafish

O zebrafish tem sido utilizado como modelo experimental para estudos de propriedades mutagênicas, carcinogênicas e teratogênicas desde o ano de 1950. Essa longa trajetória de utilização do modelo experimental se deve principalmente ao uso de diversas espécies de peixes para estudos ecotoxicológicos (Sukardi *et al.*, 2011). Na década de 60, George Streisinger iniciou os estudos sobre o desenvolvimento embrionário do zebrafish (White *et al.*, 2013). No final da década de 1980 o modelo começou a tomar espaço nos estudos de desenvolvimento e genética e, na década seguinte começou a se posicionar como um modelo para estudos na área biomédica envolvendo doenças humanas e a busca de novos medicamentos (Sukardi *et al.*, 2011). Os principais indícios da utilidade do modelo envolvem a conservação de órgãos de vertebrados (figura 1) e os estudos genéticos que identificaram uma grande quantidade de genes compartilhados com mamíferos em quase todos os tipos de células e órgãos (Howe *et al.*, 2013; White *et al.*, 2013). Os peixes possuem a maioria dos órgãos dos humanos exceto mama, próstata e pulmão (Spitsbergen and Kent, 2003).

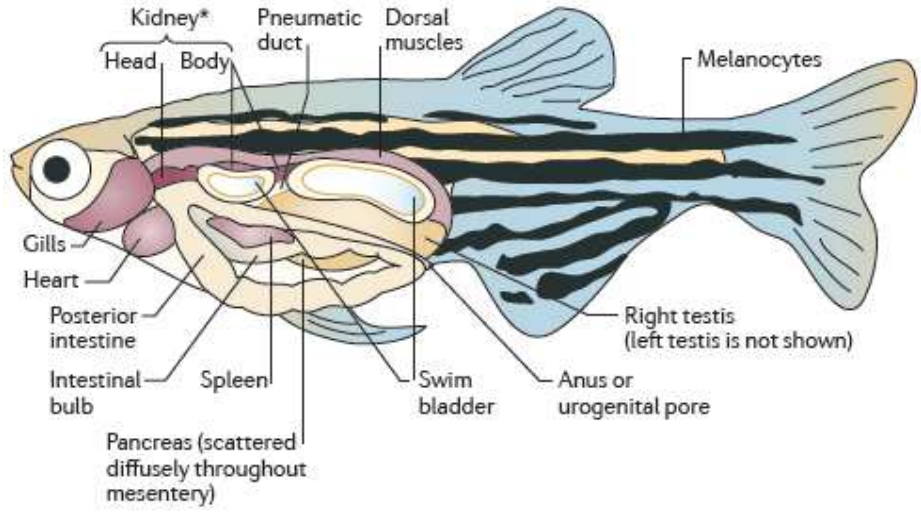

Figura 1. Anatomia do zebrafish. *A maioria dos órgãos apresentam homólogos de mamíferos como o cérebro, coração, fígado, baço, pâncreas, vesícula biliar, intestino, rim, testículos e ovários.* Reimpressão da figura de White *et al.*, 2013 com autorização do *Nature Publishing Group*.

Muitos estudos recentes têm reforçado a importância desse modelo para a compreensão do desenvolvimento humano e da patofisiologia de muitas doenças. Pesquisadores de todo o mundo têm focado suas pesquisas no desenvolvimento normal e anormal de praticamente todos os órgãos e tecidos, bem como no estudo de lesões e doenças incluindo o envelhecimento, o alcoolismo e a dependência de drogas para as quais o zebrafish pode fornecer indícios dos mecanismos nas patogêneses humanas (Spitsbergen and Kent, 2003). Há uma base de dados internacional, atualmente, chamada ZFIN (*The Zebrafish Information Network*), onde centros de pesquisa e cientistas do mundo inteiro depositam informações sobre anatomia, manutenção, criação, dados de expressão gênica, linhagens disponíveis (selvagem e transgênicas), técnicas analíticas e outras ferramentas para a pesquisa com a espécie (Sprague *et al.*, 2008).

Estudos *in vitro* utilizando cultura de células são muito utilizados e adequados para a triagem de novos fármacos quando se tem uma grande gama de moléculas a serem testadas, no entanto, poderiam ter resultados mais relevantes se apresentassem a fisiologia completa de um organismo complexo. Em contrapartida, estudos *in vivo* com mamíferos fornecem todo o tipo de evidência necessária, mas não são adequados para a triagem de um grande número de potenciais moléculas. A comunidade científica tem sugerido o zebrafish como modelo experimental para preencher essa lacuna em estudos pré-clínicos (figura 2) (Sukardi *et al.*, 2011).

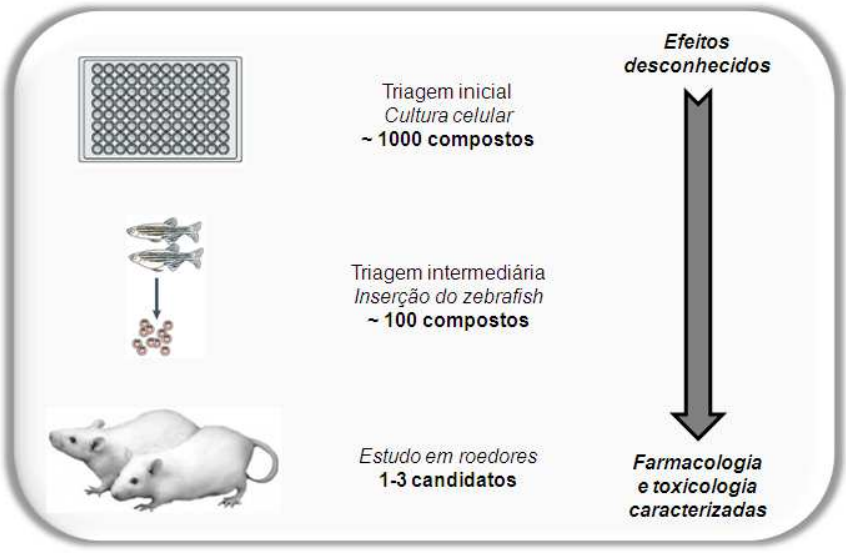

Figura 2. Utilização do zebrafish em ensaios pré-clínicos (Sukardi *et al.*, 2011). *O esquema acima mostra a inserção do zebrafish como modelo experimental complementar aos estudos que utilizam cultura celular e roedores. Também mostra de que forma o seu uso como intermediário entre os modelos experimentais pode auxiliar na caracterização farmacológica e toxicológica de novas moléculas com potencial terapêutico.*

Na legislação européia o zebrafish nas fases iniciais de desenvolvimento - antes de receber alimentação - é visto como alternativa para experimentos com mamíferos, sendo considerado como um modelo *in vitro* (Sukardi *et al.,* 2013). Segundo Sukardi *et al.* (2013), uma vez que o zebrafish possui a vantagem de ter características de estudos *in vitro* e *in vivo*, ele se torna consistente podendo ser utilizado com base na abordagem 3R (*reduction, refinement and replacement*) para estudos de toxicidade envolvendo mamíferos.

Além das características fisiológicas e da presença dos órgãos comparáveis aos dos humanos, muitos estudos têm demonstrado que o zebrafish também responde biologicamente de forma semelhante à xenobióticos como medicamentos e contaminantes ambientais. Devido à diversidade dos processos biológicos conservados entre os vertebrados, muitos genes e componentes biológicos com funções essenciais encontradas em humanos também são encontrados no zebrafish. Dentre os processos conservados estão a bioacumulação, o metabolismo de xenobióticos e a biotransformação de medicamentos. Além disso, esses autores demonstraram a presença de metabolismo de fase I e II para testosterona, dextrometorfano e fenacetina em zebrafish (Alderton *et al.*, 2010).

Dentre as vantagens da utilização desse teleósteo, principalmente de embriões e larvas, destacam-se as características favoráveis de absorção, as pequenas quantidades de compostos necessárias para os ensaios e a grande quantidade de animais e grupos experimentais que podem ser avaliados em um mesmo ensaio (Berghmans *et al*., 2008).

Em comparação com os ensaios de cultura de células, a utilização de embriões e larvas de zebrafish tem como principal vantagem a avaliação da ação de uma determinada substância sobre um organismo completo *in vivo* (McGrath and Li, 2008; Thienpont *et al.,* 2013). Podemos acrescentar que os efeitos colaterais e efeitos positivos secundários podem ser detectados, semelhantemente, às observações de que as estatinas não apenas reduzem o colesterol, como também reduzem a resposta inflamatória *in vivo* (Langheinrich, 2003). Em analogia com os ensaios *in vivo* em camundongos, os benefícios da utilização de embriões e larvas de zebrafish se caracterizam pelo maior número de animais que podem ser estudados em um determinado tempo, a transparência dos animais sem necessidade de dissecação para a observação de alguns processos, o tamanho do animal (pequenas quantidades de drogas são necessárias) e o fato de que os embriões se desenvolvem fora do corpo da mãe durante os primeiros cinco dias de desenvolvimento embrionário (importante para avaliação de efeitos cardiovasculares e circulatórios) (Langheinrich, 2003; Weinstein, 2002).

2.2 Inflamação em larvas de zebrafish

A inflamação é uma resposta imediata do organismo a danos celulares e teciduais por patógenos e estímulos químicos ou físicos. A inflamação aguda é caracterizada por uma resposta de curto prazo, que normalmente resulta em infiltração de leucócitos no sítio afetado, eliminação do estímulo e reparo tecidual. No entanto, a inflamação crônica é uma resposta prolongada e desregulada que envolve a inflamação ativa, destruição tecidual e, tentativas sucessivas de reparo. A inflamação está associada a muitas doenças crônicas, incluindo alergias, aterosclerose, câncer, artrite e doenças auto-imunes; tanto na resposta aguda quanto na crônica, citocinas e outros mediadores inflamatórios estão envolvidos nos eventos celulares principais, como interleucinas (IL-1β, IL-6, IL-8), fator de necrose tumoral-α (TNF-α), interferon- γ (IFN-γ) e ERO (Dantzer *et al*., 2008; Martinon *et al*., 2010).

O zebrafish é um modelo único na manipulação farmacológica de processos inflamatórios; eles são pequenos e permeáveis a muitos compostos moleculares. O fato de serem transparente permite a visualização e quantificação de respostas inflamatórias pela observação da migração de células transgenicamente marcadas para um foco inflamatório (Loynes *et al*., 2010).

Niethammer *et al.* (2009) demonstraram que o primeiro sinal de lesão liberado após o dano tecidual em zebrafish é a liberação de peróxido de hidrogênio (H_2O_2). O rápido recrutamento de neutrófilos para o foco inflamatório é de extrema importância para impedir a entrada de microorganismos e para ajudar a coordenar o processo de reparação tecidual. O principal papel das citocinas neste contexto é modular a amplitude das respostas imunes. Homólogos para uma variedade de citocinas de mamíferos como TNF-α, IL-1β, IL-8, IL-10, IL-11, IL-15, IL-22, e IL-26 foram identificados em peixes, sendo muitos desses observados em zebrafish (Sullivan *et al*., 2008). Martin e Feng (2009) utilizaram neutrófilos com marcação fluorescente para caracterizar a migração destas células para o foco inflamatório em um modelo de lesão caudal em larvas de zebrafish, como mostrado na figura 3.

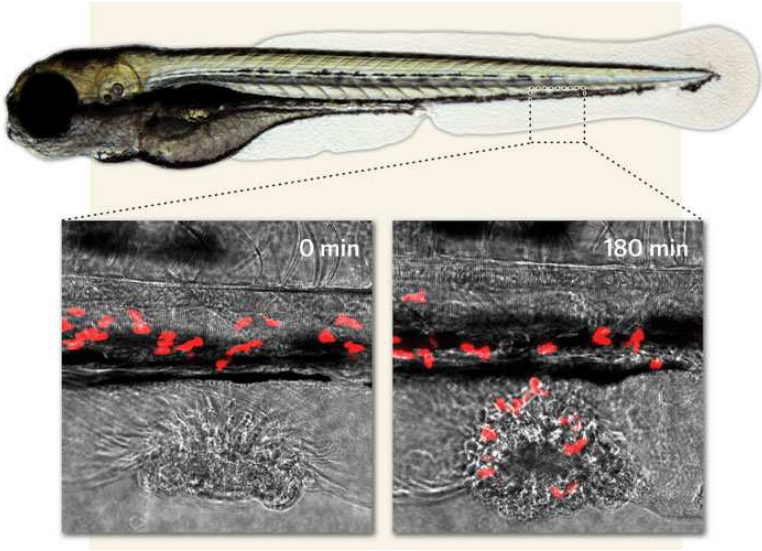

Figura 3. Migração de neutrófilos para o foco inflamatório em larvas de zebrafish. *A pequena lesão feita na região ventral da cauda de larvas de zebrafish de 4 dias, resulta na migração de neutrófilos marcados (vermelho) para foco inflamatório em poucos minutos. Os neutrófilos permanecem na lesão durante algumas horas, apresentando uma miniaturização de uma resposta de lesão inflamatória humana.* Reimpressão da figura de Martin and Feng, 2009 com autorização do *Nature Publishing Group*.

Em estudos utilizando o LPS de *E. coli* na indução da resposta inflamatória, a imersão de embriões de zebrafish em meio contendo esse agente infeccioso induziu a expressão gênica das citocinas pró-inflamatórias TNF-α, IL-β e IL-8 (Watzke *et al*., 2007; Novoa *et al*., 2009; Oehlers *et al*., 2010). Diversos outros estudos têm sido realizados utilizando como modelo experimental o zebrafish, a fim de avaliar o comportamento de outros mediadores inflamatórios como a ciclooxigenase 2 (COX-2) (Grosser *et al*., 2002), a oxido nítrico sintase induzível (iNOS) (Jin *et al*., 2010), proteínas quinases ativadas por mitógenos (MAPK) (Krens *et al*., 2006) e quimiocinas (Sullivan *et al*., 2008).

A importância dos fagócitos na imunidade inata em zebrafish também foi demonstrada em estudos *in vivo* em adultos e embriões infectados por *Mycobacterium marinum*, onde os macrófagos fagocitaram as bactérias e formaram agregados semelhantes aos granulomas (Davis *et al*., 2002). Em outro estudo semelhante, utilizando a microscopia para visualizar a atividade dos macrófagos em embriões de zebrafish infectados por *E.coli*, verificou-se que em quinze minutos de infecção, os macrófagos foram capazes de fagocitar as bactérias e, em poucas horas, as mesmas foram completamente eliminadas do sistema circulatório (Herbomel *et al*., 1999).

Na inflamação induzida pelo cobre, Olivari *et al.* (2008) demonstraram que o mecanismo de lesão do cobre inicia através do estresse oxidativo, que lesa e mata as células ciliares da linha lateral do zebrafish, como mostrado na figura 4.

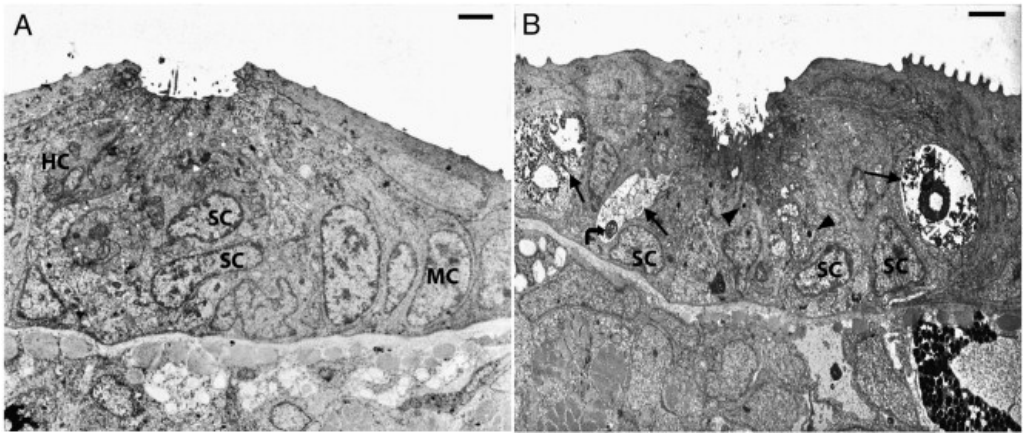

Figura 4. Microscopia de transmissão de neuromastos lesados pelo cobre. *A figura mostra as células ciliares da linha lateral (neuromastos) do grupo controle (A) e do grupo exposto a 1 µM de cobre durante 2 h (B). No grupo exposto foi observada morte celular (setas compridas), formação de vacúolos (setas curvas) e restos apoptóticos (setas curtas). Na concentração de 10 µM também ocorreu lesão nas células de suporte (SC).* Reimpressão da figura de Olivari *et al.*, 2008 autorizada pela Elsevier.

Outro estudo de d'Alencon *et al.* (2010) demonstrou que devido a esta lesão, os neutrófilos migram para o foco inflamatório e que o tratamento com fármacos anti-inflamatórios e antioxidantes é capaz de reverter a migração e reduzir a lesão induzida pelo cobre. Apesar de ser considerado um metal essencial, quando a exposição é alta e crônica ou em exposição acidental, ocupacional e ambiental o cobre pode induzir danos oxidativos. Além disso, a exposição crônica está associada a desordens de metabolismo encontradas na doença de Wilson e em doenças neurodegenerativas, como o Alzheimer (Gaetke e Chow, 2003; Brenner, 2013).

Apesar do papel do sistema purinérgico na inflamação ser parcialmente conhecido, nada se sabe sobre a patofisiologia envolvendo o cobre e o sistema purinérgico em larvas de zebrafish, dificultando os estudos para triagem de potenciais fármacos que atuem sobre este sistema. Um dos poucos relatos dos efeitos do cobre sobre o sistema purinérgico é proveniente do estudo de Rosemberg *et al*. (2007), em cérebro de zebrafish adulto, que demonstraram que o cobre induziu efeitos tóxicos ao sistema nervoso central via inibição das ectonucleotidases, importantes enzimas do sistema purinérgico.

2.3 Sistema Purinérgico

O sistema purinérgico é um sistema de sinalização, onde os nucleotídeos e nucleosídeos atuam como mensageiros extracelulares e agem a partir da sensibilização de purinoreceptores. Como mostrado na figura 5, as suas concentrações extracelulares são controladas por um conjunto de enzimas que promovem a hidrólise sequencial do ATP até adenosina, a qual pode ser captada pelas células e seguir as rotas intracelulares de salvação das purinas ou sequencialmente ser desaminada a inosina (Robson *et al*., 2006; Rosemberg *et al.*, 2007; Ramakers *et al.*, 2012).

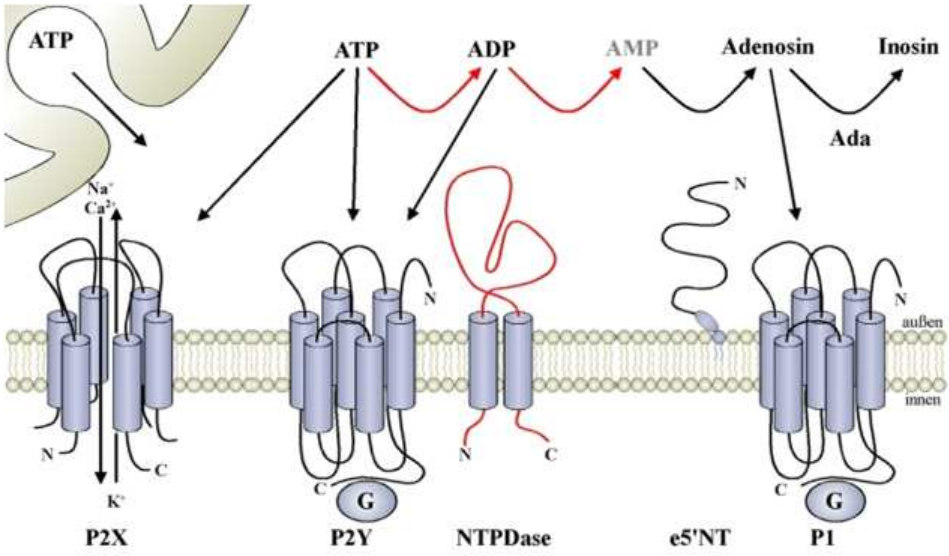

Figura 5. Sistema purinérgico. *A figura acima apresenta a liberação celular de ATP, a sua hidrólise sequencial até inosina e a ativação de cada família de receptor pelos seus respectivos ligantes.* Figura produzida por Matthias Zebisch, reproduzida com permissão (Figura adaptada).

Cronologicamente, a adenosina foi o primeiro componente do sistema purinérgico a ser identificado. Estudos que datam do ano de 1914 detectaram a adenosina no sangue, sendo sugerido que ela poderia ser encontrada na forma de adenosina monofosfato (AMP). Apenas entre 1927 e 1929 o ATP, que é composto de uma base nitrogenada (adenina), um açúcar (ribose) e uma cadeia de três grupos ionizados (fosfatos) ligados à ribose, foi descoberto em extratos de músculo. O papel do ATP intracelular como a principal fonte de energia para todas as células vivas foi descrito em 1941 (Burnstock, 1997; Bodin and Burnstock, 2001). Em 1970 Geoffrey Burnstock lançou a hipótese de que o ATP atuaria também como um neurotransmissor. O pesquisador encontrou muitas resistências, mas com estudos posteriores

a sua hipótese foi comprovada (Burnstock, 2006). A partir de 1978, o efeito diferencial entre os diferentes nucleotídeos e nucleosídeos foram a base dos estudos de Burnstock para a identificação dos receptores P1 (receptores de adenosina) e P2 (receptores de ATP e ADP). Em 1994 foram propostos 4 subfamílias de receptores P1 e os receptores P2 foram divididos em duas subfamílias: P2X (quando o receptor é ligado a um canal iônico) e P2Y (quando o receptor é ligado à proteína G) (Bodin and Burnstock, 2001).

Geralmente as purinas são liberadas por células vivas por meio de processos fisiológicos importantes como a exocitose, difusão através de canais de membrana e via transportadores. Entretanto, também podem ser liberadas por morte celular, atuando como importantes moléculas para a sinalização de dano e exercendo efeitos sobre a resposta inflamatória e imune (Lazaroswski *et al*., 1997; Burnstock, 2006; Burnstock, 2009).

O ATP é importante na secreção de citocinas e no recrutamento e diferenciação de células imunes, enquanto que a adenosina desencadeia ações contrárias àquelas do ATP, mediando uma resposta imunossupressora para proteger os tecidos saudáveis dos ataques promovidos pelas células de defesa (Ohta and Sitkovsky, 2009; Bours *et al*., 2006).

Durante o processo inflamatório, tanto o ATP quanto a adenosina são liberados no sítio de inflamação como resultado do dano celular ou lançados ao meio extracelular por células ativadas. Plaquetas e células endoteliais, por exemplo, sob condições de estresse fisiológico podem ser ativadas e secretar ATP e ADP (Luttikhuizen *et al*., 2004; Burnstock, 2006). O ATP está envolvido no desenvolvimento da inflamação por meio de uma combinação de ações, que compreendem o estímulo a liberação de histamina a partir dos mastócitos (induzindo a produção de prostaglandinas) e a produção e liberação de citocinas pelas células imunes. Ao contrário do ATP, a adenosina tem uma ação principalmente anti-inflamatória (Burnstock, 2006).

A concentração extracelular dos nucleotídeos é controlada por uma ampla família de enzimas denominadas ecto-nucleotidases (Zimmermann, 2011). Dentre elas, as E-NTPDases e ecto-5'-NT catalisam a hidrólise sequencial de ATP a adenosina e dessa forma exercem um fino controle dos efeitos desencadeados por essas importantes moléculas sinalizadoras. A expressão das ecto-nucleotidases e dos diferentes receptores purinérgicos tem sido amplamente descrita em zebrafish (Kucenas *et al*., 2003; Egan *et al*., 2007; Appelbaum *et al*., 2007). De acordo com a nomenclatura atual, pelo menos oito diferentes membros da família das E-NTPDases já foram descritos, clonados e funcionalmente caracterizados (E-NTPDases 1 a 8) (Bonan, 2012). Os membros das E-NTPDases hidrolisam seus substratos com diferentes habilidades e especificidades e podem diferir de acordo com a sua localização

celular (Zimmermann, 2001). As NTPDases 1, 2, 3 e 8 são expressas como enzimas de superfície celular e apresentam o seu sitio catalítico voltado para o meio extracelular. As NTPDases 5 e 6 estão localizadas no meio intracelular, mas podem ser secretadas para o meio extracelular. Já as NTPDases 4 e 7 são totalmente intracelulares, localizando-se voltadas para o lúmen de organelas citoplasmáticas (Robson *et al*., 2006; Rosemberg *et al*., 2010). Quanto as diferenças nas hidrólises, a NTPDase 1 hidrolisa ATP e ADP praticamente na mesma proporção, as NTPDases 2 e 8 apresentam maior atividade de hidrólise do ATP e a NTPDase 3, possui uma especificidade intermediária entre a NTPDase 1 e NTPDAse 2, com preferência de duas a três vezes maior pelo ATP (Robson *et al*., 2006; Brunschweiger *et al*., 2008).

As concentrações de adenosina podem ainda ser reguladas pela recaptação e subsequente fosforilação intracelular a AMP pela enzima adenosina quinase (AK) ou pela continuação da cascata de hidrólise do ATP no meio extracelular, sofrendo deaminação a inosina pela enzima ecto-adenosina deaminase (ADA) (Rosemberg *et al.*, 2007; Ramakers *et al.*, 2012). Essa apresenta duas subfamílias, denominadas ADA 1 e ADA 2, sendo que a segunda é subdividida em ADA 2.1 e ADA 2.2. Também já foram descritas outras ADA, conhecidas como ADAasi, uma isoforma derivada de *splicing* alternativo, e a ADA L, conhecida como adenosina deaminase *like* (Rosemberg *et al.*, 2007). As concentrações intra e extracelulares da adenosina são similares, normalmente na faixa entre 30 e 300 nM (Ballarin *et al*., 1991). Em condições de hipóxia, isquemia ou inflamação os níveis extracelulares de adenosina podem alcançar níveis entre 1 e 100 μM (Ye and Rajendran, 2009).

Os efeitos biológicos dos nucleotídeos são exercidos por meio da sensibilização das principais subfamílias de receptores purinérgicos, que são os receptores de adenosina (P1) e os receptores que reconhecem principalmente ATP, ADP, UTP, e UDP (P2). Os receptores P1 são acoplados à proteína G e são subdivididos em A_1, A_{2A}, A_{2B}, e A_3, de acordo com a estrutura molecular e bioquímica. Para o receptor A_{2A} já foram descritos dois genes em zebrafish, identificados como $A_{2A.1}$ e $A_{2A.2}$. No entanto, as suas diferenças funcionais ainda não são conhecidas (Boehmler *et al*., 2009).

Baseado em diferentes estruturas moleculares e mecanismo de transdução de sinal, os receptores P2 se dividem em duas famílias de receptores: receptores P2X (ionotrópicos) e P2Y (metabotrópicos) (Ralevic and Burnstock, 1998; Burnstock, 2006). Atualmente, sete subtipos de receptores P2X ($P2X_1$ à $P2X_7$) e oito subtipos de receptores P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) já foram identificados (Burnstock, 2006).

Os receptores P1 são potenciais alvos no tratamento de doenças inflamatórias como asma, doença pulmonar obstrutiva crônica, isquemias, artrite, sepse e na cicatrização. A ativação do receptor A_1 parece induzir a um aumento da resposta inflamatória *in vitro*. No entanto, em estudos *in vivo* em camundongos sem este receptor (*knockout*) ocorre um aumento das lesões renais e hepáticas, além do aumento da resposta inflamatória e da mortalidade elevada na sepse em comparação a camundongos selvagens. Estes efeitos são atribuídos ao possível efeito inibitório que a ativação do receptor A_1 exerce sobre o NF-κB. Os receptores A_{2A} reduzem/regulam a infiltração de células inflamatórias nos tecidos e a produção lesiva de EROs e de citocinas pró-inflamatórias. Diversos estudos apontam que entre os subtipos de receptores de adenosina, o A_{2A} é o principal mediador da inibição do TNF-α. A ativação deste receptor está ligada a liberação de IL-10 por macrófagos ativados por antígenos bacterianos de *E.coli*. Outro receptor P1 que está envolvido na mediação de processos inflamatórios é o A_{2B}. Embora, o seu papel pró e/ou anti-inflamatório seja bastante controverso, a ativação deste receptor parece estar mais relacionada com os efeitos pró-inflamatórios da adenosina, que envolvem o aumento da produção de IL-6 e a produção de fatores pro-inflamatórios, além de estimular a degranulação dos mastócitos e a liberação da histamina (Haskó *et al*., 2008; Gao and Jacobson, 2011).

Entre os agonistas do receptor A_{2A} que estão em fase clínica de estudos estão o Sonedenoson avaliado para o tratamento tópico de úlceras provenientes da diabetes, devido aos seus efeitos cicatrizantes e anti-inflamatórios e o Regadenoson, já aprovado pelo *Food and Drug Administration* (FDA) para a cintilografia de perfusão miocárdica, que está sendo avaliado para o uso no tratamento de adultos com doenças com células falciformes (Gao and Jacobson, 2011).

Os receptores P2X representam um alvo importante para a regulação da inflamação. Antagonistas de receptores $P2X_7$ podem servir como uma nova classe de compostos anti-inflamatórios, capazes não apenas de inibir o início da inflamação, mas também de auxiliar na sua resolução (Lister *et al*., 2007). Nos últimos anos, desde a clonagem do receptor purinérgico $P2X_7$, muitos estudos foram realizados, sendo comprovado que esse receptor desempenha funções importantes no sistema imunológico. O $P2X_7$ está claramente envolvido na secreção de citocinas por macrófagos, especialmente IL-1β, IL-18, TNF-α e IL-6, que desempenham um papel importante nas respostas inflamatórias. Esse receptor também regula a liberação de IL-8 a partir de eosinófilos e pode ser expresso em células polimorfonucleares, potencialmente influenciando suas funções. (Bavaresco *et al*., 2007; Rico *et al*., 2006; Braganhol *et al*., 2009).

Os receptores P2Y possuem potenciais terapêuticos, como os receptores $P2Y_1$ e $P2Y_{12}$ envolvidos na agregação plaquetária e receptores $P2Y_2$ como alvos para o tratamento de fibrose cística e de processos inflamatórios (Kügelgen and Wetter, 2000; Burnstock, 2006; Eltzschig *et al*., 2012). A ativação dos $P2Y_2$ aumenta a síntese e/ou liberação de mediadores inflamatórios importantes como prostaglandinas e NO. Foi demonstrado também que o aumento na expressão destes receptores é estimulado por TNF-α, IL-1β e IFN- γ e o aumento na sua ativação induzido por nucleotídeos promove a estimulação de proteína quinase C (PKC), COX-2 e MAPK (Burnstock, 2007).

3. JUSTIFICATIVA

O conhecimento proveniente do “Projeto Genoma Zebrafish”, aliado à capacidade de absorver rapidamente substâncias químicas diretamente adicionadas à água, fazem com que cada vez mais o zebrafish seja utilizado como modelo animal para estudos farmacológicos e para a caracterização de doenças humanas. Nesse sentido, a biologia desse teleósteo tem sido extensivamente pesquisada, resultando na caracterização de muitos genes que estão envolvidos em padrões de comportamento e nos mecanismos implicados na patogênese das doenças.

A padronização de um modelo de inflamação em larvas de zebrafish, bem como a caracterização dos sistemas envolvidos neste processo, pode contribuir para a avaliação da validade e segurança de sua utilização como modelo para a triagem de novos fármacos. Uma vez que este modelo experimental apresente resultados satisfatórios e compatíveis com os sistemas bem caracterizados em mamíferos, também poderá ser utilizado para avaliações mais completas, como em estudos que buscam elucidar mecanismos relacionados a doenças humanas e mecanismos de ação de fármacos e, consequentemente, reduzindo o uso de mamíferos e promovendo o uso consciente de animais.

Considerando que a expressão de diferentes receptores purinérgicos e das ecto-nucleotidases têm sido amplamente descritas em zebrafish e o potencial envolvimento da sinalização purinérgica na inflamação, um estudo mais aprofundado desse sistema permitirá a obtenção de avanços no conhecimento dos mecanismos da sinalização purinérgica em processos inflamatórios, fornecendo evidências para a utilização desse modelo para estudos de mecanismos de ação.

Ademais, a padronização do modelo de inflamação em larvas de zebrafish e a caracterização do envolvimento do sistema purinérgico na inflamação podem representar juntos uma nova ferramenta para a avaliação pré-clínica de novos agentes com potencial anti-inflamatório, oportunizando o estudo de novas alternativas para o tratamento da inflamação, especialmente naqueles casos refratários às terapias tradicionais.

4. OBJETIVOS

4.1 Objetivo Geral

Estabelecer e caracterizar um modelo de inflamação induzida pelo cobre em larvas de zebrafish e estudar o envolvimento do sistema purinérgico nesse modelo.

4.2 Objetivos específicos

a) Padronizar um modelo de inflamação induzido pelo cobre em larvas de zebrafish;

b) Adaptar e, se necessário, desenvolver metodologias analíticas para a determinação de marcadores inflamatórios e de estresse oxidativo em larvas de zebrafish;

c) Avaliar a absorção do cobre durante o período de exposição;

d) Determinar a correlação temporal entre a produção de citocinas, a produção de espécies reativas do oxigênio, a ativação de enzimas e a estimulação do sistema purinérgico;

e) Avaliar o perfil das atividades das E-NTPDases, da ecto 5’-NT e da ADA no processo inflamatório, bem como avaliar o papel do sistema purinérgico na exacerbação e na resolução do dano.

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6. ARTIGOS CIENTÍFICOS

6.1 ARTIGO I – Analytical Biochemistry 421 (2012) 534–540

Analytical method for determination of nitric oxide in zebrafish larvae: toxicological and pharmacological applications

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Abstract

Zebrafish are currently used at various stages of the drug discovery process and can be a useful and cost-effective alternative to some mammalian models. Nitric oxide (NO) plays an important role in physiology of zebrafish. The availability of appropriate analytical techniques to quantify the NO is crucial for studying its role in physiological and pathological conditions. This work aimed at establishing a high performance liquid chromatography method for determination of NO levels in zebrafish larvae. Attempts have also been made to assess the normal levels of NO at the first days post-fertilization, and the possible changes under pathological conditions. The method validation was quantitatively evaluated in terms of sensitivity, specificity, precision, accuracy, linearity and recovery. NO levels from zebrafish larvae at the first days post-fertilization and larvae challenged to N(G)-nitro-L-arginine methyl ester, sodium nitroprusside, *E. coli* lipopolysaccharide and copper sulphate were analyzed. The samples were derivatized with 2,3-diaminonaphthalene, and fluorescence detection was used for the indirect determination of NO. The method showed a good performance for all validation parameters evaluated and was efficient to monitor changes in NO concentration under physiological and pathophysiological conditions. This method might represent a powerful tool to be applied in NO studies with zebrafish larvae.

Keywords: zebrafish larvae; nitric oxide; validation; high performance liquid chromatography; pathological conditions.

Abbreviations: $CuSO_4$, copper sulphate; DAN, 2,3-diaminonaphthalene; Dfp, days post-fertilization; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide; L-NAME, N(G)-nitro-L-arginine methyl ester; NAT, 2,3-naphthotriazole; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; NOS, nitric oxide synthase; SNP, sodium nitroprusside.

Introduction

Drug discovery involves a complex interactive process of biochemical and cellular assays, with final validation in animal models, and ultimately in humans. Zebrafish (*Danio rerio*) is currently used at various stages of the drug discovery process and has several advantages when compared to other experimental models, such as cell culture and rodents [1; 2]. Advantages of zebrafish as a model organism include the small size of embryos and larvae, the high fecundity of adult zebrafish, the optical transparency of embryos and larvae, the speed at which they develop, and the possibility of simply adding compounds to water for screening of biomarkers, drugs and toxic agents [3; 4].

The teleost zebrafish is a small tropical fish, which produces optically transparent embryos that develop outside the mother's body [5; 6; 7]. At six days post-fertilization (dpf), a complex circulatory system and counterparts of most mammalian organs is developed. Although zebrafish proteins display less than 70% identity to their human orthologues, the conservation of functional domains, such as the substrate binding regions (often the drug-binding targets), is considerably higher, with approaching values of 100% similarity [5].

Nitric oxide (NO), a free radical and a signaling molecule, plays an important role in regulating vascular tone, neurotransmission, host immunity, nutrient metabolism, whole-body homeostasis, macrophage activity and cell proliferation, differentiation and apoptosis [1; 8; 9]. NO is formed in biological tissues from L-arginine by three major nitric oxide synthase (NOS) isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). All three major NOS isoforms are expressed during early development of zebrafish [10].

In the past few years, several additional tools have been developed, what greatly increased the utility of zebrafish as an experimental organism [6]. The availability of

appropriate analytical techniques to quantify the NO is crucial for studying its role in physiological and pathological conditions. However, because of its very short half-life in circulation and in cells (<5 s), the direct measurement of NO is extremely difficult. For this reason, the cellular generation of NO is most often assessed by measuring the nitrite (NO_2^-) and nitrate (NO_3^-) contents in biological fluids [8].

The aim of this study was to develop a simple and rapid routine method using a high performance liquid chromatography (HPLC) for determination of NO in nanomolar concentrations. The method validity was further confirmed by evaluating the basal levels of NO during the first days after fertilization, in addition to the changes of NO levels under inflammatory conditions.

Materials and methods

Chemicals

All chemicals were of HPLC grade. Methanol, chloride acid (HCl), sodium nitroprusside (SNP) and copper sulphate ($CuSO_4$) were purchased from Merck® (Darmstadt, Hessen, Germany). Sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH), 2,3-diaminonaphthalene (DAN), lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) and N(G)-nitro-L-arginine methyl ester (L-NAME) were acquired from Sigma Chemical Company® (Saint Louis, Missouri, USA).

Standards solutions

Stock solutions of SNP, $CuSO_4$ and LPS for larvae treatment were prepared in distilled/deionized water (dd-water). Stock standard solution of $NaNO_2$ (1 mM), for the HPLC assay, was prepared and added in dd-water to produce final concentrations of 20,

30, 50, 100, 250, 500, 1000 and 1500 nM. DAN solution (0.05 mg/mL) was prepared in HCl 0.65M. These solutions were made on the day of the experiments.

Zebrafish larvae and treatments

Zebrafish larvae were generated by natural pair-wise mating in aquariums with filtration system in series (Zebtec, Tecniplast®, Italy). They were staged and reared according to standard procedures [11]. Because the embryo receives nourishment from an attached yolk sac, no feeding was required until 7 dpf [12]. All protocols were approved by the Institutional Animal Care Committee (09/00135, CEUA–PUCRS) and followed the Canadian Council for Animal Care (CCAC) Guide on the care and use of fish in research, teaching, and testing.

All experiments using animals were conducted in triplicates containing a pool of 20 larvae each. The concentration of SNP was selected on the basis of the previous publication of Pelster *et al*. (2005) [9]. Survival curves for LPS and $CuSO_4$ were performed to choose a sub-lethal concentration to induce inflammation. For this purpose, two replicates were made with 15 larvae for each concentration. The treatments were performed in six-well culture plates.

NO_2^- levels were determined in zebrafish larvae at 0 (one hour post fertilization), 1, 3, 5 and 7 dpf. In a separate series of experiments, the larvae with 7dpf were incubated for 1 hour with the non-selective NOS inhibitor, L-NAME (1 µM and 5 µM), in order to confirm that NO_2^- detected in our protocol is derived from NOS.

To determine whether NO_2^- assay could be used to detect changes in NO production associated with stress situations, we performed three additional sets of experiments. Larvae with seven dpf were treated with SNP (1 mM), a nitric oxide donor used as positive control, $CuSO_4$ (10 µM), a chemical inducer of inflammation, and LPS

(150 µg/mL), an infectious stimulus. The agents used to stimulate NO release were directly added to water and NO_2^- was evaluated at 4 h for SNP and, at 4 and 24 h for LPS and $CuSO_4$.

Sample preparation

Zebrafish larvae stored at -80 ° C in 500 µl of phosphate buffered saline - PBS (pH 7.2 – 7.4) were thawed and washed four times with 1 ml of PBS to eliminate any environmental contamination of NO_2^-. After cleaning procedures, the samples were homogenized in 500 µl of cold PBS using a homogenizer glass-teflon immersed in ice. The samples were centrifuged at 13,500 x g for 5 min at 4 °C in 1.5 mL tubes and the supernatants were collected for NO_2^- analysis.

The samples were placed in 0.6 ml polypropylene tubes. DAN working solution (10 µL) was added to each polypropylene tube with 50 µl of dd-water and 50 µl samples, standards or blank (dd–water:PBS 1:1). The tubes were capped, protected from light, mixed and incubated for 15 min at room temperature. The reaction was stopped by adding 15 µl of NaOH (1 M). This reaction mixture was maintained at 4 °C in the dark and used for the chromatographic separation within 6 h. Twenty µl of this solution were injected into the HPLC system.

The total protein concentration of zebrafish larvae homogenates was determined according to the method of Bradford (1976) [13]. The total weight of larvae was not used to correct the results because the concentration of the protein has a better correlation with the quality of the homogenate, and consequently with the release of the analytes for the material used for quantification (supernatant).

Chromatographic conditions and equipments

A HPLC equipped with an isocratic pump, fluorescence detector, degasser and manual injection system (all HPLC components and software ChemStation were from Agilent Technologies® Inc. Santa Clara, California, USA) was used. Chromatographic separations were performed using a reverse-phase column (150 mm x 4 mm, 5 µm Agilent® 100 RP-18 ec). The column was protected by a guard column (4x4 mm, 5 µm Agilent® 100 RP-18 ec), and was maintained at 22 ± 2ºC. The flow rate of the acetonitrile:phosphate buffer 50 mM (pH=8.2) (35:65, v/v) mobile phase was 0.8 ml/min and the fluorescence was monitored with excitation at 355 nm and emission at 460 nm.

To confirm the specificity and accuracy of the method, a NO analyzer (model 270B NOA, Sievers Instruments Inc., USA) was used [14].

Validation of HPLC method and quality control procedures

The HPLC method was quantitatively evaluated in terms of sensitivity, specificity, precision, accuracy, linearity and recovery. We tested the levels of NO_2^- in dd-water obtained from Deionizator Permution® and water obtained from Millipore® system to choose the one with the lowest levels of NO_2^- for cleaning materials and the preparation of solutions used in the tests. The solutions for the preparation of the homogenate were also tested (saline and PBS). Six aliquots of each solvent were tested and were analyzed as the samples.

Statistical analysis

The results of validation are presented as the mean ± standard deviation and precision and accuracy are expressed as percentage. The linear relationship between concentrations of NO_2^- obtained by HPLC and NO analyzer was obtained using the

Pearson correlation coefficient. The matching (between methods) was analyzed with the Bland and Altman method. Survival curves were estimated using the Kaplan-Meier analysis. Statistical comparison of the data from NO_2^- determination in zebrafish larvae was performed by one-way ANOVA followed by Tukey's test. Values of $P < 0.05$ were considered significant.

Results

Chromatographic behavior and specificity

Laboratory plasticware and glassware and buffer solutions are common sources of contamination and considerable attention needs to be paid to the water quality. Problems may also be encountered regarding the procedures for the extraction of samples and assay reproducibility, again often arising from NO_2^- and NO_3 contamination [1]. In the preparation of solutions and curves of NO_2^-, dd-water was used, by presenting basal levels of NO_2^- 45% lower than the MilliQ ultrapure water. For the preparation of homogenates, PBS was used, which showed basal levels of NO_2^- 59% lower than sterile saline solution.

The fluorometric assay for NO_2^- detection is based on the reaction of NO_2^- with DAN under acid conditions to form fluorescent 2,3-naphthotriazole (NAT). For specific analysis of NAT, reverse-phase HPLC method has been used to separate NAT from DAN and other fluorescent substances present in biological samples [15; 16]. The retention times of DAN and NAT, were 3.0 and 5.9 min, respectively. Reliable separation of the analysis was achieved with adequate retention times for routine analysis, with runs less than 7 min. Figure 1 shows representative chromatograms of NAT from blank (A) and zebrafish samples with 247 nM (B) of NO_2^-.

Specificity was evaluated by determining the retention time of DAN, NAT and, also by analyzing of six homogenates (n=6) from different reproductions to check endogenous interference components. All tested zebrafish larvae homogenates were free for endogenous interference in this assay. Moreover, to confirm the specificity of our method for NO_2^-, three different curves were made and analyzed using the method described in this article and in a NO analyzer. Figure 2 shows a high correlation between NO_2^- measured by HPLC and NO_2^- measured by NO analyzer (The Pearson correlation coefficient= 0.9619; $P < 0.0001$). The slope of the regression lines between the two assays was 1.028. Moreover, Bland-Altman analysis (figure 3) showed that the concentrations of NO_2^- measured by two methods are not significantly different. Applying the Bland-Altman plot to measure the difference between NO_2^- by HPLC and NO Analyzer, we found a bias mean difference of 7.07 nM and a 95% limit of agreement from -98.85 to 112.98 nM.

Precision and accuracy

The precision and accuracy were determined by analysis of four different concentrations, covering the low, medium and higher ranges of the calibration curves. The intra and inter-day precision and accuracy data are shown in Table 1. Precision was expressed as percent coefficient of variation (CV) and accuracy was expressed as a percentage of the added concentration (found value x 100/added value). The inter-day precision and accuracy were determined over a period of one week.

Linearity

The calibration curves were linear using different calibrating standards containing known amounts of NO_2^- in the range of 30 to 1500 nM. The calibration equation was y =

0.04543138x + 0.1075868 (y is the peak area and x is the concentration in nM) with a correlation coefficient of r = 0.9996.

Sensitivity

The limit of detection (LOD) was determined as being the detected concentration with an area greater than that presented in the blank prepared with dd-water (8.0 nM). The limit of quantification (LOQ) is the lowest analyzed amount, which can be measured with defined precision and accuracy and, reproducible with CV less than 20% and accuracy of 80–120% [17]. The LOQ were found to be 21 ± 4 nM with CV of 18.58% and accuracy of 103.98% for NO_2^-.

NO_2^- recovery

The recovery for NO_2^- was determined by spiking known quantities of standards into 7 dpf larvae homogenate to obtain low, medium and high ranges of the calibration curve. The results were obtained by calculating the difference between NO_2^- before and after spiking standards in samples. Results of samples were divided by added concentrations and the results were multiplied by hundred. The recoveries of NO_2^- to 30, 250, 1000 and 1500 nM were expressed as mean ± standard deviation, revealing the following results: 90 ± 4; 93 ± 2; 95 ± 2; and 97 ± 1, respectively.

NO levels, blockade with L-NAME and stimulation with SNP, LPS and $CuSO_4$

Firstly, zebrafish larvae of 0, 1, 3, 5 and 7 dpf were evaluated to determine the NO_2^- levels in the early days of development. The results show that the concentration of NO_2^- increased gradually after fertilization, as shown in figure 4. In the inbox of figure 4, it is shown that L-NAME treatment decreased the basal concentrations of NO_2^-,

demonstrating that the NO increase until 7 dpf is likely the result of a gradual augmentation in the activity of NOS.

The survival curves to choose sub-lethal concentrations of LPS and $CuSO_4$ to induce inflammation are shown in figure 5. The concentration chosen was the one that induced mortality between 10 and 20% (150 µg/ml and 10 µM induced 16.7% and 11.5% of mortality for LPS and $CuSO_4$, respectively). The SNP was assessed only at 4 h, as this agent is short-acting NO donor [18]. For LPS and $CuSO_4$ groups, the time-points of 4 and 24 h were used to determine the best periods for the establishment of an acute inflammatory response. The zebrafish larvae challenged to SNP for 4 h (5200 ± 340 nmol/ mg protein), LPS for 4 h (1040 ± 50 nmol/ mg protein) and $CuSO_4$ for 24 h (1300 ± 160 nmol/ mg protein) showed a significant increase of NO_2^- levels when compared to the controls groups (790 ± 50, 790 ± 90 and 760 ± 90 nmol/ mg protein, respectively), with significance levels of $P<0.001$, $P<0.01$ and $P<0.01$, respectively, as shown in figure 6.

Discussion

Several animal models have been used in toxicological and pharmacological studies. Zebrafish has emerged as a promising experimental model, and developing methods for the quantification of different biochemical and physiological markers are extremely important for its consolidation [19].

In this study, we present a new, simple, rapid and suitable HPLC method with fluorescence detection for determination of NO_2^- in homogenates of zebrafish larvae. The method presented herein employs an extremely simple sample preparation, which does not need internal standard, using a small sample volume. Furthermore, we have achieved satisfactory separation under isocratic conditions. These characteristics are easily found

in many laboratories. Moreover, as we are working with low levels of protein and a very sensitive method, a simple dilution was able to prevent any interference from chlorine or protein. Thus, it was not necessary to use processes of precipitation or ultrafiltration of proteins and/or removal of chlorine from samples using cation-exchange chromatography, precipitation with silver reagents, or silver-based solid-phase extraction C18 [1; 15].

When we compare the data quantified by HPLC and by NO analyzer no significant differences were found in the results, confirming the specificity and accuracy of the method developed. Moreover, the curve determined by the analyzer showed sensitivity only above 100 nM, which makes the new method described here more sensitive. Other methods for the determination of NO in zebrafish larvae employ procedures based on the use of chemical probes or molecular biology assays [19; 20]. Although these methods are efficient, not always there is availability of reagents and equipment in the laboratories. Thus, we provide a new sensitive, safe and fast methodology for quantifying NO_2^-. A disadvantage of the proposed method is the impossibility of directly assessing NO_3^- levels, which might be reached through NO_3^- reduction, and the subsequent determination according to the technique described here. During inflammatory challenges not only NO formation is increased but also the level of superoxide and other reactive oxygen species is increased providing multiple routes for oxidative break-down of NO (e.g. by peroxynitrite formation or myeloperoxidase catalyzed nitrogen dioxide formation in the presence of nitrite and hydrogen peroxide) leading to NO_3^- formation. Therefore, a limitation of the present technique is that NO ending up as NO_3^- is not detected, which could lead to underestimation of NO under inflammatory conditions (e.g. LPS treatment). Among the possible techniques used to escape of NO_3^- reduction are the fluorescence probes, such as 4,5-diaminofluorescein

(DAF-2). The fluorescein reacts with an oxidation product of NO to the highly fluorescent DAF-2T triazol fluorescein by the known reaction mechanism. This probe is used to measure NO extracellularly and could be adapted to other experimental models [21].

Rico *et al*. (2011) recently suggested further investigations about the functional role of NO in zebrafish to evaluate changes in this system induced by pharmacological approaches and toxic agents [22]. In addition to validation of a NO_2^- determination method, a series of biological assays, including unpublished results, demonstrating the efficiency and applicability of the new method. The determination NO_2^- in zebrafish larvae of different dpf showed the difference in NO concentrations according to the stage of development. These findings are consistent with the fast development of these animals, as demonstrated by Lepiller *et al*. (2007) [20]. One proposed mechanism for the effects of NO in developmental processes is a suppressive influence on DNA synthesis, whereby NO acts as a negative regulator on precursor cells, affecting the balance of cell proliferation, differentiation and apoptosis [10; 23]. The differentiated expression of NOS isoforms in certain tissues at different developmental stages indicates that temporal and spatial NO-mediated activities may be regulated by distinct NOS-producing systems [10]. For example, the expression of NOS in zebrafish embryos was detected at 16 hpf in the hypothalamus, and it was present in discrete central nervous system locations after 3 dpf [22]. Additional studies are still required to better characterize the NOS isoforms related to zebrafish post-fertilization NO production.

Several studies show the multifunctional properties of NO in various normal and pathophysiological events in early life processes [10; 24; 25; 26]. NO production is increased in a variety of diseases, and several classic signs of inflammation are reversed by NOS inhibitors. Zebrafish is a good model for studying inflammation and infectious

diseases; for instance, Watzke *et al*. (2007) and Novoa *et al*. (2009) showed that LPS was able to induce the expression of pro-inflammatory cytokine genes as part of the innate immune responses in zebrafish embryos and larvae by immersion in bacteria or bacterial LPS [2; 27]. D'Alençon *et al*. (2010) showed that exposure of fish larvae to sublethal concentrations of $CuSO_4$ selectively damages the sensory hair cell population inducing infiltration of leukocytes, release of reactive oxygen species and a robust acute inflammatory response [28; 29; 30]. The method proposed herein was efficient to monitor changes in NO production in zebrafish larvae at first days post-fertilization when stimulated with NO donor or under inflammation induced by infection and chemical agents. NO_2^- levels were found significantly increased in LPS-induced inflammation at 4 h, whereas inflammation induced by $CuSO_4$ showed a significant increase at 24 h. This probably occurred because the inflammation induced by LPS involves an infectious process and NO formation is one of the first-line defenses against invading microbial organisms including parasites, bacteria, and viruses [31]. Additionally, the later release of NO induced by $CuSO_4$, compared to LPS, might likely occur because the copper is uptaken from water and accumulates in zebrafish in a time- and concentration-dependent manner [32]. Accordingly, Craig *et al*. (2007) showed that the highest concentrations of copper in the liver and gills of zebrafish are found after 24 h of exposure to this metal [33]. The low response using LPS might be explained by difference in functionality of TLR-4 in this model, which has a known weak responsiveness to LPS.

In summary, NO_2^- was determined with high sensitivity, accuracy and precision using a very simple, quick and inexpensive method. This method showed to be efficient to monitor changes in physiological NO production during the development phase and pathophysiological conditions, such as inflammation. It is tempting to propose that this method is a powerful tool to be applied for nitric oxide studies in zebrafish larvae.

Additionally, NO levels in the early stages of development showed a gradual increase from fertilization until the seventh day of life and, the larvae exposed to inflammatory agents and nitric oxide donor showed a significant release of nitric oxide.

Acknowledgments

This work was supported by DECIT/SCTIEMS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Proc. 10/0036-5 – PRONEX/ Conv. 700545/2008). S.C.C. is an undergraduate Pharmacy student receiving grants from FAPERGS. We would like to thank Mr. Fabiano Peres Menezes for his excellent technical assistance and Dr. Rafael Zanin and Msc. Denis Broock Rosemberg for technical advices**.**

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Table 1. Intra- and inter-day precision and accuracy for the NO_2^- quantification by HPLC.

NO_2^- Added (nM)	Intra-assay (n=6)			NO_2^- Added (nM)	Inter-assay (n=6)		
	Found (nM) (mean±[a]SD)	Precision [b]CV (%)	Accuracy (%)		Found (nM) (mean±[a]SD)	Precision [b]CV (%)	Accuracy (%)
30	32 ± 2	5.12	106.1	30	32 ± 2	7.14	108.3
250	249 ± 15	5.98	99.73	250	251 ± 12	4.88	100.34
1000	998 ± 21	2.08	99.76	1000	1003 ± 25	2.53	100.28
1500	1495 ± 23	1.51	99.68	1500	1499 ± 33	2.17	99.91

[a]*SD: Standard deviation;* [b]*CV: Coefficient of variation*

Figures

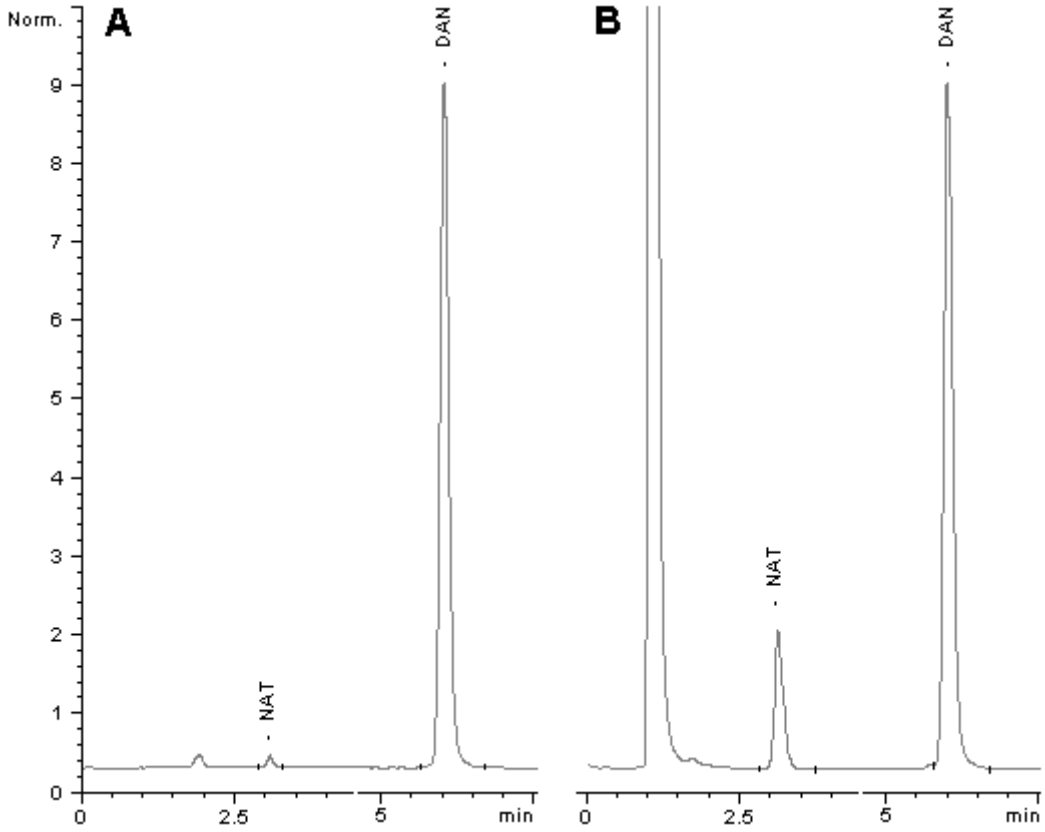

Figure 1. Typical chromatograms of NAT from blank (A) and zebrafish sample with 247 nM (B) of NO_2^-.

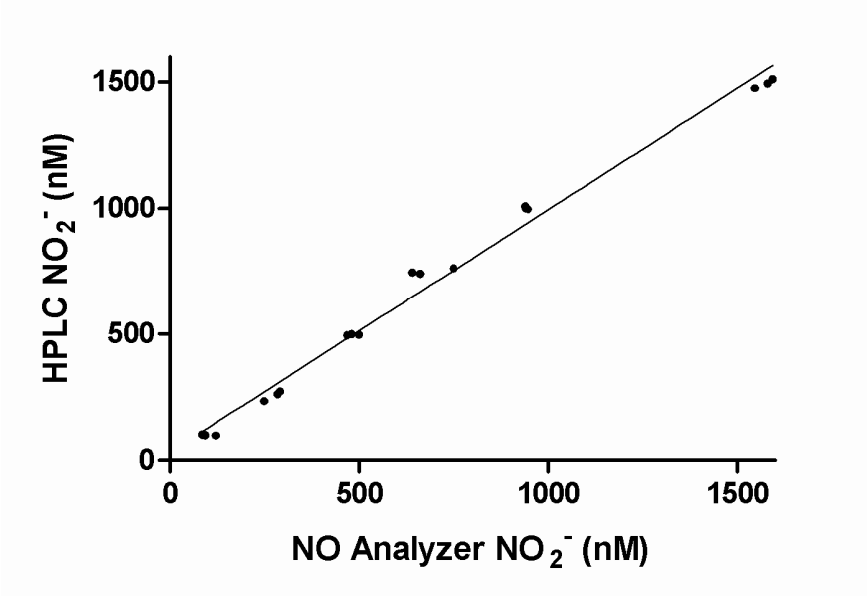

Figure 2. Correlation between the concentrations of NO_2^- obtained by HPLC and obtained by NO analyzer. The linear relationship between concentrations was obtained using the Pearson correlation coefficient.

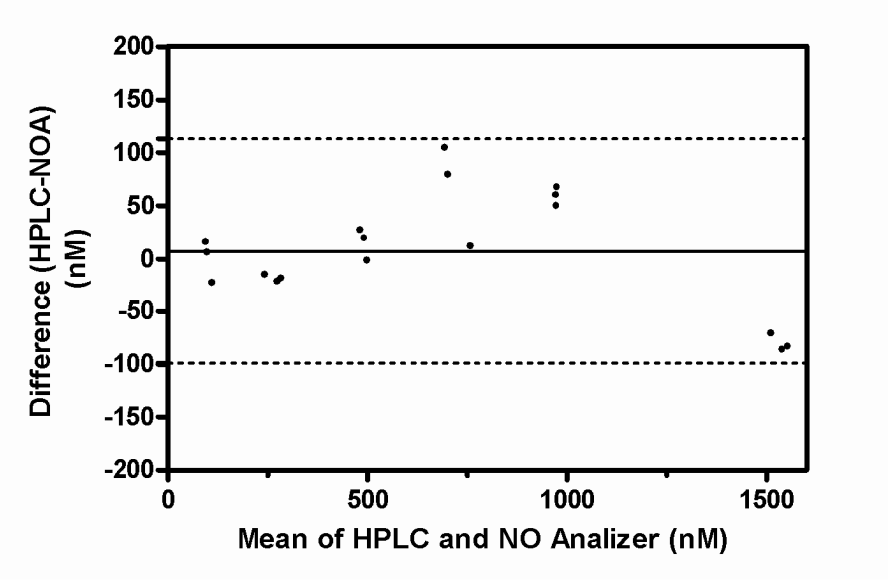

Figure 3. Bland-Altman bias plot of the difference between the NO_2^- concentrations obtained by HPLC and NO Analyzer vs. the mean of the two measurements. The thick solid line represents the bias between the assays, and the dashed lines represent 95% limits of agreement.

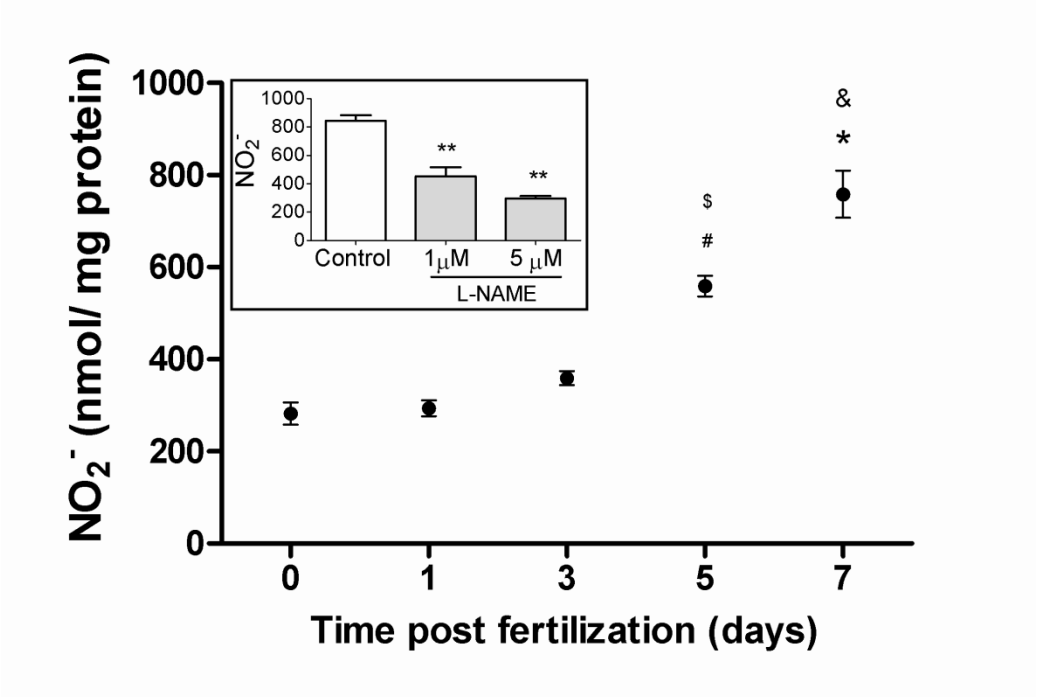

Figure 4. NO_2^- levels in the early days of development. Each point represents the mean of 3 homogenates (20 zebrafish larvae each) and the vertical lines show the S.D.: * indicates $P < 0.001$when compared to 0, 1, and 3 dpf; & indicates $P < 0.01$ when compared to 5 dpf; # indicates $P < 0.001$ when compared to 0 and 1 dpf; $ $P < 0.01$ when compared to 3 dpf. In the box is shown the effect of L-NAME on the NO_2^- concentrations in zebrafish larvae of 7 dpf. Asterisks denote the significance levels in comparison to control values: *$P<0.05$; **$P<0.01$; Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

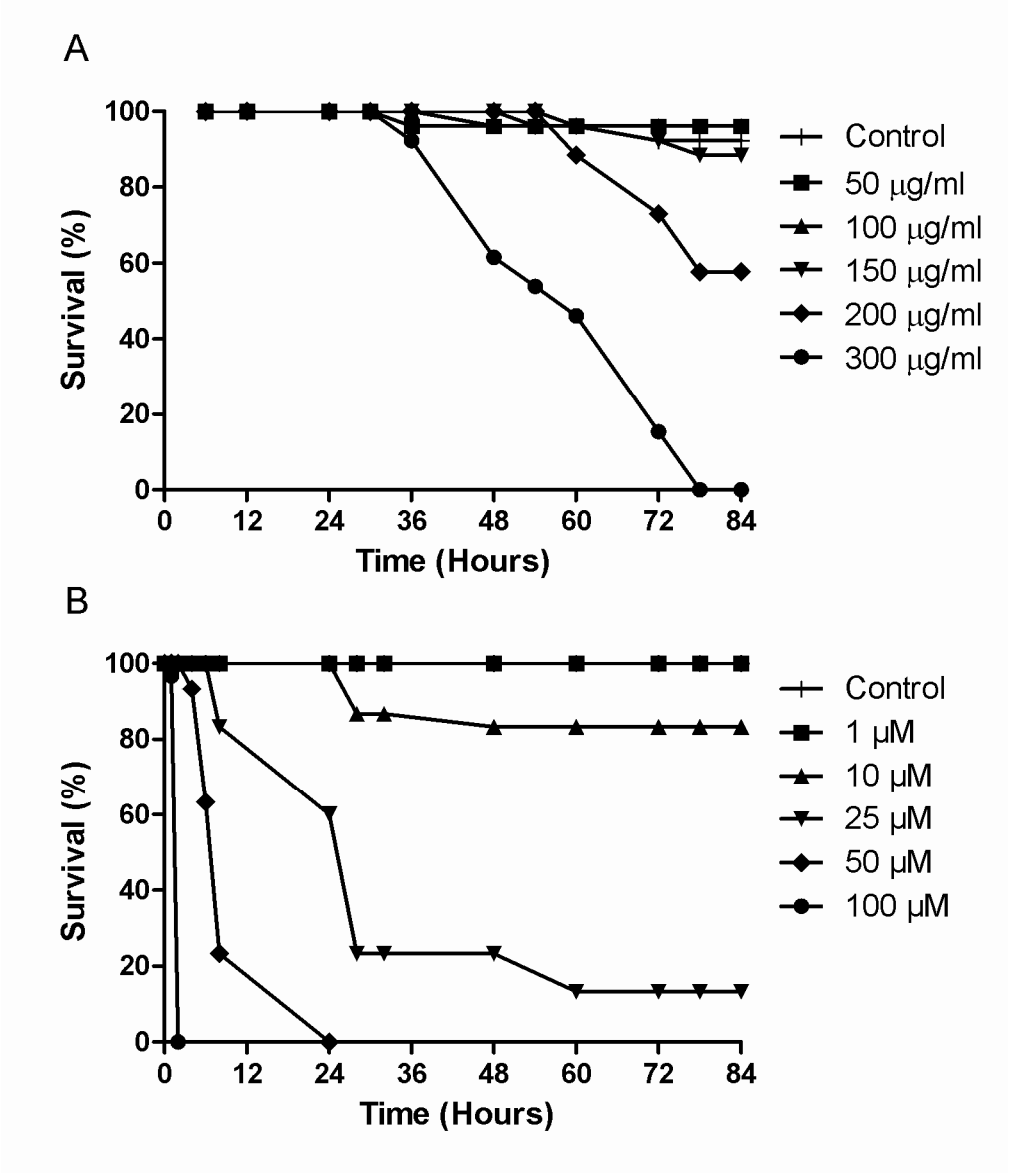

Figure 5. Kaplan-Meier survival curves from zebrafish larvae challenged with different concentrations of LPS (A) and $CuSO_4$ (B).

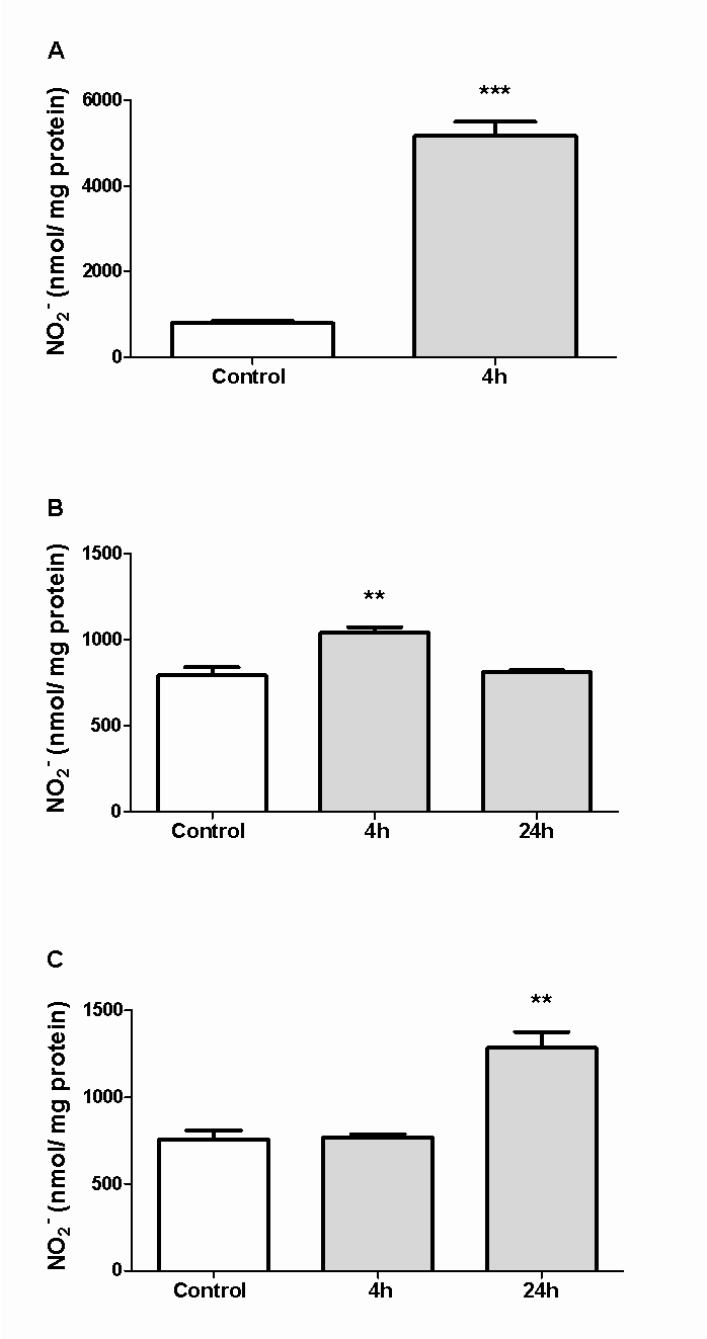

Figura 6. Zebrafish larvae were treated with 1 mM of SNP (A), 150 µg/ml of LPS (B) and 10 µM of $CuSO_4$ (C). Each column represents the mean of 3 homogenates (20 zebrafish larvae each) and the vertical lines show the S.D. Asterisks denote the significance levels in comparison to control values: **P*<0.05*;* ***P*<0.01; ****P*<0.001. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

6.2 ARTIGO II – Toxicology and Applied Pharmacology 272 (2013) 681-689

Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae

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ABSTRACT

The use of zebrafish (*Danio rerio*) is increasing as an intermediate preclinical model, to prioritize drug candidates for mammalian testing. As the immune system of the zebrafish is quite similar to that of mammals, models of inflammation are being developed for the screening of new drugs. The characterization of these models is crucial for studies that seek for mechanisms of action and specific pharmacological targets. It is well known that copper is a metal that induces damage and cell migration to hair cells of lateral line of zebrafish. Extracellular nucleotides/nucleosides, as ATP and adenosine (ADO), act as endogenous signaling molecules during tissue damage by exerting effects on inflammatory and immune responses. The present study aimed to characterize the inflammatory status, and to investigate the involvement of the purinergic system in copper-induced inflammation in zebrafish larvae. Fishes of 7 days post fertilization were exposed to 10 µM of copper for a period of 24 h. The grade of oxidative stress, inflammatory status, copper uptake, the activity and the gene expression of the enzymes responsible for controlling the levels of nucleotides and adenosine were evaluated. Due to the copper accumulation in zebrafish larvae tissues, the damage and oxidative stress were exacerbated over time, resulting in an inflammatory process involving IL-1β, TNF-α, COX-2 and PGE_2. Within the purinergic system, the mechanisms that control the ADO levels were the most involved, mainly the reactions performed by the isoenzyme ADA 2. In conclusion, our data shed new lights on the mechanisms related to copper-induced inflammation in zebrafish larvae.

Keywords: zebrafish; inflammation; copper; purinergic system; adenosine.

ABBREVIATIONS

ADA – Adenosine deaminase

ADO - Adenosine

ADP - Adenosine diphosphate

AMP - Adenosine monophosphate

ATP - Adenosine triphosphate

CAT – Catalase

COX-2 - Cyclooxygenase 2

Ecto-5'-NT - Ecto-5'-nucleotidase

E-NTPDase - Ecto-nucleoside-trifosfo difosfoidrolases

GSH - Reduced glutathione

HPLC-FLD - High performance liquid chromatography with fluorescence detector

ICP-MS - Inductively coupled plasma mass spectrometry

IL-10 - Interleukin 10

IL-1β - Interleukin 1β

INO - Inosine

MPO - Myeloperoxidase

NO - Nitric oxide

PGE_2 - Prostaglandin E_2

SOD - Superoxide dismutase

TNF-α - Tumour necrosis factor α

UHPLC – Ultra high performance liquid chromatography coupled with mass spectrometry

DAD – Diode array detector

INTRODUCTION

Zebrafish (*Danio rerio*) is a vertebrate that has been widely used in studies of the immune system. Toxicology and safety pharmacology are broadly assessed by *in vitro* assays, but the results might not be predictive of *in vivo* effects. Thus, the use of zebrafish is increasing as an intermediate step, together with the cell culture evaluation, to prioritize drug candidates for mammalian testing, allowing a reduction in the number and cost of studies (McGrath and Li, 2008; Thienpont et al., 2013). Among other advantages of using this teleost, especially embryos and larvae, it is possible to remark the favorable absorption characteristics, small amounts of compounds necessary for testing and, animals and experimental groups that can be evaluated in a single trial (Berghmans *et al.*, 2008).

The immune system of zebrafish is quite similar to that of mammals, including the presence of T and B lymphocytes, antigen presenting cells, phagocytic cells, and humoral immunity, together with the complement components. The similarities between the immune system of fishes and mammals, a fully sequenced genome and an increase in the production of transgenic and knockout zebrafish have encouraged many researchers to establish models of bacterial infections (Vojtech *et al.*, 2009; Loynes *et al.*, 2010) and chemical inflammation, such as induced by *Escherichia coli* and copper, respectively (d'Alencon *et al.*, 2010; Leite *et al.*, 2012).

Copper is an essential metal obtained from diet sources, chelated by amino acids, absorbed in the small intestine and transported in the blood in a binding form (Gaetke and Chow, 2003; Rosemberg *et al.*, 2007b). Oxidative damage has been linked to chronic copper overload and/or exposure to excessive concentrations caused by accidents, occupational hazards, and environmental contamination. Additionally, oxidative damage induced by copper has been implicated in disorders associated with abnormal metabolism (Wilson disease) and neurodegenerative diseases (Alzheimer disease) (Gaetke and Chow, 2003; Brenner, 2013). Olivari *et al*. (2008) described events induced by copper via oxidative stress, such as cell death by apoptosis and necrosis in hair cells of lateral line of zebrafish. These events, also described in cell cultures, were related to the participation of this metal in Fenton chemistry (Prousek, 2007; Olivari *et al.*, 2008). Recently, d'Alencon *et al.* demonstrated that copper exposure induces neutrophil migration to the inflammatory focus, due to the damage induced in hair cells of lateral line of zebrafish larvae, pointing out this model as a potential tool for screening of anti-inflammatory

compounds (d'Alencon *et al.*, 2010). Another interesting study described the toxicity induced by copper in the central nervous system of adult zebrafish, via inhibition of ecto-nucleoside-trifosfo difosfoidrolases (E-NTPDase) and ecto-5'-nucleotidase (ecto-5'-NT) (Rosemberg *et al.*, 2007). These enzymes belongs to ecto-nucleotidase family and are responsible for controlling the levels of nucleotides and nucleosides by sequential hydrolysis of adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and, sequentially the desamination of AMP to adenosine (ADO) is carried out by the enzyme adenosine deaminase (ADA) (Rico *et al.*, 2003; Senger *et al.*, 2004; Rosemberg *et al.*, 2008; Rosemberg *et al.*, 2010)

The extracellular nucleotides act as endogenous signaling molecules in tissue damage by exerting effects on inflammatory and immune response (Lazarowski *et al.*, 1997; Burnstock, 2006). The ATP is important in the secretion of cytokines and, recruitment and differentiation of immune cells, whereas ADO commonly displays immunosuppressive responses to protect healthy tissues from stressful stimuli (Bours MJ, 2006). In the inflammatory process, ATP and ADO are released at the site of inflammation as a result of cell damage or through the release to extracellular medium in consequence to cell activation (Luttikhuizen *et al.*, 2004; Burnstock, 2006).

Considering that: (I) zebrafish is a model widely used in toxicological studies; (II) copper model of inflammation has emerged as an important tool for drug screening; (III) purinergic system can play a key role on inflammation homeostasis, modulating ATP and ADO levels and; (IV) there is no data relating copper inflammation model in zebrafish larvae with purinergic system, the major aim of this study was to standardize the time-related biochemical and molecular markers related to inflammation, and to investigate the involvement of the purinergic system in the inflammatory status induced by copper in larvae of zebrafish.

MATERIALS AND METHODS

Animals

Zebrafish larvae were generated by natural pair-wise mating in aquariums with filtration system in series (Zebtec, Tecniplast®, Italy). They were staged and reared according to standard procedures (Westerfield, 2000). Because the embryo receives nourishment from an attached yolk sac, no feeding was required until 7 days post

fertilization (dpf) (Lewis, 2010). All protocols were approved by the Institutional Animal Care Committee (09/00135, CEUA–PUCRS).

Chemicals

Copper, added as copper sulfate pentahydrate ($CuSO_4.5H_2O$) were purchased from Merck (Darmstadt, Hessen, Germany). All other reagents used were purchased from Sigma (St. Louis, MO, USA).

Treatments

All treatments were performed in 6-well culture plates and, the copper ($CuSO_4.5H_2O$) was added directly to water. First, we used the oxidative stress as a parameter for evaluating the extent of damage caused by 1, 10 and 25 µM of copper during 24 hours of treatment. The concentration of 10 µM of copper, considered harmful without excessive mortality, was used to characterize the inflammation model and, to determine its association with the purinergic system in zebrafish larvae. The observation times were tested and based on knowledge of the activation cascade of inflammation. The parameters assessed and times of observations were: oxidative stress (0, 4, 6 and 24 h) neutrophil migration to the damaged tissues (0, 0.5, 1, 4 and 24 h), release of prostaglandin E_2 (PGE_2) (0, 1, 2, 4 and 24 h), expression of inflammatory mediators (0, 4 and 24 h), extracellular ATP hydrolysis (0, 4 and 24 h) and ecto-nucleotidase and ADA activities (0 and 24 h). The enzyme gene expressions were assessed in 0 and 24 h. The temporal determination of copper absorption by zebrafish larvae was performed in 0, 1, 2, 4, 6 and 24 h to evaluate the relationship of the concentration of copper with the effects at different times.

Oxidative stress and antioxidative defenses

In order to determine the antioxidant defenses and oxidative stress, we have measured catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD), and nitric oxide (NO). Initially, the experiments were performed using n=6, containing a pool

of 20 larvae each, after 24 h of treatment with 1, 10 and 25 µM of copper. The concentration of 10 µM of copper was chosen to continue the study and, it was evaluated in 0 h (control), 4, 6 and 24 h after copper treatment as described above. After the different concentrations and times of treatments the larvae were homogenized in 500 µl of phosphate buffered saline - PBS (pH 7.2 - 7.4). All samples were centrifuged at 13 500 x g for 5 min at 4 °C in 1.5 mL tubes, and the supernatants were collected for analysis. CAT activity was assessed through the hydrogen peroxide concentration decrease, according to the method described previously (Aebi, 1984). GSH levels were determined by using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), according to the method described previously (Shim *et al.*, 2010). SOD activity was assayed by measuring the adrenaline auto-oxidation inhibition, according to the method described previously (Bannister and Calabrese, 1987). NO was determined by high performance liquid chromatography with fluorescence detector (HPLC-FLD) using 2,3-diaminonaphthalene to derivatize the samples, according to the method described previously (Leite *et al.*, 2012).

Determination of PGE_2 by UHPLC-MS/MS

PGE_2 was tested at 0 h (control), 0.5, 1, 4 and 24 h, since it is one of the first mediators to be released during the inflammatory process. The experiments were conducted using n=6, containing a pool of 35 larvae each. Homogenates were prepared in 500 µl of phosphate buffered saline - PBS (pH 7.2 - 7.4). A 400 µl aliquot of the homogenate was transferred into a 9 ml glass tube and subjected to extraction using a modified method described previously (Shim *et al.*, 2010). Briefly, 80 µl of 1M nitric acid were added to the samples. Fifty microliters of BHT 1% was added to each tube. PGE_2 was then extracted with 2 ml of hexane: ethyl acetate (1:1, v/v) and mixed for 1 min. Samples were centrifuged at 800 x *g* for 5 min at 4 °C. The upper organic layer was collected, and the organic phases from three extractions were pooled and then evaporated to dryness under a stream of nitrogen at room temperature. Samples were then reconstituted in 100 µl of methanol before analysis by ultra high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS). Five microliters were injected into the UHPLC 1290/ MS 6460 TQQQ – Agilent (all UHPLC components and software MassHunter were from Agilent Technologies®). Chromatographic separations were performed using a Zorbax Eclipse Plus Phenyl-Hexyl 4.6x50mm 1.8 µm column. The flow rate of formic acid 0.1%: acetonitrile (formic acid 0.1%) 50:50 v/v mobile phase was 0.4 ml/min with a column temperature of 45°C. PGE_2 was detected

using electrospray negative ionization and multiple-reaction monitoring of the transition ions. The collision energy was 14 V for transition 351>271 (quantifier) and 6 V for 351>315 (qualifier). The results were expressed as nanograms of PGE_2 per mg protein.

Neutrophil myeloperoxidase assay

Neutrophil migration was assessed by means of myeloperoxidase activity (MPO), according to the method described previously, with slight modifications for zebrafish (Passos *et al.*, 2004). MPO was tested at 0 h (control), 1, 2, 4 and 24 h following copper treatment. Experiments were conducted using n=6, containing a pool of 20 larvae each. All samples were homogenized in 500 µL of EDTA/NaCl buffer (pH 4.7) and centrifuged at 5 000 × *g* for 20 min at 4°C. The pellet was suspended in 200 µL of hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were frozen. Upon thawing, the samples were re-centrifuged and, the supernatant was used for MPO assay. The results were expressed as optical density per milligram of tissue.

Analysis of extracellular ATP metabolism by HPLC

ATP hydrolysis and its degradation products (ADP, AMD, ADO, and INO) were analyzed in the groups at 0 h (control), 4 h and 24 h after copper exposure. Experiments were conducted using n=4 containing a pool of 35 larvae each. The membranes were prepared in 500 µl of chilled Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4 adjusted with citric acid), centrifuged at 800 × *g* for 10 min and the pellet was discarded. The supernatant was centrifuged for 25 min at 40 000 × *g* and, the pellet was frozen in liquid nitrogen, thawed, suspended in 500 µl of Tris–citrate buffer and, used for analysis. All membranes were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$. All samples were preincubated for 10 min at 37 °C before starting the reaction by the addition of substrate ATP to a final concentration of 100 µM. To stop the reaction, an aliquot of the incubation medium was transferred to a pre-chilled tube and centrifuged at 4°C for 15 min at 14 000 x *g*. Twenty microliters were injected into the HPLC system (all HPLC components and software ChemStation were from Agilent Technologies®). Chromatographic separations were performed using a reverse-phase column (150 mm x 4 mm, 5 µm Agilent® 100 RP-18 ec). The column was protected by a guard column (4x4 mm, 5 µm Agilent® 100 RP-18 ec), and was maintained at room temperature. The flow ramp of methanol: 60 mM KH_2PO_4 with 5 mM tetrabutylammonium chloride (pH 6.0) (13:87, v/v) mobile phase was maintained

between 1.2 and 2.0 ml/min according retention time of each compound. The mobile phase was prepared daily due to low stability. Absorbance was monitored at 260 nm with diode array detector (DAD).

Ecto-nucleotidase assay

E-NTPDase and ecto-5'-NT assays were performed as described previously (Rico *et al.*, 2003; Senger *et al.*, 2004). Activities were analyzed in 0 h (control) and after 24 h of exposure to copper in zebrafish larvae. Zebrafish larvae membranes (3–5 μg protein) prepared as described above (analysis of ATP metabolism) were added to the reaction mixture containing 50 mM Tris–HCl (pH 8.0) and 5 mM $CaCl_2$ (for the ATPase and ADPase activities) or 50 mM Tris–HCl (pH 7.2) and 5 mM $MgCl_2$ (for the AMPase activity) in a final volume of 200 μl. All membranes were preincubated for 10 min at 37 °C before starting the reaction by the addition of substrate (ATP, ADP or AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min by the addition of 200 μl of trichloroacetic acid 10% (final concentration 5% w/v). Samples were chilled on ice for 10 min before assaying for the release of inorganic phosphate (Pi), according malachite green method, using KH_2PO_4 as a Pi standard (Chan *et al.*, 1986). The non-enzymatic Pi released from nucleotides into the assay medium was subtracted from the total Pi released during the incubation, giving net values for enzyme activity. Specific activity was expressed as nanomol of Pi released per minute per milligram of protein. All enzyme assays were run at least in triplicate.

Determination of total adenosine deaminase activity

Adenosine deaminase (ADA) activity was determined spectrophotometrically as described previously (Rosemberg *et al.*, 2008). The activity was analyzed in control group (0 h) and 24 h after exposure the larvae to copper. To assess the total activity of ADA the homogenate was centrifuged to 1 000 x *g* and, the supernatant was used for activity assay. Zebrafish larvae supernatants (10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) in a final volume of 200 μl. The samples were preincubated for 10 min at 37 °C and the reaction was initiated by the addition of adenosine to a final concentration of 1.5 mM. After incubation for 120 min, the reaction was stopped by adding the samples on 500 μl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml). Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to

correct non-enzymatic deamination of substrate. The reaction mixtures were mixed to 500 µl of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125 % available chlorine, in 0.6 M NaOH), vortexed and incubated at 37 °C for 15 min for colorimetric assay at 635 nm. The ADA activity was expressed as nmol of NH_3 released per minute per milligram of protein.

Quantitative real time RT-PCR (qRT-PCR)

The gene expression of important elements in inflammation as IL-1β (interleukin 1β), IL-10 (interleukin 10), TNF-α (tumour necrosis factor α) and COX-2 (cyclooxygenase 2) and, expressions of ADA subfamilies (ADA 1, ADA 2.1, ADA 2.2) including an alternative splicing isoform (ADAasi) and, an adenosine deaminase like related gene (ADA L) were determined. The total RNA was isolated with Trizol® reagent, according to the manufacturer's instructions. Pools of 15 zebrafish larvae with 7 dpf were used. Total RNA was quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System from 1 µg total RNA in accordance with the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I to detect double-strand cDNA synthesis. The reactions were done in a volume of 25 µl using 12.5 µl of diluted cDNA (1:50), containing a final concentration of 0.2 x SYBR Green, 100 µl dNTP, 1 x PCR Buffer, 3 mM $MgCl_2$, 0.25 U Platinum® Taq DNA Polymerase and 200 nM of each reverse and forward primers (Table 1). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 ^oC for desnaturation, 35 s at 60 ^oC for annealing and 15 s at 72 ^oC for elongation. At the end of the cycling protocol, a melting curve analysis was included and fluorescence measured from 60 ^oC to 99 ^oC. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems) and, determined using the $2^{-\Delta\Delta ct}$ method. The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl) and the stability of the reference genes EF1α, RIp13α and β-actin (M-value) and its optimal number according to the pair wise variation (V) were analyzed by GeNorm 3.5 Software (http://medgen.ugent.be/genorm/).

Analysis of temporal absorption of copper by ICP-MS

The levels of copper absorbed for 24 h were assessed by inductively coupled plasma mass spectrometry (ICP-MS), according to the method described previously, with minor modifications (Ashoka *et al.*, 2009). ICP-MS (all ICP-MS 7700x series

components and software MassHunter from Agilent Technologies®) were used. Briefly, a pool of 10 larvae was washed two times with the water of Zebtec system and three times with 1 ml of Ringer's solution (Ringer's) to eliminate any environmental contamination of copper. After cleaning procedures, the samples, in 100 µl of cold Ringer's solution, were digested with 0.3 ml of concentrated nitric acid and 0.2 mL of hydrogen peroxide in a screw cap polypropylene tube. The cap was tightened and the tube was placed in a water bath at 85 ^{o}C. After digestion, each sample was diluted to 5 ml with a 1 % solution of nitric acid in a volumetric flask for analysis.

Protein quantification

The total protein concentrations of zebrafish larvae homogenates were determined according to the Coomassie blue method (Bradford, 1976).

Statistical analysis

Data were expressed as mean ± standard deviation and were subjected to Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's test. The statistical comparison of data regarding extracellular ATP hydrolysis was carried out at each time-point of incubation and over-time of incubation. For assessing the global over-time changes, the area under the curve were obtained for each homogenate. The test used was one-way ANOVA followed by Tukey's test. Values of $P < 0.05$ were considered significant.

RESULTS

The biological markers of oxidative stress were used as an initial tool to characterize and evaluate the inflammation model induced by copper in zebrafish larvae. Initially, we have made a curve using concentrations of 1, 10 and 25 µM of copper, challenging the larvae for a period of 24 h. As shown in Table 2, the concentrations of 10 and 25 µM induced a significant reduction in GSH and in the activities of CAT and SOD. Since 10 µM of copper showed a significant increase of oxidative stress with less mortality than 25 µM, as demonstrated in a previous report from our group (Leite *et al.*, 2012), we chose the respective concentration for further experiments. After determining the optimal concentration, the oxidative stress was evaluated at different time-points of 0,

4, 6 and 24 h (Table 3). SOD and CAT activities displayed a significant reduction after 24h of exposure to copper when compared to control, whereas GSH decrease was significant in 6 and 24 h of exposure to copper. NO levels were also significantly elevated after 24 h of copper exposure.

The release of PGE_2 and the neutrophil migration to the inflammatory site occurred before the increase of oxidative stress. The Fig. 1 shows copper promotes an increase in PGE_2 levels (29.2 ± 9 % and 32.6 ± 5 %, at 1 and 24 h, respectively) when compared to the control group. Neutrophil migration, as indirectly measured by MPO activity, displayed a significant increase of 69.3 ± 18 % after 4 h of exposure to copper (Fig. 2).

The nucleotide hydrolysis revealed a rapid catabolism of ATP and ADP (Fig. 3 A and B), in which both nucleotides were completely consumed after 1 h of incubation. No significant difference was found between the control and the treated groups. However, it was observed a significant reduction in ADP hydrolysis only at 30 min of incubation in the group treated for 24 h with 10 µM of copper. The extracellular metabolism of AMP, ADO, and inosine (INO) (Fig. 3 C, D, and E) occurred slowly during the incubation period of 2 h. The AMP and ADO were less metabolized in the group treated with 10 µM of copper for 24 h when compared to the control groups, showing a significant difference from 10 min to 180 min of incubation for AMP and, from 30 min to 180 min for ADO. INO levels were significantly decreased according to evaluation after 24 h of challenge to copper, probably due to the inhibition of ADA activity. The areas under the curve were calculated for all groups and the statistical analysis confirmed the data described above (Fig. 3 - Inset).

Considering the results of HPLC analysis, which showed that ATP and ADP hydrolysis presented a partial, but not significant decrease after copper treatment, the ATPase and ADPase activities were also assessed by Pi released to ensure the reliability of data. The results showed that there were not significant changes for ATP and ADP hydrolysis after 24h of treatment with 10 µM of copper. Additionally, as AMP hydrolysis and ADO deamination (Fig. 3) were significantly reduced after the same treatment, ecto-5'-NT and ADA activities was also evaluated by measuring Pi and NH_3 released, respectively. As expected, ecto-5'-NT and ADA activities decreased by ~ 20 % (Fig. 4), confirming data from HPLC hydrolysis assay.

The RT-qPCR quantification (Fig. 5) showed a significant increase of IL-1β and TNF-α expression at 4 and 24 h after copper exposure. IL-10 was found significantly

decreased at 4 h, whereas COX-2 was significantly increased at 24 h treatment. Ecto-5'-NT gene expression showed a significant decrease following 24 h of copper exposure. The genes of ADA showed different expressions after 24 h of exposure to copper: expressions of ADA 1 and ADAasi were not affected by copper; nevertheless, ADA 2.1 showed decreased expression, while ADA 2.2 and ADA L demonstrated an increased expression when compared to control (Fig. 6).

To evaluate the absorption levels of copper over the exposure time, an absorption curve of copper until 24 h of treatment was performed. We verified that the copper uptake was significantly increased over time, from 4 to 24 h, as shown in Fig. 7.

DISCUSSION

Inflammation is a physiological process that represents the body immediate response to cellular and tissue damage by pathogens and chemical or physical stimuli. In some cases, it is associated with chronic pathological states, including allergies, atherosclerosis, cancer, arthritis and autoimmune diseases. Both in acute and chronic responses, cytokines and other inflammatory mediators are involved in key cellular events such as the production of reactive oxygen species (ROS) (Dantzer *et al.*, 2008; Martinon, 2010).

In a previous study from our group (Leite *et al.*, 2012), we assessed the dose-related mortality induced by copper in zebrafish larvae, and it was demonstrated that copper kills 15-20% of the animals at 10 µM, after 24 h of exposure. In the same study, it was demonstrated that lower concentrations of copper did not induce mortality, whereas 25 µM induced 75-80% of deaths. Supporting these data, a previous study conducted by Olivari *et al.* (2008) revealed that 1 µM copper is able to induce injury of lateral line hair cells of zebrafish larvae. Furthermore, the authors demonstrated that the concentration of 10 µM also induced a partial damage of the supporting cell layer. The concentration of 10 µM of copper, assessed by oxidative stress in the present study, leads to moderate toxicity, what might be allied to the biochemical and molecular inflammatory changes, while the concentration of 1 µM was not effective and 25 µM induces a high mortality. Production of ROS is crucial in regulating the innate immune response. The activation of phagocyte cells generates ROS that drive their toxicity to phagocytized microorganisms. ROS are also important in signaling damage to the immune system, and its production

regulates the activation of transcription factors and cytokine production (Martinon, 2010). Our results using 10 µM copper demonstrated that oxidative stress increases slowly during the first hours of exposure to copper, but it is rather evident after 24 h, presumably due to the continuous exposure and increased uptake of copper by zebrafish larvae. The oxidative stress showed herein can occur by two mechanisms: direct effect of copper, which induces DNA and cell damage, or the increase of ROS release due to the activation of phagocytic cells and increased tissue damage (Olivari *et al.*, 2008). The antioxidant defenses, including CAT and SOD activation, may be reduced after copper exposure due the high levels of ROS present. Reduced activity of CAT may also be related to excessive superoxide anion radical resulting from reduced activity of SOD (Liu *et al*., 2008). During the inflammation process, there are two important groups of cytokines: (i) the pro-inflammatory series, secreted primarily by activated macrophages, and (ii) the anti-inflammatory ones, which are involved in the reduction of the inflammatory response. Homologs for a variety of mammalian cytokines have been identified in zebrafish, such as: TNF-α, IL-1β, IL-8, IL-10, IL-11, IL-15, IL-22 and IL-26 (Sullivan and Kim, 2008). The immersion of zebrafish embryos in LPS induced the gene expression of pro-inflammatory cytokines TNF-α, IL-8 and IL-β (Watzke *et al.*, 2007; Novoa *et al.*, 2009; Oehlers *et al.*, 2010). Our data showed that in zebrafish larvae of 7 dpf challenged to copper presented a significant increase in IL-1β and TNF-α expression, and a reduction of the expression of IL-10 when compared to the control groups. These altered expressions, associated with a significant increase of PGE_2, MPO and ROS production provides a general scenario of the inflammatory response induced by copper.

PGE_2 is known for presenting multiple functions in the immune system. Its actions are related to the classical signals of inflammation, including vasodilation, increased vascular permeability, in addition to the chemotaxis and activation of neutrophils, macrophages, and mastocytes (Kalinski, 2012). Chronologically, the results of our research showed a remarkable increase of PGE_2 release after 1 h of exposure to copper, a return to normal levels, followed by a second peak after 24 h, where it is likely important for resolution. The first wave of PGE_2 production probably occurs due to the activation of the inflammatory process induced by copper, which is necessary for cell migration and subsequent activation. This migration was confirmed by increased MPO activity after 4 h of exposure. Furthermore, it preceded the increase in the expression of important inflammatory mediators released by activated cells (IL-1β and TNF-α).

The PGE_2 levels are controlled via the cyclooxygenase 1 and 2 (COX-1 and COX-2) pathways and its degradation by 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (Kalinski, 2012). Expression of COX-2 showed a significant increased after 24 h of exposure to copper, which is likely responsible for the increase in the production of PGE_2 at this time-point. This second increase in PGE_2 can be related to its role in the late phases of inflammation, which might be linked to IL-10 release and suppression of pro-inflammatory cytokines (Kalinski, 2012). The elevation of IL-10 observed in our study at 24 h (in relation to 4 h), together with the second peak of PGE_2, might be indicative of the activation of pro-resolution mechanisms in the experimental model described herein.

The extracellular concentration of the nucleotides is controlled by E-NTPDase and ecto-5'-NT that sequentially catalyze the hydrolysis of ATP to ADO and thereby exert a tight control of the effects triggered by these important signaling molecules (Robson *et al.*, 2006). ATP is implicated in the development of inflammation through a combination of actions, such as histamine release from mast cells (inducing the production of prostaglandins) and the production and release of cytokines from immune cells (Burnstock, 2006). To assess the ATP hydrolysis, the nucleotide hydrolysis was registered at different times and analyzed by HPLC. We observed that hydrolysis of either ATP or ADP was not altered by 24 h of exposure to copper. However, the hydrolysis of AMP was reduced at 24 h of exposure, with the consequent increase of AMP levels (the results were confirmed by decrease of ecto-5'-NT activity). In addition, RT-PCR analysis indicates that changes in the ecto-5'-NT activity could be related to the decrease of the expression of this enzyme. Ramarkers *et al.* (2012) demonstrated that the peak TNF-α production was coincident with the reduction of ecto-5'-NT activity in peripheral blood mononuclear cells, in an experimental model of endotoxemia.

Excessive collateral damage in healthy tissues threatens its functions, and should be stopped by resolution mechanisms, which are activated to avoid excessive cell damage. ADO shows anti-inflammatory effects and its extracellular levels are found increased in inflammatory pathologies such as asthma and sepsis (Ohta and Sitkovsky, 2009). Thus, ADO is an important pharmacological target during the resolution of inflammation. The concentrations of ADO can be regulated by reuptake and subsequent phosphorylation to AMP by adenosine kinase or deamination to INO by the enzyme ADA (Rosemberg *et al.*, 2007; Ramakers *et al.*, 2012). The results presented in the Fig. 3 show that there was an increase in ADO concentration which would be, at least in part, due to inhibition of ADA activity. We confirmed its reduction by determining the total

activity of this enzyme, which controls the high systemic levels of ADO. Elevated ADO levels and reduction of ADA activity have been already described in LPS-induced endotoxemia in humans, demonstrating similarities of adenosinergic modulation mechanisms in these two models (Ramakers *et al.*, 2012). Nevertheless, this modulation of ADO levels in inflammatory process had never been described in zebrafish larvae before.

To assess ADA subfamilies involved in the inflammatory process, we determined the gene expression of ADA 1, ADA 2.1, ADA 2.2 and ADAasi (the adenosine deaminase "alternative splicing isoform" of ADA 2.1). The expression of another similar group ADA L (adenosine deaminase "Like") was also investigated. Three of the assessed genes showed changes in their expression, with a reduction in the expression of ADA 2.1 and increase in the expression of ADA 2.2 and ADA L. This modulation in the gene expression is likely responsible for the resultant total ADA activity.

ADA 2 can be released at the sites of inflammation by macrophages, as demonstrated by Conlon and Law (2004) using "in vitro" experiments or "*in vivo*" model of sepsis in rodents. In humans, it was demonstrated that ADA 2 stimulates the proliferation of $CD4^+$ T cells, and induces the differentiation of monocytes into macrophages (Zavialov *et al.*, 2010). Furthermore, a differential release of isoenzymes by macrophages might exist and ADA also may have distinct functional role according to the tissue in which they are expressed (Conlon and Law, 2004; Rosemberg *et al.*, 2007). Considering that macrophages release ADA at the sites of inflammation, a fine adjustment of the levels of different isoenzymes may occur. In the present study, we detected increased ADO levels, decreased ADA activity and significant different modulations in the expression of ADA 2.1 and ADA 2.2. Thus, we showed the first evidence that ADA 2 is closely linked to the control of ADO levels during inflammation induced by copper in zebrafish larvae. This event most likely occurs over the 24 h period and involves mainly ADA 2.1 (Fig. 5).

The functional role of ADA L still remains unclear, but it was demonstrated by Rosemberg *et al.* (2007) that ADA L mRNA levels were more abundant in the liver and kidney of zebrafish, suggesting that it has some physiological role in these tissues. Chronic exposure to copper primarily affects the liver, because this organ is the first site of copper deposition after entering the blood. The toxicity of copper in humans is typically manifested by the development of liver cirrhosis and damage of kidney and brain tissues (Gaetke and Chow, 2003). Considering that our results showed an increase

in the expression of ADA L and, that the concentrations of copper in larvae exposed over 24 h were higher than in control samples, we might suggest that the increased expression of ADA L is related to liver and kidney toxicity induced by copper. However, additional studies should be conducted to elucidate the role of this ADA subtype in toxicity induced by copper.

In this study, we demonstrated that copper induces oxidative stress in zebrafish larvae and that this element is absorbed throughout the exposure period of 24 h. Due to the accumulation of copper, the damage and oxidative stress are exacerbated over the time, resulting in an inflammatory process involving COX-2 and PGE_2, and relying on the regulation of pro-inflammatory and anti-inflammatory cytokines. Concerning the purinergic system, the adenosinergic mechanisms might be the most involved in these events, and we could infer that this system is implicated in the resolution phase of inflammation induced by copper. In conclusion, this work brings novel advances to the characterization of inflammatory responses induced by copper in zebrafish larvae. Moreover, we provide additional knowledge on the role of purinergic system in copper-evoked inflammation.

ACKNOWLEDGMENTS

This work was supported by DECIT/SCTIEMS through Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, proc. 10/0036-5–PRONEX/conv. 700545/2008) and “Implantação, Modernização e Qualificação de Estrutura de Pesquisa da PUCRS” (PUCRSINFRA) #01.11.0014-00 and PPG-CM, UFRGS. L.O.M. is undergraduate Biology student and F.F.C. is undergraduate Pharmacy student receiving grants from FAPERGS. We thank Fabiano Peres Menezes for his excellent technical assistance and Rafael Zanin for his outstanding technical advices.

Tables

Table 1. PCR primer design and PCR product

Enzymes		Sequences (5´–3´)	PCR product (bp)	GenBank Accession number	ZFIN ID (ZBD-GENE)
IL-1β	F	TCGCCCAGTGCTCCGGCTAC	178	NM_212844	040702-2
	R	GCAGCTGGTCGTATCCGTTTGG			
IL-10	F	TTCAGGAACTCAAGCGGGATATGG	73	NM_001020785	051111-1
	R	GCTGTTGACTTCAAAGGGATTTTGG			
TNF-α	F	AGGAACAAGTGCTTATGAGCCATGC	157	NM_212859	050317-1
	R	AAATGGAAGGCAGCGCCGAG			
COX-2	F	AACTAGGATTCCAAGACGCAGCATC	207	ENSDART00000093609 *	-
	R	AAATAAGAATGATGGCCGGAAGG			
Ecto-5'-NT	F	TGGACGGAGGAGACGGATTCACC	149	BC055243.1	040426-1261
	R	GGAGCTGCTGAACTGGAAGCGTC			
ADA 1	F	GCACAGTGAATGAGCCGGCCAC	168	AAH76532	040718-393
	R	AATGAGGACTGTATCTGGCTTCAACG			
ADA 2.1	F	TTCAACACCACACGTATCGGGCAC	161	AAL40922	030902-4
	R	ATCAGCACTGCAGCCGGATGATC			
ADA 2.2	F	TTGCAATTGTTCATCATCCCGTAGC	186	XP_687719	041210-77
	R	TCCCGAATAAACTGGGATCATCG			
ADAasi	F	CTTTGTGGTACTTCAAGGACGCTTTG	121	AAL40922	030902-4
	R	TTGTAGCAGATAAAAGAAGCGAGACG			
ADA L	F	CTCTAATGTGAAAGGTCAAACCGTGC	108	NP_001028916	050913-145
	R	AAGACGCCCTTATCATCCGTGC			

*Ensembl Data base

Table 2. Effect on oxidative stress after 24 hours of exposure to 1, 10 and 25 µM of copper

		CAT (µmol/min/mg protein) (Mean±SD)	GSH (µmol/mg protein) (Mean±SD)	SOD (U/mg protein) (Mean±SD)
Control		127.4 ± 5.9	9.0 ± 0.9	1.7 ± 0.2
Copper – 24 h	1 µM	119.2 ± 4.5	7.0 ± 0.5	1.6 ± 0.5
	10 µM	64.1 ± 5.2 *	4.2 ± 0.8 *	0.9 ± 0.2 *
	25 µM	65.7 ± 6.7 *	3.6 ±1.2 *	1.4 ± 0.3

* Mean value was significantly decreased compared to control group ($P<0.05$).
Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

Table 3. Effect of copper 10 µM on oxidative stress after 4, 6 and 24 hours of exposure

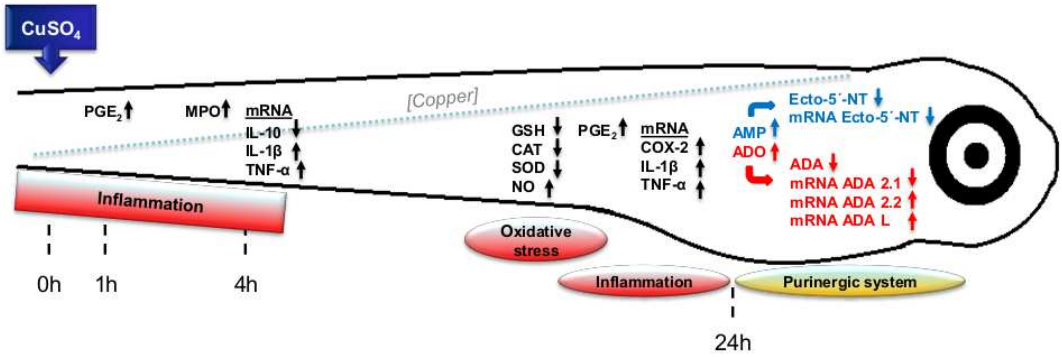
		CAT (µmol/min/mg protein) (Mean±SD)	GSH (µmol/mg protein) (Mean±SD)	SOD (U/mg protein) (Mean±SD)	NO (nmol/mg protein) (Mean±SD)
Control		118.4 ± 16.9	9.0 ± 1.2	1.7 ± 0.1	758.2 ± 88.5
Copper 10 µM					
	4 h	95.2 ± 13.3	6.9 ± 0.6	1.8 ± 0.5	771.9 ± 28.71
	6 h	90.2 ± 12.8	3.0 ± 0.2 *	1.4 ± 0.3	894.1 ± 70.9
	24 h	58.3 ± 7.9 *	3.7 ± 0.4 *	0.8 ± 0.3 *	1373.0 ± 68.2 †

* Mean value was significantly decreased compared to control group (P<0.05).

† Mean value was significantly increased compared to control group (P<0.05).

Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

Figures



Graphical abstract. This scheme provides a chronological proposition for the biochemical events induced by copper in zebrafish larvae. The dashed line shows the absorption of copper over the exposure time. After 1 h of exposure to copper, the release of PGE_2 occurs, followed by an increase of MPO (as a consequence of neutrophil migration), increased expression of genes involved in inflammatory events (IL-1β and TNF-α) and, reduction of the anti-inflammatory cytokine IL-10 at 4 h. At 24 h, the copper concentration is found highly increased, what is coincident with oxidative stress. Regarding the purinergic system, it is possible to observe an inhibition of ecto-5'-NT and ADA, with the consequent increase of AMP and ADA, respectively, at 24 h. The expression of enzyme-related genes shows a decrease in the expression of ecto-5'-NT and variable expressions of ADA subfamily enzymes.

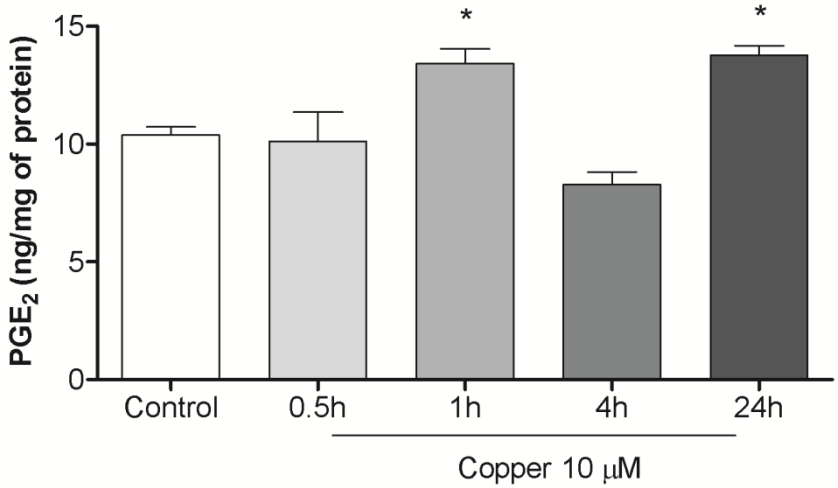


Fig. 1. Profile of PGE_2 release in 24 hours of exposure to 10 µM of copper. The levels of PGE_2 were significantly higher at 1 and 24 hours of exposure than controls. Each column represents the mean of six homogenates and, the vertical lines show the standard deviations. Asterisks (*) denote the significance level ($P<0.05$) in comparison with control values. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

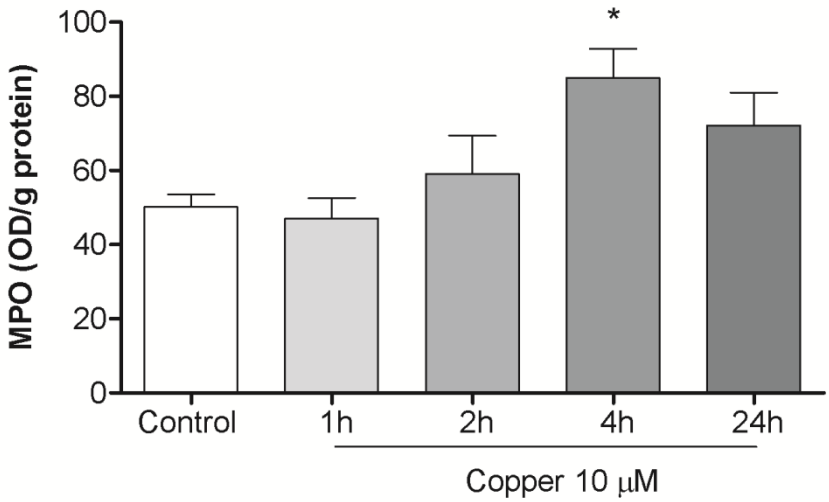


Fig. 2. MPO profile in 24 hours of exposure to 10 μM of copper. The levels of MPO were significantly higher at 4 hours of exposure than controls. Each column represents the mean of six homogenates and, the vertical lines show the standard deviations. Asterisks (*) denote the significance level ($P<0.05$) in comparison with control values. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

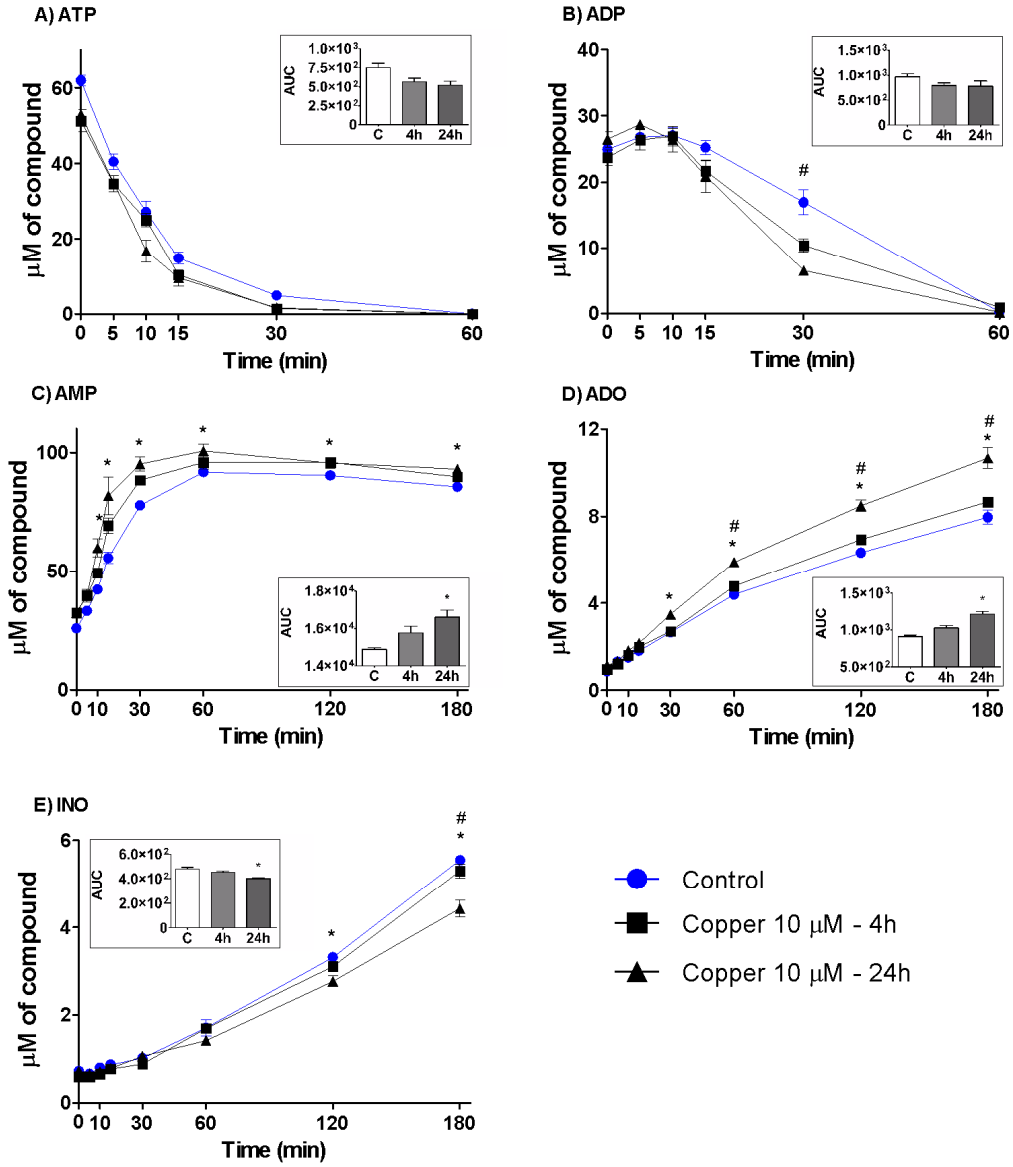


Fig. 3. Copper effects on extracellular ATP hydrolysis and, its degradation products. The zebrafish larvae were exposure to copper for 4 and 24 hours. ATP (A), ADP (B), AMP (C), ADO (D) and INO (E) were assayed by HPLC-DAD. The data are mean ± S.D. of four homogenates. Asterisks (*) denote the significance ($P<0.05$) in comparison with control values; a pound sign (#) denote the significance ($P<0.05$) in comparison with 4 hours group. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test. The groups were compared at each time of incubation (lines) and over time of incubation (inset). For assess over time, the area under the curves were obtained for each homogenate.

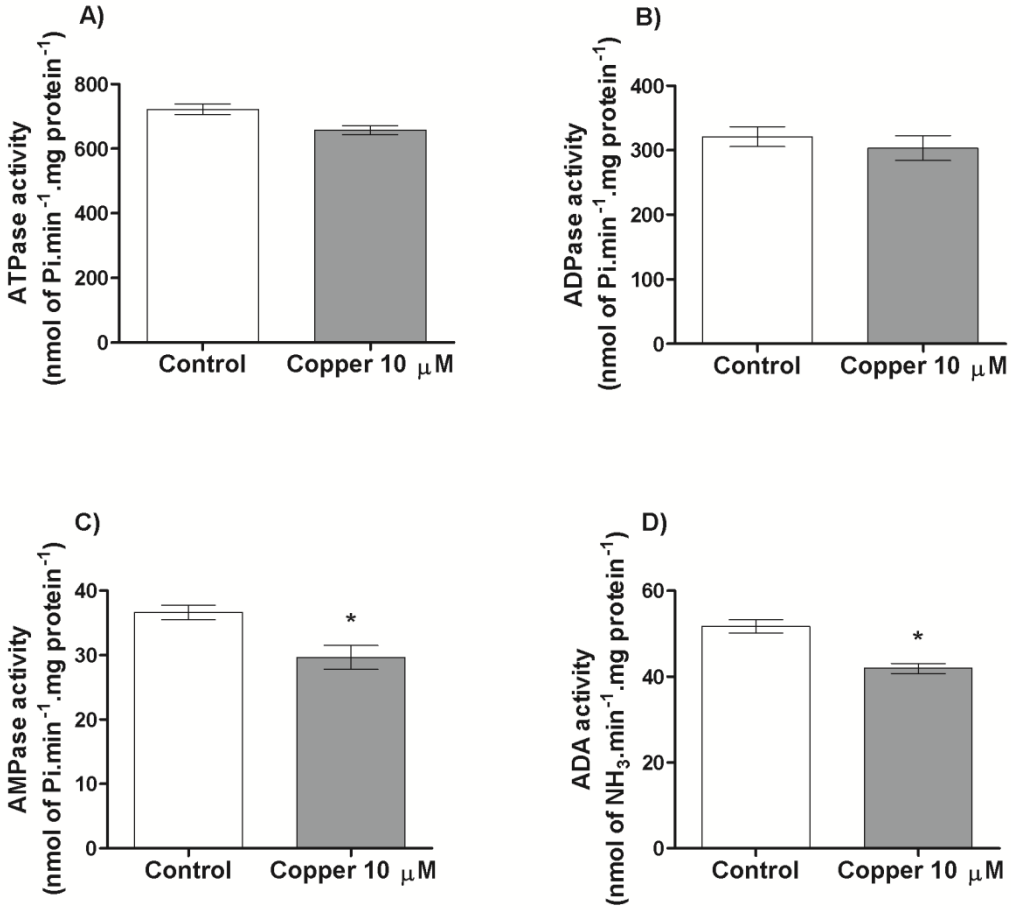


Fig. 4. Nucleotide hydrolysis analyzed by Pi liberation after 24 hours of exposure to 10 μM of copper. ATPase (A), ADPase (B), AMPase (C) and ADA (D) activities were determined as described in Material and Methods. Asterisks (*) denote significant difference from control group by Student's t-test analysis ($P<0.05$).

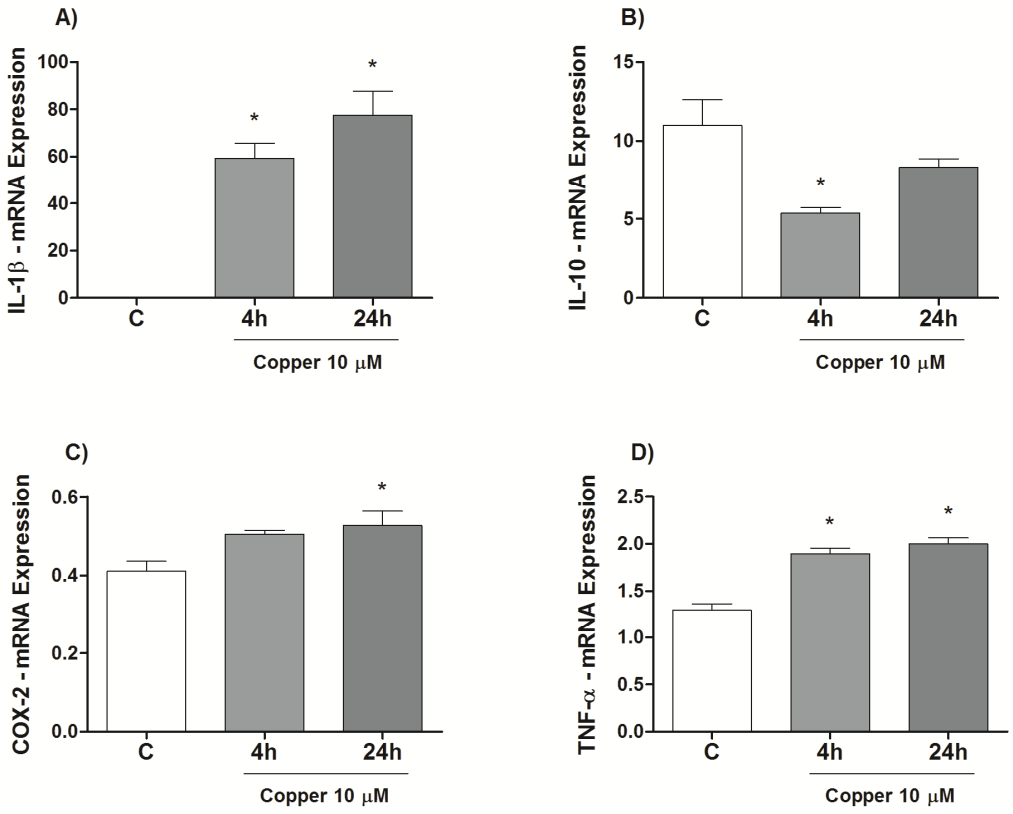


Fig. 5. Copper effect on inflammatory markers after 24 hours of exposure. The figure shows IL-1β (A), IL-10 (B), COX-2 (C) and TNF-α (D) mRNA expression in zebrafish larvae. Asterisks (*) denote the significance level ($P<0.05$) in comparison with control values. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

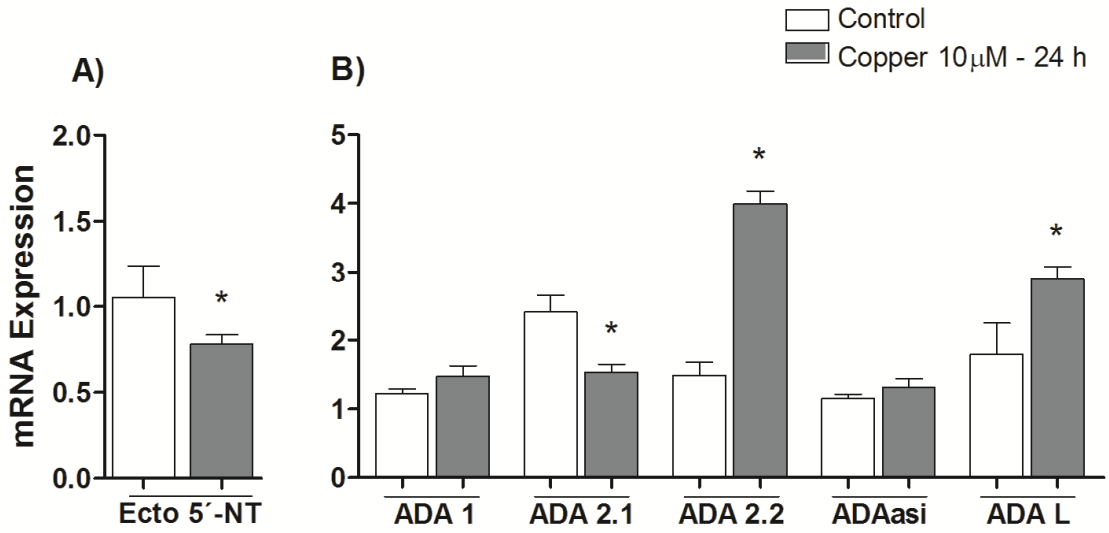


Fig. 6. Copper effect on enzymes transcripts after 24 hours of exposure. The figure shows ecto-5´-NT (A) and ADA isoforms (B) mRNA expression in zebrafish larvae. Asterisks (*) denote significant difference from control group by Student's t-test analysis ($P<0.05$).

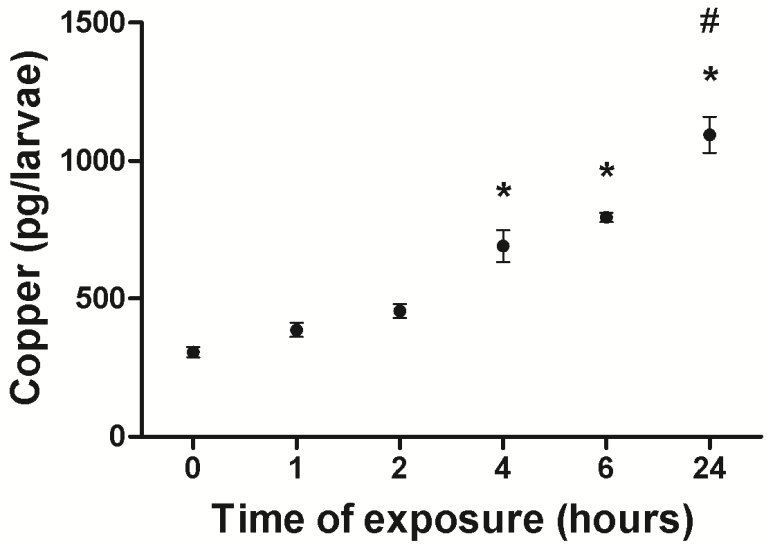


Fig. 7. Uptake of copper by zebrafish larvae over time in 24 hours. Asterisks (*) denote the significance ($P<0.05$) in comparison with control values; a pound sign (#) denote the significance ($P<0.05$) in comparison with 4 hours group. Statistical comparison of the data was performed by one-way ANOVA followed by Tukey's test.

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7. RESULTADOS ADICIONAIS

7.1 Expressão gênica dos receptores P1 em larvas expostas ao cobre

A adenosina é muito conhecida por atuar como uma molécula de sinalização extracelular. Em condições de sofrimento celular, como hipóxia ou inflamação, a produção e degradação deste componente são alteradas, gerando profundas mudanças nas suas concentrações e resultando em um aumento rápido e prolongado da adenosina (Martin *et al*., 2000). Esses níveis têm efeito direto sobre os seus receptores e de uma forma geral resulta em redução da inflamação e da lesão celular. Os quatro subtipos de receptores de adenosina são denominados A_1, A_{2A}, A_{2B} e A_3, sendo que para o receptor A_{2A} foram descritos dois genes em zebrafish, identificados como $A_{2A.1}$ e $A_{2A.2}$ (Boehmler *et al*., 2009). A ativação desses receptores induz uma grande variedade de efeitos, incluindo a vasodilatação, a inibição da agregação plaquetária, a modulação da atividade do sistema nervoso simpático e a modulação da resposta inflamatória (Ramakers *et al*., 2011; Haskó *et al*., 2008).

Os efeitos da adenosina sobre os seus receptores dependem das suas concentrações. Os níveis basais desse nucleosídeo são muito baixos, encontrando-se a níveis de nanomolar, mas que são suficientes para ativar três dos seus receptores: A_1, A_{2A} e A_3 (Fredholm *et al*., 1999). O Receptor A_{2B} por possuir menor afinidade pela adenosina é ativado por concentrações em nível de micromolar. Uma vez que em processos inflamatórios os níveis extracelulares de adenosina podem alcançar concentrações entre 1 e 100 μM, todos os receptores são passíveis de serem ativados nesse modelo (Ye and Rajendran, 2009).

A análise de expressão dos receptores de adenosina A_1, $A_{2A.1}$, $A_{2A.2}$ e A_{2B} foi realizada 4 e 24 horas após a exposição ao cobre utilizando a técnica de RT-PCR. A avaliação da expressão do receptor A_3 não foi realizada devido à falta de evidências da sua atuação na inflamação. Foi utilizado um n=4, com um pool contendo 15 larvas de zebrafish por grupo. Para o isolamento de RNA total foi utilizado o reagente Trizol (Invitrogen®) e a síntese de cDNA foi realizada de acordo com as instruções do kit Super Script First Strand III (Invitrogen®). O RNA total foi quantificado por espectrofotometria e o cDNA sintetizado com ImProm-II™ Reverse Transcription System (Promega®) a partir de 1 µg de RNA total, de acordo com as recomendações do fabricante. O estudo do padrão transcricional dos diferentes genes foi realizado utilizando primers específicos desenhados a partir de referências encontradas no banco de dados GenBank (tabela 1). O PCR quantitativo foi realizado utilizando SYBR Green I (Invitrogen®) para detectar a síntese da dupla-fita de cDNA. Níveis

de expressão relativos foram determinados com o software Fast Real-Time System Sequence Detection v.2.0.5 (Applied Biosystems®). A eficiência por amostra foi calculada usando o software LinRegPCR 11.0 (http://LinRegPCR.nl). Os níveis de expressão relativa de RNA foram determinados utilizando o método $^{2-\Delta\Delta CT}$. A estabilidade dos genes de referência EF1α, RIp13α e β-actina (M-valor) e o número ótimo de genes de referência, calculado de acordo com a variação pareada (V), foram analisados pelo software GeNorm 3.5 (http://medgen.ugent.be/genorm).

A exposição das larvas de zebrafish ao cobre durante 4 e 24 horas, apresentou um aumento na expressão gênica dos receptores A_1, $A_{2A.1}$, $A_{2A.2}$ e A_{2B}, como mostrado na figura 1.

O receptor A_1 e o A_{2B} apresentam efeitos controversos atuando em alguns casos como anti e outros como pró-inflamatórios. Já o receptor $A_{2A,}$indubitavelmente, atua na inibição do processo inflamatório. Uma vez que a ativação de cada receptor desencadeia um efeito diferente, os resultados parecem concordar com os encontrados nas expressões dos diferentes subtipos de ADA, aonde constatou-se que as enzimas com atividades pró e anti-inflamatórias estão envolvidas na inflamação induzida pelo cobre. O interessante é que o aumento da expressão dos receptores já ocorre no tempo de 4 h e que a alteração na atividade da ADA foi constatada apenas no tempo de 24 h. Consequentemente, fica demonstrado que o sistema adenosinérgico já está ativado e agindo sobre o processo inflamatório nas primeiras fases da inflamação induzida pelo cobre.

Tabela 1. Primers utilizados e produtos de PCR.

Receptor		Sequência (5´–3´)	Produtos de PCR (bp)	Número de acesso ao GenBank	ZFIN ID (ZBD-GENE)
A_1	F	GTTCCTCATTTACATTGCCATTCTGC	180	NM_001128584.1	081105-156
	R	TGGTTGTTATCCAGTCTCTCGCTCG			
$A_{2A.1}$	F	GCGAACTGTACGCCGAGCAGAG	178	AY945800	-
	R	TTATTCCCAGTGAGCGGCGACTC			
$A_{2A.2}$	F	GGATTGGGTCATGTACCTGGCCATC	160	AY945801.1	-
	R	GCTGTTTCCAATGGCCAGCCTG			
A_{2B}	F	GTTTGTTCGCTCTCTGTTGGCTGC	178	AY945802.1	-
	R	CTAAAAGTGACTCTGAACTCCCGAATG			

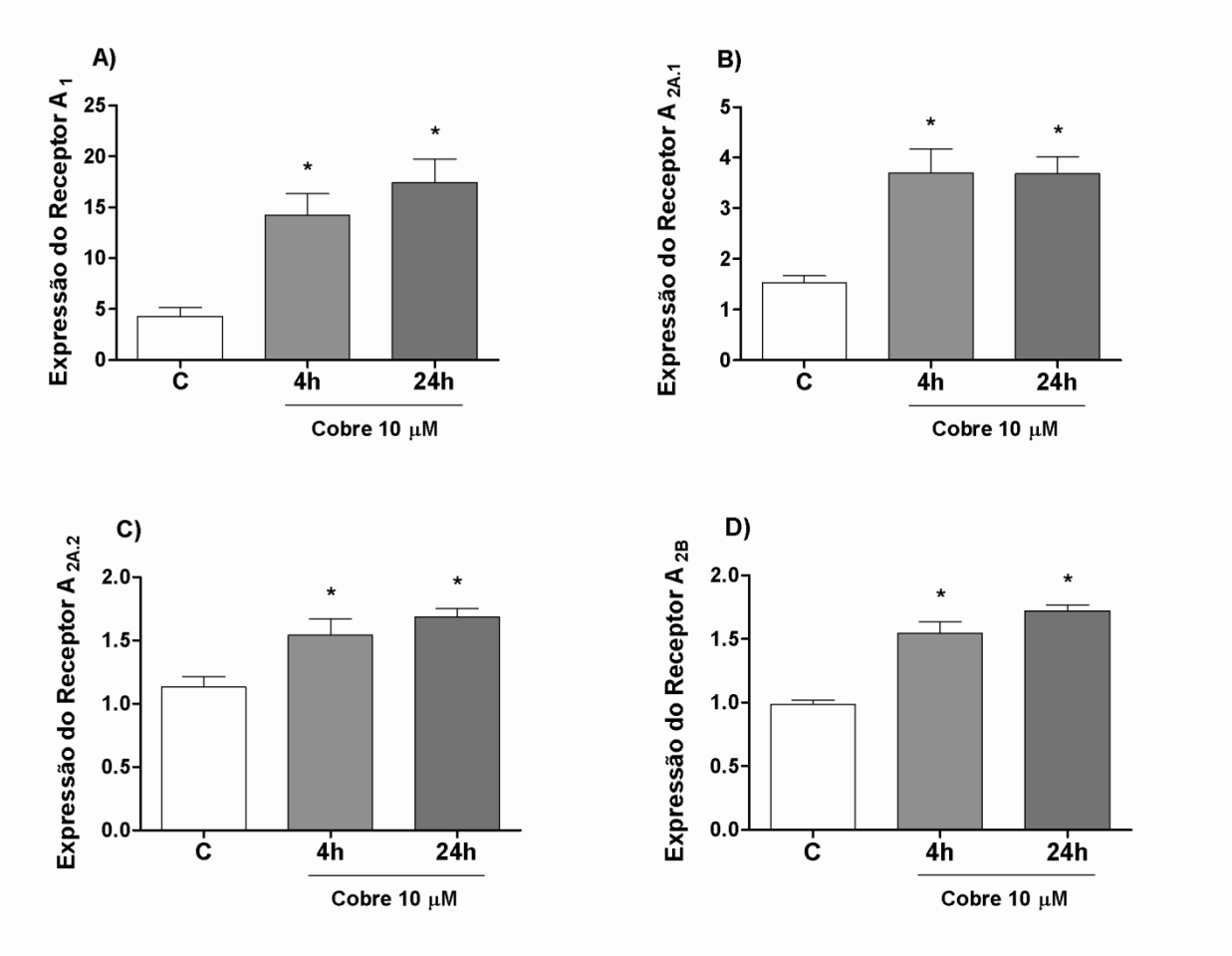

Figura 1 - Expressão dos receptores de adenosina após 4 e 24 horas de exposição das larvas de zebrafish a 10 µM de cobre. A figura apresenta a expressão dos receptores A_1 (A), $A_{2A.1}$ (B), $A_{2A.2}$ (C) e A_{2B} (D). Os asteriscos (*) representam a diferença significativa ($P<0.05$) em relação ao grupo controle. A análise estatística foi realizada utilizando a análise de variância de uma via, seguida do teste de Tukey.

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7.2 Avaliação de morte celular de células dos neuromastos

Como citado anteriormente, o cobre age diretamente sobre os neuromastos da linha lateral e das regiões crânio-faciais das larvas de zebrafish induzindo estresse oxidativo, lesão e morte celular. Utilizamos a metodologia descrita por Tucker e Lardelli (2007), fazendo o uso do marcador de laranja de acridina para avaliação da morte celular em consequência da lesão induzida pelo cobre sobre os neuromastos. Embora o uso deste marcador fluorescente não apresente especificidade para a marcação da apoptose, ele é utilizado para a avaliação deste tipo de morte celular em larvas e embriões de zebrafish.

Larvas de 7 dias pós-fertilização foram expostas à 10 µM de cobre durante o período de 1 hora (n=10). Após foram realizas 3 lavagens para retirada do agente inflamatório e a solução de laranja de acridina foi aplicada no meio na concentração final de 2 µg/ml. Após 30 minutos o meio foi retirado e os poços contendo as larvas foram lavados 3 vezes, sendo 10 minutos para cada lavagem. Posteriormente, foi adicionada tricaína aos poços e a marcação tecidual foi avaliada em microscopia óptica.

A morte celular de células pertencentes aos neuromastos foi avaliada e confirmada visualmente tanto na região do tronco, como na craniana (figura 2). Os dados corroboram o estudo de Olivari *et al.* (2008) que demonstram que a morte celular é consequência do estresse oxidativo induzido pelo cobre. Essa morte pode ocorrer tanto por apoptose como por necrose dependendo da concentração e do tempo de exposição ao metal. Ademais, a confirmação foi utilizada para que continuássemos com a utilização do modelo para avaliação da inflamação e do papel do sistema purinérgico. Em estudos futuros, a metodologia poderá ser utilizada para a avaliação do efeito de fármacos com potencial anti-inflamatório ou antioxidante contra a ação do cobre nos neuromastos..

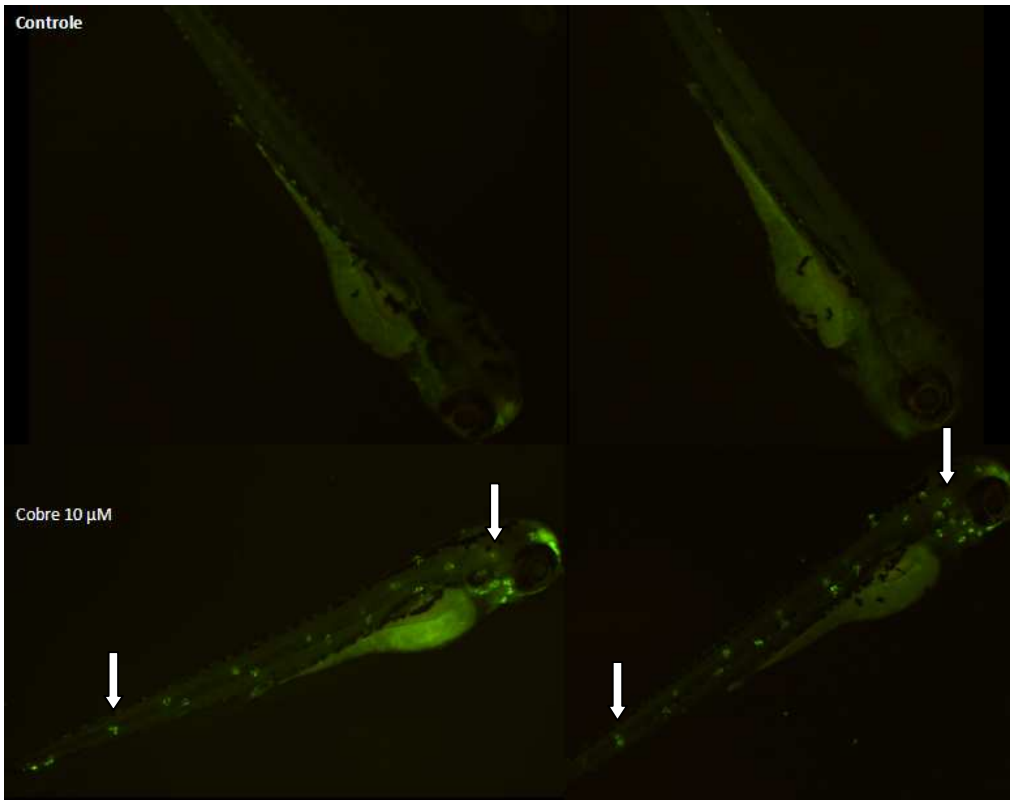

Figura 2. Marcação fluorescente nas células apoptóticas dos grupos controle e exposto a 10 µM de cobre. As setas brancas sinalizam as células apoptóticas dos neuromastos.

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8. CONSIDERAÇÕES FINAIS

Devido à falta de ensaios validados para quantificar diretamente marcadores bioquímicos e avaliar a inflamação em larvas de zebrafish, aperfeiçoamos e desenvolvemos metodologias para a determinação do estresse oxidativo (CAT, SOD, GSH e NO), PGE_2, MPO e para a determinação do cobre absorvido pelas larvas. A metodologia utilizada para a quantificação do NO foi totalmente desenvolvida pelo nosso grupo e devido a isso oferecemos à comunidade científica uma nova metodologia com alta sensibilidade, exatidão e precisão para a quantificação deste analito, como mostrado no primeiro artigo. A metodologia foi validada seguindo o Guia para Validação de Métodos Analíticos e Bioanalíticos presente na Resolução RE nº 899, de 29 de maio de 2003 da Agência Nacional de Vigilância Sanitária (ANVISA). Para uma maior confiabilidade do método, os resultados encontrados foram comparados com os resultados obtidos a partir de um equipamento comercial utilizado para quantificação do NO. O novo método apresentou resultados satisfatórios, inclusive com maior sensibilidade que o equipamento disponível no mercado para esse fim. Além disso, a metodologia apresenta baixo custo e pouca geração de resíduos, sendo facilmente adaptável aos cromatógrafos encontrados em muitos laboratórios de pesquisa e de rotina, tem um baixo custo e pouca geração de resíduos.

Em uma revisão do ano de 2011, Rico *et al.* sugeriram mais investigações sobre o papel funcional do NO em zebrafish. Devido a isso e a constatação da real falta de conhecimento do comportamento do NO nas primeiras fases de desenvolvimento do zebrafish, utilizamos a nova metodologia para avaliar as variações fisiológicas desde a fecundação até o sétimo dia após a fertilização. Verificamos que houve um aumento tempo-dependente na produção de NO nas larvas durante o período avaliado. Existem duas hipóteses plausíveis para explicar o aumento do NO. A primeira é que nessa fase o crescimento é muito acelerado e o NO tem um papel de regulador negativo para o controle de proliferação celular, diferenciação e apoptose. A outra possibilidade baseia-se na expressão diferencial das NOS, onde as enzimas podem iniciar a produção do NO em alguns tecidos já em dezesseis horas após a fertilização e em outros apenas após o terceiro dia.

Essa metodologia também foi utilizada para a comparação entre um modelo de inflamação induzido pelo cobre, o qual estava sendo padronizado para a continuidade do estudo e outro induzido pelo LPS de *E.coli*, mostrando-se adequada para a avaliação dos níveis de NO em eventos inflamatórios para ambos os agentes. O estudo inicialmente tinha como objetivo a avaliação da inflamação induzida pelo LPS de *E. coli* como agente

inflamatório. No entanto, durante os ensaios iniciais encontramos dificuldades devido à falta de reprodutibilidade dos experimentos, provavelmente pela não sensibilização das larvas pelo agente. Mais tarde, em um estudo de Novoa *et al.* (2009), encontramos suporte para os resultados obtidos. Segundo o estudo, após o primeiro contato com a bactéria *E. coli* ou via reatividade cruzada por contato com outras bactérias, o sistema imune inato do zebrafish desenvolve tolerância ao LPS bacteriano, fazendo com que em um segundo contato não apresente a mesma intensidade de resposta ao estímulo. Supomos que a falta de reprodutibilidade poderia estar ocorrendo devido ao processo de reprodução das larvas, onde os embriões recém-fecundados ficam em contato com os dejetos e restos de alimentos dos adultos até o momento da coleta. A partir desses resultados, iniciamos os estudos apresentados no segundo artigo, utilizando o cobre como agente inflamatório. A resposta a esse agente foi reprodutível e mais estável, desta forma iniciamos a sua utilização e a caracterização das respostas bioquímicas induzidas pelo cobre, para posterior estudo do sistema purinérgico.

Após a realização de curvas de mortalidade, realizamos a avaliação da absorção do cobre pelas larvas de zebrafish e uma série de parâmetros inflamatórios e relacionamos ao comportamento do sistema purinérgico dentro desse modelo inflamatório. Cronologicamente, após uma hora de exposição ao cobre, ocorreu a liberação de PGE_2, seguida por um aumento da migração neutrófilos e da expressão de genes envolvidos em eventos pró-inflamatórios como a IL-1β and TNF-α e, redução da citocina IL-10 (que apresenta efeitos anti-inflamatórios) após 4 horas de exposição. Após 24 horas em contato com o cobre, as concentrações do metal aumentaram significativamente nas larvas, coincidindo com o aumento do estresse oxidativo. Neste mesmo período, as atividades das enzimas ecto-5´-NT e ADA estavam reduzidas, com consequente aumento de AMP e adenosina, respectivamente. As enzimas responsáveis pela hidrólise do ATP e ADP não apresentaram qualquer alteração nas suas atividades. Posteriormente as avaliações dos genes dos diversos elementos da subfamília da ADA demonstraram que a ADA 2.1, ADA 2.2 e ADA L apresentaram alteração nas suas expressões, demonstrando que o sistema adenosinérgico é o mais envolvido na inflamação induzida pelo cobre.

Corroborando os dados acima, a avaliação da expressão gênica dos receptores A_1, $A_{2A.1}$, $A_{2A.2}$ e A_{2B} mostrou que da mesma forma que os transcritos para as enzimas que controlam os níveis de adenosina extracelular estão alterados, a expressão dos receptores responsáveis pelos efeitos finais da adenosina tiveram suas expressões elevadas após a exposição ao cobre. Um estudo mais aprofundado sobre a resposta dos receptores deve ser

feito, mas é possível que mecanismos anti e pró-inflamatórios estejam atuando concomitantemente e, por isso, todos os receptores possam estar ativados.

O objetivo de caracterizar um sistema dentro de um modelo experimental envolve uma série de etapas para que o mesmo seja alcançado. O presente trabalho apresentou dados consistentes do envolvimento do sistema purinérgico na inflamação induzida pelo cobre em larvas de zebrafish, mas sempre ficam perguntas a serem respondidas e novas perguntas surgem. Inicialmente acreditávamos que principalmente as E-NTPDases e o ATP estavam ligados aos eventos inflamatórios, mas o sistema adenosinérgico mostrou-se mais ativo nas nossas condições de estudo e na execução dos experimentos. Desta forma, acreditamos que uma maior exploração da ação da adenosina sobre os seus receptores, bem como a elucidação do papel de cada receptor e dos mecanismos envolvidos na ativação dos mesmos na inflamação induzida pelo cobre seja o próximo passo para complementar o conhecimento do comportamento do sistema purinérgico neste modelo de inflamação.