

UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CAMPUS DE JABOTICABAL

REMODELAÇÃO MIOCÁRDICA PÓS-LESÃO CARDÍACA
INDUZIDA POR VIDEOCIRURGIA, EM TILÁPIAS-DO-NILO

Roberta Martins Crivelaro
Médica Veterinária

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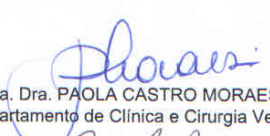
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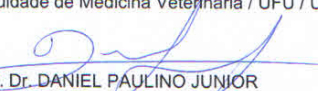
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DADOS CURRICULARES DA AUTORA

ROBERTA MARTINS CRIVELARO – nascida na cidade de São Paulo, Estado de São Paulo, em 10 de setembro de 1987. Ingressou na graduação em medicina veterinária na Universidade Estadual Paulista, campus de Jaboticabal, no ano de 2006. Em 2007, foi bolsista de iniciação científica da FAPESP, trabalhando com análise seminal e morfologia externa em veado-mateiro (*Mazama americana*), sob a orientação do professor Maurício Barbanti. Em 2009, realizou sua segunda iniciação científica junto ao setor de oftalmologia veterinária, sob a orientação do professor José Luiz Laus, trabalhando com diferentes midriáticos e cicloplégicos em ovelhas Santa-Inês. Graduiu-se em Medicina Veterinária em dezembro de 2010. Ingressou no Programa de Pós-Graduação em Cirurgia Veterinária em março de 2012, sob orientação do Prof. Dr. José Luiz Laus. Defendeu seu Mestrado em janeiro de 2014. Ingressou no curso de Doutorado em março de 2014, sob orientação da Profa. Dra. Paola Castro Moraes. É integrante do corpo clínico de atendimento do Serviço de Oftalmologia do Hospital Veterinário “Governador Laudo Natel”, da referida Faculdade e do Serviço de Endoscopia e Vídeocirurgia no mesmo hospital.

**“Só se pode alcançar um grande êxito quando nos mantemos fiéis a nós
mesmos”**

(Friedrich Nietzsche)

**“Nunca atribua unicamente a você o sucesso desta ou aquela tarefa,
compreendendo que em todo trabalho há que considerar o espírito de
equipe”**

(Chico Xavier pelo espírito André Luiz)

**“A compaixão para com os animais é das mais nobres virtudes da
natureza humana”**

(Charles Darwin)

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CERTIFICADO

Certificamos que o Protocolo nº 5222/15 do trabalho de pesquisa intitulado "Mecanismos de lesão cardíaca e de remodelação ventricular remanescente em tilápias do nilo: Estudo por videocirurgia, microscopias especiais e eletrocardiograma", sob a responsabilidade da Profª Drª Paola Castro Moraes está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 01 de abril de 2015.

Jaboticabal, 01 de abril de 2015.

Prof.ª Dr.ª Mirela Tinucci Costa
Vice-Coordenadora – CEUA

REMODELAÇÃO MIOCÁRDICA PÓS-LESÃO CARDÍACA INDUZIDA POR VIDEOSQUIRURGIA, EM TILÁPIAS-DO-NILO

RESUMO – As doenças cardiovasculares constituem grande problema de saúde pública. A necessidade do entendimento do processo de remodelação cardíaca tem acarretado em aumento do número de pesquisas envolvendo terapias biológicas. Neste contexto, peixes teleósteos emergem como opção de modelo experimental, pois possuem homologia morfofuncional e imunológica com mamíferos. Tilápias-do-Nilo (*Oreochromis niloticus*) já vêm sendo empregadas no estudo de diversas doenças que afetam seres humanos. Com a presente pesquisa, objetivou-se desenvolver técnica de acesso videocirúrgico e induzir lesão cardíaca mecânica, em tilápias-do-Nilo; avaliar a eficácia da metadona no controle da dor associada à lesão; e caracterizar os mecanismos de remodelação do miocárdio ventricular, empregando-se avaliações por eletrocardiograma e por microscopias. As avaliações foram conduzidas antes, 1 h depois, e transcorridos 7, 14, 30, 60 e 90 dias da lesão cardíaca. O acesso à cavidade celomática foi realizado utilizando-se um *punch* de biópsia de 3 mm, permitindo a introdução de endoscópio para posterior amputação do ápice do coração. O procedimento foi executado rapidamente e com baixo índice de mortalidade transoperatória (5,60%) e pós-operatória (3,38%). Aproximadamente 6% de tecido cardíaco, apresentando epicárdio e miocárdio, foi removido com a pinça de biópsia, sendo que a videocirurgia permitiu boa visualização interna, padronização do tamanho da lesão e alta precisão. A avaliação da analgesia fornecida pela metadona foi realizada por meio de parâmetros fisiológicos e comportamentais. Os resultados demonstraram que, na dose empregada, a metadona não possuiu efeito analgésico em tilápias-do-Nilo, contudo, o tempo de recuperação da anestesia foi maior entre os animais que receberam metadona, sugerindo um possível efeito sedativo do fármaco. Os ventrículos da tilápia-do-Nilo, após lesão, não apresentaram evidências de regeneração e evoluíram para um processo de remodelamento caracterizado pelos espessamentos das paredes do ventrículo e do epicárdio, pela hipertrofia dos cardiomiócitos, e por alterações em atividade elétrica. Mudanças no estado ordenado de agregação do colágeno intersticial, no tamanho e no formato dos núcleos dos cardiomiócitos, e na supraorganização da cromatina nuclear dos cardiomiócitos, bem como presença de células poliplóides, de células necróticas e de células apoptóticas foram observadas. Em conclusão, a tilápia-do-Nilo parece ser um bom organismo modelo para estudos em cardiologia experimental, uma vez que seu ventrículo, após amputação do ápice do coração, acumula muitas das alterações vistas em corações de pacientes humanos cardiopatas. Vantagens adicionais referentes ao emprego de tilápias-do-Nilo em pesquisas incluem o tamanho dos animais, o que possibilita a utilização de técnicas menos onerosas; e a facilidade de alojamento e de manutenção de um grande número de indivíduos num espaço reduzido, com baixa taxa de mortalidade.

Palavras chave: cardiopatias; coração; regeneração; teleósteo; ventrículo; endoscopia.

MYOCARDIAL REMODELING AFTER VIDEO SURGERY-INDUCED CARDIAC INJURY IN NILE TILAPIA

ABSTRACT – Cardiovascular disease are a public health big problem. The need for understanding cardiac repair mechanism has increased the number of researches biologic therapy field. In this context, teleost fish show as a experimental model, as they have morpho-functional and immunologic homology whit mammals. Nile tilapia (*Oreochromis niloticus*) has been used to study several diseases that affect humans. The present study aimed to develop a video surgery access and mechanically induce cardiac injury in Nile tilapia; to evaluate the analgesic activity of methadone in effectively control the pain associated with the injury; and characterize myocardial repair mechanisms by microscopy and electrocardiogram analysis. Data were recorded before cardiac injury and then 1 hour, 7, 14, 30, 60 and 90 days after cardiac injury. The access to coelomatic cavity was made with a 3-mm biopsy punch, allowing the passage of an endoscope for amputation of the cardiac apex. The procedure was quickly performed with intraoperative (5.6%) and post-operative (3.38%) low mortality rates. Approximately 6% cardiac tissue, including epicardium and myocardium were removed with the biopsy forceps, once video surgery allowed good visualization, standard lesion areas and great precision. To evaluate the analgesic effect of methadone, physiological and behavioral parameters were recorded. Results show that in the dose used, methadone did not show analgesic effect in Nile tilapia. However, recovery time was longer in fish that received methadone, suggesting a sedative effect of the drug in the specie. The ventricles of Nile tilapia, after lesion, did not showed any evidence of regeneration, progressing for a remodeling process, characterized by a thicker ventricle wall and epicardium, by cardiomyocytes hypertrophy, and changes in electrical activity. Changes in the interstitial collagen aggregation state, size and shape of cardiomyocytes nuclei, and in the supra-organization of nuclear chromatin of cardiomyocytes, as well as presence of ploidy, necrotic and apoptotic cells, were also observed. It was concluded that Nile tilapia seems to be a good experimental model organism for experimental cardiology studies, once its ventricle, after have its apex amputated, shows many of the alterations observed in hearts of humans cardiac patients. Additional advantages regarding the use of Nile tilapia in research include the size of the animals, that allows the use of cheaper techniques; and the practical way to keep a great number of animals in small spaces, with low mortality rates.

Keywords: cardiopathies; heart; regeneration; teleost; ventricle; endoscopy.

CAPÍTULO 1 - Considerações Gerais

1. Introdução

As doenças cardiovasculares, como o infarto agudo do miocárdio (IAM), figuram como causas principais de mortes, entre seres humanos. Diante deste cenário, para ampliar entendimentos sobre elas, emergem estudos envolvendo animais não mamíferos, e nunca antes empregados em pesquisas biomédicas.

O peixe-zebra (*Danio rerio*) é um exemplo de organismo modelo, cujas aplicações em estudos sobre IAM só crescem. Diferentemente dos mamíferos, ele é capaz de regenerar órgãos lesados, incluindo o coração. O peixe-zebra é excelente para se investigarem vias de regeneração miocárdica, e pode fornecer subsídios para o desenvolvimento de novos fármacos ou de intervenções voltadas ao tratamento de IAM. Ele, no entanto, justamente pela plasticidade cardíaca, não é modelo mimético de algumas doenças do coração. Para este escopo, outras espécies de Teleostei estão sendo investigadas.

Especula-se que tilápia-do-Nilo (*Oreochromis niloticus*) constitui organismo modelo para estudo das bases moleculares das doenças cardiovasculares, pois, aparentemente, sua habilidade para regeneração de órgãos é muito menor que a do peixe-zebra. Se assim for, a espécie pode ser útil em ensaios experimentais envolvendo modulação de genes do desenvolvimento cardíaco, pois possui genoma totalmente sequenciado. Como há evidências de que o coração de tilápia resiste às situações de hipóxia e de anóxia, ela, futuramente, pode ser utilizada em pesquisas sobre a ação de soluções cardioplégicas.

A presente pesquisa almeja validar o emprego de tilápia-do-Nilo como organismo modelo para se estudarem doenças cardiovasculares humanas, dentre elas o IAM. Para tal, desenvolveu-se protocolo de acesso videocirúrgico ao coração de tilápia, a fim de induzir lesão mecânica no miocárdio. Visando-se o bem-estar animal, a eficácia de metadona no controle da dor pós-operatória foi avaliada. Diferentes aspectos funcionais e estruturais do coração lesado foram investigados.

2. Revisão da Literatura

2.1 Cardiopatias e remodelação miocárdica ventricular

As cardiomiopatias são um grupo heterogêneo de doenças associadas com dilatação e alteração na função contrátil do ventrículo esquerdo, ou de ambos os ventrículos. Elas podem ser idiopáticas (resultando, por exemplo, de lesões mecânicas), genéticas, virais e/ou autoimunes, tóxicas, isquêmicas, ou decorrentes de outras doenças cardiovasculares reconhecidas (DUTRA, 2006). Frequentemente, as cardiopatias ensejam remodelação ventricular, que pode culminar ou não em falência cardíaca.

A remodelação ventricular, portanto, corresponde ao conjunto de alterações que ocorrem após injúrias cardíacas (*v.g.*, IAM), lesões iatrogênicas, sobrecargas crônicas de pressão (*v.g.*, estenose aórtica ou hipertensão arterial) e de volume (*v.g.*, insuficiência de valva aórtica ou mitral), processos inflamatórios, ou ainda, a expressão de genes relacionados às cardiomiopatias (REMME, 2003; KESSLER *et al.*, 2014; UYGUR; LEE, 2016; SILVA *et al.*, 2017). Trata-se de processo tempo-dependente, que enseja modificações moleculares, neuro-humorais, celulares e intersticiais no coração, e cujas manifestações clínicas decorrem de mudanças no tamanho, no formato e na função do miocárdio (DIETZ *et al.*, 1999; UYGUR; LEE, 2016; SILVA *et al.*, 2017). Sua etiologia está associada com mudanças no trânsito de cálcio, na via beta-adrenérgica, nas proteínas contráteis do miocárdio, no estresse oxidativo, no déficit energético, e nas proteínas do citoesqueleto, da membrana plasmática e da matriz extracelular (DIETZ *et al.*, 1999; COHN; FERRARI; SHARPE, 2000; SUTTON; SHARPE, 2000; COHN, 2002; REMME, 2003).

Alguns hormônios e certos mediadores autócrinos ou parácrinos, como noradrenalina, angiotensina II, proteína G, endotelina-1 e as citocinas pró-inflamatórias, promovem a ativação das proteíno-quinases C (PKC) e das proteíno-quinases ativadas por mitógenos (MAPK), o que favorece a expressão de genes que elevam a síntese proteica e causam hipertrofia de cardiomiócitos (PASSIER *et al.*, 2000; LIM *et al.*, 2005; STREICHER *et al.*, 2010). Interessantemente, apesar de as citocinas, incluindo o fator de necrose tumoral alfa (TNF- α), contribuírem para a remodelação ventricular negativa, evidências

recentes sugerem efeitos paradoxais, pois elas, também, desempenham funções importantes na adaptação do miocárdio às condições de isquemia e reperfusão (JARRAH; TARZAMI, 2015).

A hipertrofia está relacionada à replicação de sarcômeros, à redução de estresse parietal por aumento da espessura da parede (i.e., hipertrofia concêntrica) e à elevação da fração de volume ejetado em função do aumento do volume diastólico final (i.e., hipertrofia excêntrica) (SIMONE, 2004; MIHL; DASSEN; KUIPERS, 2008; MAILLET; VAN BERLO; MOLKETIN, 2013; MARSHALL *et al.*, 2017). Adjunto, há aumento da deposição de colágeno intersticial (ie. fibrose) e da densidade numérica de células não contráteis (HUDLICKA; BROWN, 1996; TAI; WANG; ZHANG, 2014; MARSHALL *et al.*, 2017). Em consonância, eventos apoptóticos ocorrem, contribuindo para a remodelação ventricular (ABBATE; BIONDI-ZOCCAI; BALDI *et al.*, 2002). A apoptose eleva a carga funcional sobre os cardiomiócitos remanescentes, que tendem a sofrer necrose. Fármacos, como os inibidores da enzima conversora, os betabloqueadores e os antagonistas de receptores de angiotensina, ajudam a reduzir a perda celular; entretanto, o bloqueio exacerbado de apoptose pode desencadear tumorigênese (CLERK; COLE; CULLINGFORD, 2003).

Alterações na geometria do ventrículo esquerdo, em seres humanos, estão associadas ao pior dos prognósticos relacionados à lesão cardíaca, com prevalência elevada de rotura da aorta, de arritmias e de formação de aneurismas (RASHID; SAUCEDO; HENNEBRY, 2005). O evento acarreta redução do volume de sangue (ie. déficit de perfusão) que vai do coração para os tecidos periféricos e, no estágio final, pode culminar em insuficiência cardíaca congestiva (KONSTAM *et al.*, 2011). Um dos maiores desafios da ciência é tentar evitar este último desfecho. Na atualidade, as opções disponíveis se voltam para o controle da hiperativação do sistema neuro-humoral; o tratamento da etiologia, quando possível; e a prevenção de morte súbita, com cardioversor desfibrilador implantável (GUO *et al.*, 2005; FERNANDES; SILVA, 2007; UYGUR; LEE, 2016). Admite-se que o ideal seria modular os mecanismos moleculares, genéticos e celulares da remodelação ventricular, por meio de terapias biológicas, inclusive com aplicações de células tronco.

Há evidências contrárias ao paradigma de que o miocárdio humano não pode se regenerar. Ratos neonatos, de um dia, quando submetidos à ressecção ou ao infarto ventricular recuperam a função cardíaca; porém, quando esses mesmos procedimentos são realizados no sétimo dia pós-natal, não há reparação (PORRELO *et al.*, 2011). Em mamíferos adultos, o coração preserva linhagem de células capazes de reingressarem ao ciclo celular e de replicarem DNA (BELTRAMI *et al.*, 2001). Cardiomiócitos mitóticos já foram detectados em áreas peri-lesão e remotas de ventrículos esquerdos infartados, nas proporções de 4% e de 1%, respectivamente (BELTRAMI *et al.*, 2001). Ademais, foi demonstrado que o coração em disfunção ventricular contém cardiomiócitos hibernados (BELTRAMI *et al.*, 2001), cuja ativação pode ser conseguida por revascularização miocárdica. Neste caminho, terapias biológicas, como as que empregam o fator de crescimento endotelial vascular (VEGF), têm potencial para induzir angiogênese no miocárdio e para estabelecer circulação colateral (KIM; KINI; SHARMA, 2002; GUPTA; TONGERS; LOSORDO, 2009; OBERMAIR; PIERINGER, 2015).

2.2 Terapias biológicas no tratamento de doenças do coração

Relativamente às células tronco, ensaios clínicos, envolvendo pacientes com disfunção ventricular isquêmica avançada, e com contraindicação para revascularização miocárdica, mostraram que injeções intracardíacas de células mononucleares, derivadas da medula óssea, reduzem defeitos de perfusão avaliados à cintilografia miocárdica, além de melhorarem a capacidade funcional do coração, no interregno de um ano (PERIN *et al.*, 2003; PERIN *et al.*, 2004). Em cães, um importante estudo revelou que células mesenquimais autógenas se diferenciam em cardiomiócitos, quando implantadas no coração com isquemia crônica (SILVA *et al.*, 2005). O potencial cardiomiogênico de células mesenquimais foi, também, comprovado por estudo envolvendo corações de ratos imunodeficientes (TOMA *et al.*, 2002).

Terapias biológicas para regeneração cardíaca são alvos constantes de pesquisas e considerações. Amontoam-se dados com enfoque em biossegurança e em eficácia. Todavia, muitos aspectos sobre a disponibilidade

de vetores plasmidiais, bacteriófagos e virais, com tropismo pelo miocárdio (LEE *et al.*, 2014); os critérios para escolha de sequências de DNA para silenciamento ou inserção, ou de RNAs de interferência (LEE *et al.*, 2014; GIACCA, 2015); os requerimentos para obtenção e manipulação de células tronco; e a padronização de doses e de vias de implantação celular (PAWANI; BHARTIYA, 2013) permanecem sob investigação. Para preencher lacunas entre terapias biológicas, regeneração miocárdica e mecanismos de lesão cardíaca, empregam-se, nas pesquisas, organismos modelo de doenças cardiovasculares humanas (RUSSELL, 2001; MARSHALL, *et al.*, 2017).

2.3 Teleósteos como modelos de cardiopatias humanas

Animais mamíferos, notadamente os da ordem Rodentia, até pouco tempo atrás eram prioritários em pesquisas sobre doenças cardiovasculares; porém, esse cenário mudou. Primeiramente, a tecnologia do DNA recombinante e a criação de organismos transgênicos ensejaram redução importante do quantitativo de animais empregados em pesquisas. Segundo, vertebrados não mamíferos estão emergindo como modelos para uso em pesquisas, desde a descoberta que teleósteos da família Cyprinidae e anfíbios urodelos (v.g., salamandras e tritões) podem regenerar órgãos ou membros lesados (POSS; WILSON; KEATING, 2002; NYE *et al.*, 2003; ALIBARDI, 2010; STOCUM, 2011; MARSHALL, *et al.*, 2017). Por se tratarem de animais de fácil adaptação, com custo reduzido de manutenção e com baixa variabilidade genética intraespecífica, teleósteos e anfíbios oferecem vantagens, comparativamente aos mamíferos.

Teleósteos representam o maior grupo de Chordatas viventes do planeta, com mais de 32.000 espécies (www.fishdatabase.org). O emprego de peixes Teleostei em pesquisas sobre doenças cardiovasculares humanas está aumentando, uma vez que as vias regulatórias do desenvolvimento e do controle autônomo do coração dos vertebrados atendem a padrões ontogenéticos evolutivamente conservados (LYONS, 1996; TAYLOR *et al.*, 2014).

O ventrículo cardíaco dos teleósteos pode apresentar-se com diferentes formatos, a saber: sacular, tubular ou piramidal (SANCHEZ-QUINTANA *et al.*, 1995; AGNISOLA; TOTA, 1996). Esses formatos relacionam-se com a mioarquitetura ventricular e variam em função dos hábitos natatórios das espécies. Estudos sobre mioarquitetura ventricular mostram que a organização miocárdica difere entre os peixes; ou seja, as espécies podem apresentar ventrículo constituído apenas por camada esponjosa, ou além da camada esponjosa, uma camada compacta. Espécies ativas apresentam ventrículo piramidal e miocárdio misto (constituído por uma camada compacta externa e uma camada esponjosa interna), enquanto espécies sedentárias apresentam ventrículo sacular ou tubular, constituído apenas por camada esponjosa (SANCHEZ-QUINTANA *et al.*, 1995; AGNISOLA; TOTA, 1996).

O coração das tilápias-do-Nilo (Teleostei: Cichlidae: *Oreochromis niloticus*), por se tratar de espécie ativa, possui formato piramidal e mioarquitetura mista, com tecido compacto na periferia e tecido esponjoso no restante do parênquima (MUMFORD *et al.*, 2007; GENTEN *et al.*, 2009; ALMEIDA *et al.*, 2015). Em tilápias-do-Nilo, o átrio cardíaco possui lúmen maior que o ventrículo; no entanto, as arquiteturas dos miocárdios do átrio e do ventrículo são iguais. O miocárdio da tilápia está externamente protegido por um epicárdio discreto, constituído de tecido conjuntivo frouxo e de epitélio pavimentoso simples. Internamente, ele é revestido por um endocárdio formado de epitélio pavimentoso simples. O coração da tilápia apresenta uma estrutura denominada de bulboarterioso, o qual é formado de tecido conjuntivo elástico e de fibras musculares lisas, sendo totalmente envolto pelo epicárdio (ALMEIDA *et al.*, 2015).

Em relação ao processo de excitação-contração, os corações dos teleósteos, não dependem do efluxo adicional de Ca^{+2} proveniente do retículo sarcoplasmático (SHIELS & FARRELL, 1997). Nos teleósteos existe uma entrada de cálcio proveniente de canais do tipo Ca^{+2} lentos, os quais elevam a concentração citoplasmática de cálcio desencadeando assim a contração (COSTA *et al.*, 2000). Isso ocorre, porque os corações dos teleósteos possuem cardiomiócitos com tamanhos reduzidos, comparativamente aos mamíferos. Outrossim, os cardiomiócitos dos teleósteos possuem retículos

sarcoplasmáticos pouco desenvolvido e em menor número, em comparação aos animais de sangue quente (FARREL & JONES, 1992).

O peixe-zebra (Teleostei: Cyprinidae: *Danio rerio*) possui a notória capacidade de restaurar a morfofuncionalidade do coração, após lesão (POSS; WILSON; KEATING, 2002; WILKINSON; JOPLING; VAN EEDEN, 2014; KIKUCHI, 2015; SILVA *et al.*, 2017). Seu emprego, em pesquisas, contribui para a compreensão dos fatores que ativam vias de regeneração cardíaca, para posterior mimetização em mamíferos, na tentativa de entender por que o processo regenerativo não ocorre em seres humanos. O peixe-zebra pode fornecer diretrizes para o tratamento das doenças do coração. No entanto, ele não é modelo mimético de doenças cardiovasculares (exceto quando transgênicos), justamente pela alta plasticidade cardíaca. Ademais, o escore corporal do peixe-zebra (± 5 cm de comprimento e peso médio de 0,19 g) pode limitar seu emprego, para certos fins experimentais.

Tilápias-do-Nilo criadas em tanque medem entre 20 e 25 cm em comprimento, pesam aproximadamente 350 g e, possuem genoma totalmente sequenciado (GUYON *et al.*, 2012). Elas apresentam homologia fisiológica, imunológica e molecular com mamíferos (COSSINS; CRAWFORD, 2005; GUYON *et al.*, 2012) e estão sendo utilizadas em estudos sobre os mecanismos da inflamação e da sepse (MARTINS *et al.*, 2008; PEREIRA *et al.*, 2010). A tilápia é hábil em regenerar ou reparar o rim cranial, os ovários, a nadadeira caudal e os músculos estriados esqueléticos, após lesão (ILAR, 2001; CAMARGO *et al.*, 2004). Seu coração, apesar de, aparentemente, possuir capacidade regenerativa limitada, resiste às situações de hipóxia e de anóxia, o que torna a espécie ideal para se avaliarem ações de soluções cardioplégicas (SPEERS-ROESCH, 2010; LAGUE *et al.*, 2012).

Tilápias-do-Nilo já são usadas para estudarem-se doenças relacionadas ao metabolismo lipídico, as quais, em seres humanos, representam fatores preditivos de risco de obesidade, de arteriosclerose, de diabetes, de esteatose hepática e de IAM (WRIGHT *et al.*, 1998; WRIGHT *et al.*, 1999; HAN *et al.*, 2011; WU *et al.*, 2015). É factível especular que tilápias poderiam ser usadas como organismos modelo em cardiologia experimental. Porém, por enquanto,

estratégias para induzir lesão cardíaca e remodelação miocárdica não foram propostos para a espécie.

2.4 Indução experimental de lesão cardíaca

A oclusão da artéria coronária esquerda, com fio de sutura 5-0, à aproximadamente 2 mm da origem, entre a borda do átrio esquerdo e o sulco da artéria pulmonar, é a estratégia mais difundida para indução de IAM em ratos (HEINBURGER, 1946; JOHNS; OLSON, 1954; KAUFMAN; GAVAN; HILL, 1959; SELYE *et al.*, 1960). Alternativamente, Huwer e colaboradores (2000) descreveram um modelo de IAM promovido por crioinjúria. Em mamíferos, a lesão cardíaca por crioinjúria nem sempre é transmural; às vezes, manobras para obtenção de hipotermia acarretam assistolia definitiva (TUCCI, 2011). Em peixes-zebra, o procedimento de crioinjúria culmina com a morte de aproximadamente 20% dos cardiomiócitos do ventrículo (CHABLAIS; JAZWINKA, 2012; GONZÁLEZ-ROSA; MERCANDER, 2012).

Outra estratégia para induzir lesão cardíaca em peixes-zebra seria a ressecção mecânica do ápice ventricular (SINGH *et al.*, 2010; LAFLAMME; MURRY, 2011; DICHOVER; ZHANG; CHI, 2013). Tal ressecção, de até 2/3 do ventrículo, é feita com tesoura, em cirurgia a céu aberto, e requer abertura ampla da superfície ventral, para exposição e exteriorização do coração (SINGH *et al.*, 2010; LAFLAMME; MURRY, 2011; DICHOVER; ZHANG; CHI, 2013). Recentemente, procedimentos em ablação genética e métodos para indução de hipóxia transitória foram propostos para criar lesões no coração do peixe-zebra (DICKOVER *et al.*, 2013); porém, essas estratégias danificam apenas o miocárdio, sem afetar os tecidos que o reveste.

Procedimentos em videocirurgia, executados com endoscópio munido de canal de trabalho, permitem que pinças, tesouras e outros equipamentos de corte e de apreensão de tecidos possam ser guiados com segurança ao órgão alvo, possibilitando remoção com menor morbidade (TAYLOR, 1993; TAYLOR 1994).

As primeiras descrições do uso de equipamentos endoscópicos, em medicina, foram feitas nas décadas de 1950 e de 1960 (TAYLOR, 1993). Na década de 1970, as técnicas e os equipamentos para videocirurgia foram adaptados para aplicações em veterinária, e permitem a consecução de procedimentos diagnósticos e de cirurgias complexas (TAYLOR, 1993; BRUN, 2015). Os endoscópios da rotina veterinária foram desenvolvidos a partir de citoscópios pediátricos e possuem diâmetro reduzido (± 3 mm), o que possibilita seu uso em animais de pequeno porte (TAYLOS, 1993). Em peixes, há relatos de que o acesso vídeo-laparoscópico permite excelente visibilização das vísceras (FERANTI *et al.*, 2015). O fígado, as gônadas e o baço podem ser vistos na posição caudal. O coração, em movimento, pode ser visto em posição rostral (TAYLOR *et al.*, 2008).

O acesso videocirúrgico é recurso que traz benefícios ao paciente, por minimizar o trauma tecidual e o desconforto pós-operatório (MACPHAIL *et al.*, 2001; TEIXIERA *et al.*, 2014). O procedimento está associado com redução marcante no uso de analgésicos, tanto na fase aguda pós-operatória, quanto para o controle de dores crônicas relacionadas à cirurgia aberta (WALKER; CRAIG, 1996; MACPHAIL *et al.*, 2001).

2.5 Analgesia em peixes

Relativamente ao uso de analgésicos, peixes apresentam respostas nociceptivas e possuem os componentes sensoriais necessários à percepção de estímulos dolorosos (SNEDDON, 2002, 2003; SNEDDON *et al.*, 2003 a,b), apesar de alguns autores propagarem o conceito de que eles não sentem dor (KEY, 2016). Ocorre que o conhecimento sobre como peixes respondem, com mudanças comportamentais, aos estímulos nocivos agudos é limitado e, aparentemente, há diferenças entre as espécies de Teleostei.

Há poucos estudos sobre o efeito dos opioides em espécies de peixes. Contudo, já se detectou receptores μ e kappa no sistema nervoso central de salmão (*Oncorhynchus kisutch*) (EBBESSON *et al.*, 1996) e de peixe-zebra (*Danio rerio*) (VELASCO *et al.*, 2009), sendo estes funcionalmente análogos aos de mamíferos. Sendo assim, os efeitos da morfina, um forte agonista de

receptores μ , têm sido avaliados em diferentes espécies de peixes (SNEDDON, 2003; NORDGREEN *et al.*, 2009; BAKER *et al.*, 2013).

Há relatos de que a truta-arco-íris (*Oncorhynchus mykiss*), após aplicação de uma dose de 0,1 mL/10g de solução de morfina (0,3 g/mL), pela via intramuscular, seguida pela aplicação de solução de ácido acético 0,1% nos lábios, para o estímulo nóxico, reduz seu comportamento de roçar os lábios no fundo como nas laterais do aquário, comparativamente ao grupo controle (SNEDDON, 2003).

Em peixinhos-dourados (*Carassius auratus*), a aplicação de 40 mg/kg de morfina, intramuscular, aumentou significativamente o limiar térmico, após 30 minutos da injeção, mas não aos 60 e 90 minutos. Houve uma grande variação individual na resposta entre os animais, portanto os autores concluíram que a morfina não causou analgesia na espécie estudada (NORDGREEN *et al.*, 2009).

Em carpas (*Cyprinus carpio*), a injeção intramuscular de 5 mg/kg de morfina ou de 10 mg/kg de butorfanol (um agonista kappa) ensejou algum efeito analgésico, causando mudanças de comportamento na espécie. A morfina causou menos efeitos adversos do que butorfanol (HARMS *et al.*, 2005; BAKER *et al.*, 2013).

SCHROEDER & SNEDDON (2017) estudaram, em peixes-zebra, os efeitos de três analgésicos diluídos em água, sendo eles o ácido acetilsalicílico (1mg/L e 2,5 mg/L), a lidocaína (2 mg/L e 5 mg/L) e o butorfanol (0,2 mg/L e 0,5 mg/L). Como estímulo álgico, procedeu-se a amputação de metade das nadadeiras caudais. Tanto a lidocaína quanto o ácido acetilsalicílico apresentaram algum grau de eficácia analgésica, pois diminuíram a incidência e a redução de comportamentos que exprimem dor. Quanto ao butorfanol, os autores concluíram que ele não foi um analgésico eficaz. O nível de cortisol também foi quantificado em todos os espécimes, porém, diferenças não foram encontradas entre os grupos de tratamentos.

A metadona é um opioide comumente utilizado para promover analgesia em pequenos animais. Ela é um potente agonista de receptores μ (KRISTENSEN, 1995), possuindo efeitos e potência similares aos da morfina

(KUKANICH & WIESE, 2015). Ademais, a metadona possui um efeito antagonista nos receptores N-metil-D-aspartato, o que torna este fármaco eficiente no controle de alguns tipos de dor em mamíferos (CALLAHAN, 2004).

Não há pesquisas sobre respostas nociceptivas em tilápias-do-Nilo. O potencial efeito anti-nociceptivo da metadona em peixes não foi ainda estabelecido.

3. Hipóteses

Quatro hipóteses alicerçaram a presente investigação: (1) tilápias-do-Nilo podem ser utilizadas, em cardiologia experimental, como organismos modelo de doenças humanas; (2) lesões mecânicas podem ser criadas no miocárdio de tilápias-do-Nilo, por procedimentos videocirúrgicos, sem necessidade de cirurgia a céu aberto, para exteriorização do coração [Capítulo 2, pg. 28]; (3) peixes podem sentir dor e, portanto, estratégias em analgesia devem ser estudadas para promover conforto pós-cirúrgico a esses animais [Capítulo 3, pg. 36]; (4) em tilápias-do-Nilo, lesões cardíacas podem ensejar reorganização miocárdica negativa, induzindo alterações funcionais e morfológicas tempo-dependentes e permanentes [Capítulo 4, pg.49].

4. Objetivos

Objetivou-se, com a pesquisa, estabelecer uma rota de acesso videocirúrgico ao coração de tilápia-do-Nilo, para induzir lesão mecânica, por ressecção parcial do ápice ventricular. Por conseguinte, validar o uso de tilápia-do-Nilo como organismo modelo para o estudo de doenças cardiovasculares. Ademais, estudar a eficácia de metadona, um analgésico de uso crescente na clínica de pequenos animais, para o controle da dor, no pós-operatório imediato da videocirurgia. Por fim, avaliar e comparar diferentes parâmetros funcionais e estruturais do miocárdio ventricular de tilápias-do-Nilo, antes e em diferentes tempos após a indução de lesão cardíaca guiada por videocirurgia.

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CAPÍTULO 2 – Artigo Científico²

An endoscopic route to access the pericardial cavity and induce experimental mechanical injury in the heart of teleost fish

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Disponível em:
<https://us.sagepub.com/en-us/nam/journal/laboratory-animals#submission-guidelines>.

Abstract

Studies on the mechanisms of heart injury have involved a variety of animal models, from mammals to amphibians and fishes. Usually, the methods to induce heart injuries in fish models involve exteriorizing the heart for ventricle resection. In this study, we described an endoscopic route to access the pericardial cavity (coelom) of teleost fish (Nile tilapia, *Oreochromis niloticus*) and induce heart injury using a 3-mm oval cup biopsy forceps. Endoscopy has several advantages such as heart visualization, standardized biopsy size, and high precision. The procedure is executed rapidly and is associated with low rates of intraoperative (5.60%) and postoperative (3.38%) mortality. Approximately 6% of the fish heart was removed with biopsy forceps.

Keywords

Biopsy, lesion, myocardium, Nile tilapia, video surgery

Background

Heart failure (HF) after myocardial infarction (MI) is a costly human condition characterized by an increased risk of death.¹ Studies on the mechanistic basis of HF have involved a variety of animal models, from mammalian to amphibians and piscine. The heart of mammalian models, like that of human, has a limited capacity of regeneration following an acute MI.² Conversely, the hearts of urodele amphibians and cyprinid fishes (ex. zebrafish) have an impressive capacity for regeneration.² Relative to the heart of other teleost fishes, such as Nile tilapia, studies in HF are limited and controversial.² Nile tilapia has been used as a model for studying inflammation, immunity, and sepsis.³ Its exploration in research on HF may provide valuable information about adaptive molecular processes of the heart due to its genome having been fully sequenced.⁴

Various methods have been used to induce heart injuries in fish models. The most common technique is the mechanical resection,⁵ where an incision is performed in the ventral/chest of the animal to expose the heart for mechanical ventricular apical transection with scissors. Alternatively, heart injury can be induced by cryocauterization, genetic ablation, or transient hypoxia.^{6,7}

Although the mentioned methods create a robust and reproducible injury to the fish heart, they can potentially damage other organs/tissues due to the larger opening of the skin, wide exposure of coelom (pericardial and pleuroperitoneal cavities), and the existence of water microorganisms. We hypothesized that injury could be safely induced in the heart of tilapia by troughing an endoscopic route, thereby avoiding open surgery.

Endoscopy is a much less invasive technique than open surgery. There are reports of its use to access the pleuroperitoneal cavity of teleost fishes.⁸ This work describes an endoscopic route to get access to the pericardial cavity of Nile tilapia and to induce heart injury using a biopsy forceps. Data relative to mortality were collected intraoperatively and postoperatively for a follow-up time of 30 days.

Surgical protocol

The series of surgical procedures included 107 Nile tilapias, male, with a mean weight of 121 ± 31 g ($\pm$ standard deviation) and measuring from 18 to 22 cm in length. The study protocol was approved by the Institutional Ethics Committee on Animal Use (protocol number 5222/15). Approximately 24 h before the endoscopy procedures, the tilapias were transported to the laboratory and placed in tanks (60 L of water per fish) containing 0.6% of sodium chloride.

All tilapias were operated consecutively (during 2 days) by a single operator. Anesthesia induction was obtained immersing fish in tanks containing 10 L of the same water from maintenance tanks where was added propofol (Cristália, Itapira, Brazil) at 5 mg/L. Individuals were considered anesthetized when positioned at the bottom of the aquarium, exhibiting reduced operculum movements, and showing no righting reflex and absence of response to pressure applied to caudal peduncle. The tilapias were positioned in left-lateral recumbence on a surgical table covered with moist compress gauze cotton. Anesthesia was maintained using a recirculation system, using the same water from the induction tank, via normograde flow (mouth to gills).

The access point for endoscopy was created just below the operculum and immediately above the pelvic fins. The scales were removed with a clipper blade (**Fig. 1A**). Thereafter, a punch biopsy (Miltex, Plainsboro, NJ, USA) measuring 4-mm in diameter and 7-mm in depth was pushed through the skin by rotatory movements until it crossed the layers of tissue below, which were removed *en bloc* (**Fig. 1B** and **C**). A sterile swab was inserted into the access point for cleaning and hemostasis of the area (**Fig. 1D**).

A 4.2-mm laparoscopic trocar (Karl Storz, Tuttlingen, Germany) was inserted into the pericardial cavity (**Fig. 1E**). The coelom was examined and the heart located (**Fig. 1F**). A biopsy forceps (**Fig. 1G**), flexible, 5 Fr outer diameter, 3-mm oval cup jaw, 600-mm working length, ergonomic handle and autoclavable, was introduced through the working channel of the endoscope and guided towards the heart. The heart apex was amputated during the ventricle diastole by twisting the tip of the forceps on its axis and then pulling.

The access point for endoscopy was mass-closed with a simple interrupted suture technique with non-absorbable suture material (3-0 Nylon) (Shalom, São Luis de Montes, Brazil). The fishes were maintained in tanks containing 0.6% of salt, under temperature (28°C) and pH (7.3) controlled, and continuous oxygenation.

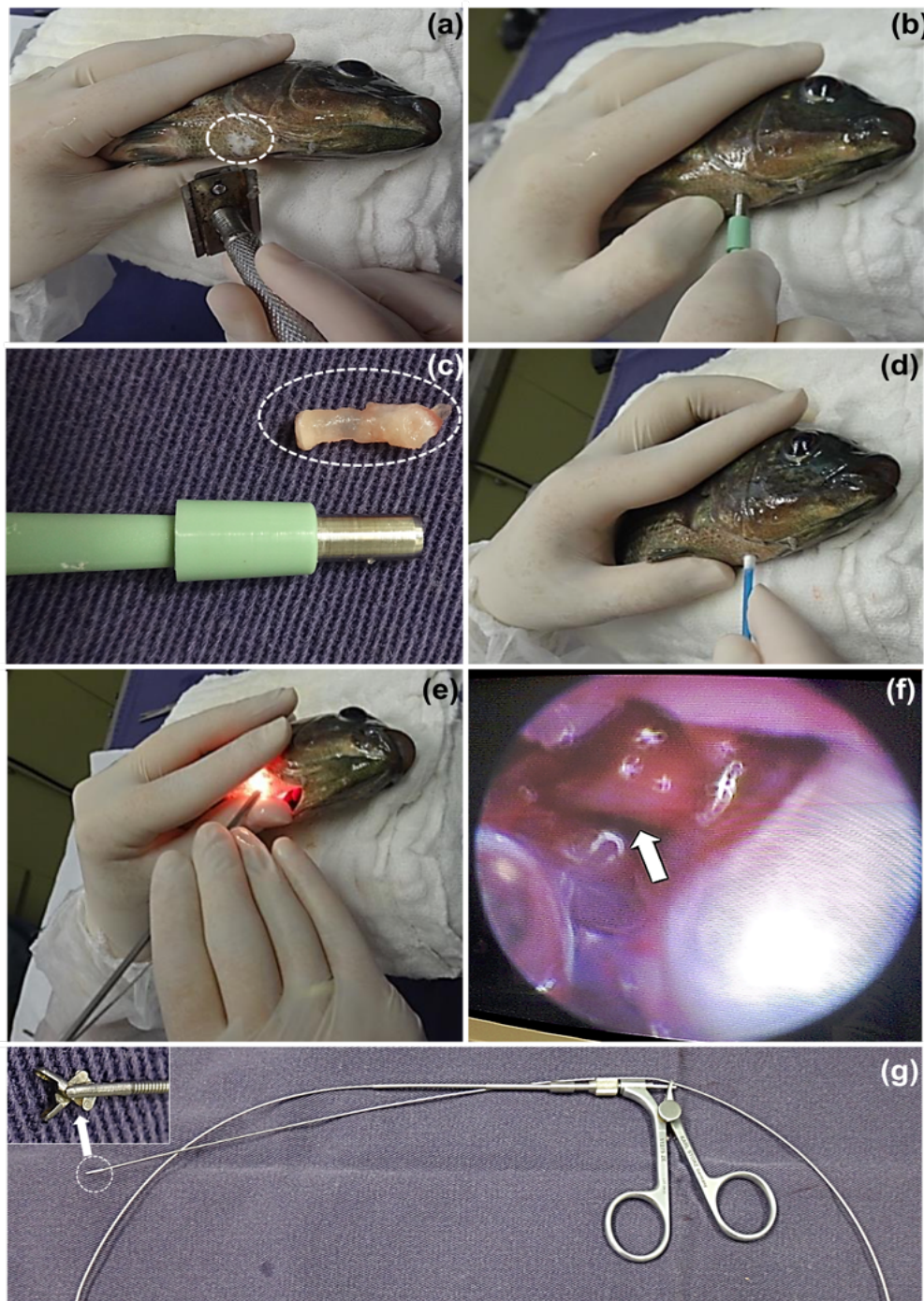


Figure 1. The endoscopic route to get access to the heart in Nile tilapia. The scales from access area (circle) were removed (a). An access point created with a punch (b). Tissues removed *en bloc* (circle) (c). Hemostasis of the access area with a swab (d). Laparoscopic trocar inserted into the pericardial cavity (e). The heart (arrow) was located (f). The ventricle apex was amputated using forceps biopsy (g).

Surgical and postoperative data

Mean surgical time was longer (10 ± 4 min) for the first 20 tilapias receiving endoscopy (early phase of the learning curve). Subsequently, the mean surgical time reduced to 3.26 ± 1.28 min, which can be attributed to increasing experience of the surgeon. In mice, the time for inducing heart injury by left coronary occlusion varies from 2 to 23 min depending on the technique used.⁹ No data on the surgical time for inducing heart injury in zebrafish was found in the literature.

Intraoperative mortality rate was 5.60% (6/107). Four deaths occurred at the beginning of the surgical series when punches used to create the access points for endoscopy procedures were replaced every 5 animals. We suspect that deaths could be related to the reuse of punches and therefore modified the protocol for use of tools. After using one punch per animal, the intraoperative mortality rate reduced to zero.

The survival rate at 30 days postoperative was 96.62%. Two tilapias died in the first 24 h and one died in the first 48 h after the procedure. Survival rate observed in this work was similar to those described by other authors in experiments with mice, rats, and zebrafish.^{5,6,9}

With the technique proposed in this work, about 6% of the heart was removed. Biopsies were fixed in 10% buffered formalin and processed for routine inclusion in paraffin. Histopathological examination revealed the presence of subepicardial myocardium in all samples (**Fig. 2**), indicating that the biopsy forceps-induced lesion was deep enough to injure the cardiac muscle.

In conclusion, this work showed that mechanical injury can be induced in the heart of Nile tilapia by using biopsy forceps through an endoscopic route. The procedure is rapidly executed and is associated with low rates of mortality. Endoscopy has several advantages such as heart internal visualization, standardized biopsy size, and high precision. Morpho-functional and molecular features of hearts injured by the technique proposed here are being studied.

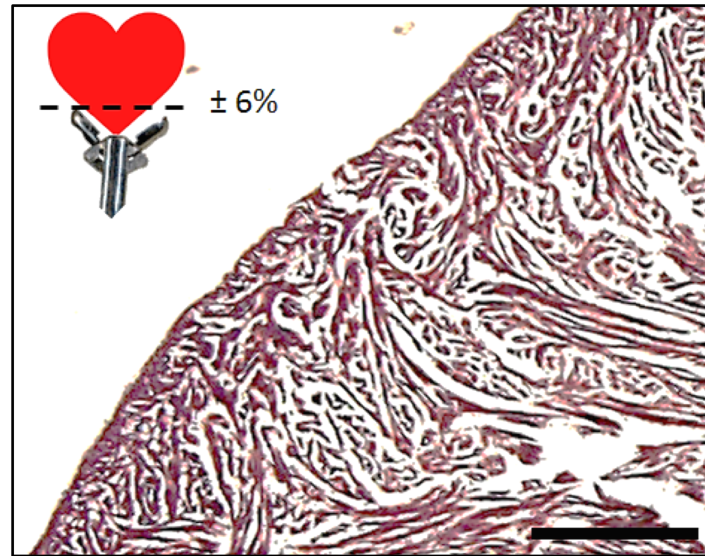


Figure 2. Photomicrography of tissue ($\pm 6\%$ of the heart) removed by the biopsy forceps. Note a significant area of subepicardial myocardium. Bar = 200 μm (Masson's trichrome stain).

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CAPÍTULO 3 – Artigo Científico³

Behavioral and physiologic effects of methadone in the perioperative period in Nile tilapia (*Oreochromis niloticus*)

Running title: Methadone in Nile tilapia

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Through the analysis of behavioral changes, this study demonstrates that methadone has behavioral, but not analgesic, effects in Nile tilapia. It provides information that can suggest a sedative action of the drug in the species, as the recovery time was higher in animals receiving methadone. Future research with different doses and different stimuli could give more information about analgesia.

Keywords: analgesia, pain, teleost, video-surgery

The use of several different species of Teleost fish in biomedical research has increased much over the last decades, as models for studies about the pathogenesis of diseases that affect humans, such as acute myocardial infarction, epilepsy, and sepsis (Poss, *et al.*, 2002; Chablais & Jaźwińska, 2012; Dickover, *et al.*, 2013). Frequently, highly invasive surgical procedures, including heart apex amputation, are performed in Teleost, however without mention the use of any analgesics during the perioperative period (Poss, *et al.*, 2002; Chablais & Jaźwińska, 2012).

Almost all components of the mammalian pain system are described for fish (Bromm, 2016). Teleost fish have nociceptors very similar to those of mammals, besides neuro-anatomic and neuro-physiologic elements, and behavioral responses related to pain perception (Velasco *et al.*, 2009; Rose, 2002; Sneddon *et al.*, 2003; Rose *et al.*, 2012). Fish and mammals have in common 17 out of the 18 criteria for pain perception listed by Sneddon *et al.* (2014). Fish show physiologic and behavioral responses related to pain and have a bigger brain activity when painfully stimulated (Sneddon, 2015).

Few studies about opioid analgesic effects in fish are available, although μ and kappa opioid receptors, functionally analogous to those of mammals, have been detected in the central nervous system of salmon (*Oncorhynchus kisutch*) (Ebbesson *et al.*, 1996) and zebrafish (*Danio rerio*) (Velasco *et al.*, 2009). Morphine, a strong agonist of μ receptors, had been tested in different species. Rainbow trout (*Oncorhynchus mykiss*) that received 0.1 ml 10 g⁻¹ of morphine, intramuscular (i.m.), reduced “rocking” (move from side to the side) and rubbing behavior (rub their lips into the gravel and against the tank walls) when noxious stimulated (Sneddon, 2003). In goldfish (*Carassius auratus*), 40

mg kg⁻¹ of morphine i.m. increased significantly the thermal threshold after 30 min of injection, but not at 60 and 90 (Nordgreen *et al.*, 2009). In koi (*Cyprinus carpio*), i.m. injection of 5 mg kg⁻¹ of morphine or 10 mg kg⁻¹ of butorphanol, a kappa agonist, appeared to have an analgesic effect, causing behavioral changes in the species, with morphine causing fewer adverse effects (Harms *et al.*, 2005; Baker *et al.*, 2013).

Methadone is commonly used to promote analgesia in small mammals. It is an agonist of μ opioid receptors (Kristensen, 1995), having effects and potency similar with those of morphine (Kukanich & Wiese, 2015). Additionally, methadone also has antagonist activity in N-methyl-D-aspartate receptors, making it more effective than morphine in controlling some types of pain in mammals (Callahan, 2004). Potential anti-nociceptive effects of methadone in fish are unknown. Therefore, the aim of this study was to evaluate if the administration of 30 mg kg⁻¹ of methadone i.m., before heart apex amputation (Poss, *et al.*, 2002) in Nile tilapia (*Oreochromis niloticus*), causes alterations in behavioral responses indicative of perioperative analgesia.

Propofol (2,6-diisopropylphenol) is an anesthetic agent commonly used in mammals and humans. In fish, propofol had demonstrated good anesthetic effects in rainbow trout (Gomulka *et al.*, 2015), goldfish (GholipourKanani and Ahadizadeh, 2013), Gulf of Mexico sturgeon (Fleming *et al.*, 2003), silver catfish (Gressler *et al.*, 2012), and zebrafish (Valentim *et al.*, 2016). In Nile tilapia, low doses of propofol did not show genotoxic or mutagenic effects (Valença-Silva *et al.*, 2014). Also, propofol has been tested diluted in different concentrations for immersion anesthesia in Nile tilapia, suggesting that is a feasible anesthetic for the species (Roberto Thiesen, personal communication, 25 October 2017).

This study was approved by the Ethics Committee on Animal Use of São Paulo State University – Unesp (registration no. 5222/15).

Sixteen adult Nile tilapia, male, weighing 210.5 ± 54.6 g (mean $\pm$ SD), ranged in length from 18 to 22 cm, free from macroscopic injuries in eyes, mouth, tegument, gills, and fins were used. Twenty-four hours before surgery, fish were taken to the lab and randomly distributed in two groups (n=8), assigned as control (CG) and methadone (MG) groups. They were kept individually in 60L glass tanks with constant aeration. One-quarter of the tank wall was covered with a black lid to give shelter (Fig. 1) (Baker *et al.*, 2013).

Food was withheld 20 h before the procedure. Variables of water condition were temperature 25 ± 2 °C, pH 8.1 ± 0.1 and dissolved oxygen over 5 mg L^{-1} . Marine salt specific for fish was added to the water at 0.6%, to reduce osmotic stress.



Figure 1. Photographic image showing the 60L tank where recovery was recorded and analyzed, which has been divided into four equal zones with one zone covered. A blue air pump is positioned over the tank.

Surgeries for ventricular partial resection (Poss *et al.*, 2012) were performed with fish anesthetized by immersion in water containing propofol (Cristália, Itapira, Brazil), at 5 mg L^{-1} . The concentration was extrapolated from studies with other species and from preliminary assays performed in our laboratory. Surgeries were accomplished with fish out of the water and over wet cotton gauze, positioned over the induction tank. A recirculating system made with a submersible pump was used to deliver aerated anesthetic water from the mouth through the gills (normograde) at a 1 L kg^{-1} flow. After induction and at least 5 min before surgery, fish in MG received an intramuscular injection of 30 mg kg^{-1} of methadone (Cristália, Itapira, Brazil) in the dorsal muscle right below the dorsal fin, while fish in CG received sterile saline solution (Cristália, Itapira, Brazil) calculated and administered in the same way as methadone. The methadone dose chosen was based on the morphine dose used by Sneddon (2003) in rainbow trout, and by Nordgreen *et al.* (2009) in goldfish, since these two drugs, in mammals, have similar analgesic potency. For induction of noxious stimulus, the coelom (pericardial cavity) was accessed through an endoscopy access point created just below the operculum and immediately above the pelvic fins. A 4.2-mm laparoscopic trocar was inserted into the

coelom and the heart apex was amputated (tissue fragment measuring 3 mm x 3 mm, corresponding to 6% heart) with an endoscopy forceps (Marshall *et al.*, 2017). Afterwards, the cavity access was closed and the fish moved to the recovery tank, that was continuously aerated and with water at same conditions as of maintenance tanks. Data of surgical time, water pH, anesthetic induction time, opercular rate before anesthesia, after induction and at the end of surgery, as well as heart rate (with a Doppler) after induction and at the end of surgery were recorded. The time necessary for animal to resume spontaneous opercular beats was also recorded.

To evaluate behavioral responses, recovery was video recorded, and videos evaluated by a single observer, unaware of the treatment administered. The videos (5 min each) were recorded before anesthesia induction and at 1, 2, 3, 6, 12, and 24 h after moving the fish to the recovery tank. At these time points, opercular beat rate, caudal fin beat rate, and use of cover (percentage of time spent undercover) (Johnston *et al.* 1994; Sneddon, 2003; Reilly *et al.* 2008) were recorded. Recovery time, i.e., the time needed to restore full equilibrium, was also recorded. Also, at the end of each video recording, one commercial pellet with 28% protein was offered, to test for food consumption.

All data were tested to normality and subjected to one-way repeated measures ANOVA (for comparing values over time among the group) with Tukey post-test, and to unpaired t-test (comparisons between groups). A Pearson correlation test (*r*) was applied to selected variables. Results are presented as the mean $\pm$ standard deviation ($\pm$ SD). Differences with $P < 0.05$ were significant. Three fish (two from MG and one from CG) died during the procedure due to surgical complications and were excluded from analysis. All calculations were performed with GraphPad Prism® 6.0c.

In this study, it was observed that, even though relatively insoluble in aqueous solutions (Berry, 2015), propofol easily homogenized with water. Initial water pH was 8.21 ± 0.15 , and 8.24 ± 0.15 after propofol addition ($P = 0.695$), keeping the values within the optimal range (6 to 9) for Nile tilapia (Lim & Webster 2006).

Surgical time did not differ between groups ($P = 0.787$), being 747.3 ± 491.8 sec for CG and 676.7 ± 416.6 sec for MG, assuring that this variable did not affect postoperative evaluation. Induction times were 439.4 ± 179 sec for

CG and 338.3 ± 110 sec for MG ($P = 0.255$). Individuals were considered anesthetized when lying down at the bottom of the tank and by lack of response to a firm squeeze at the base of the tail (Johnston *et al.*, 1994; Sneddon, 2003; Brown *et al.*, 2005; Gressler *et al.*, 2012).

Table I shows data of basal and post induction opercular beat rates, heart rate post-induction and post-surgery, time to resume spontaneous opercular beats, and recovery time.

Table I. Mean values and standard deviation of basal and post-induction opercular beat rate (per minute), recovery time (seconds), heart rate post-induction and post-surgery (beats per minute), and time to return spontaneous opercular beats (seconds) in Nile tilapia (*Oreochromis niloticus*) anesthetized with propofol and treated or not with methadone.

Group	OBR basal	OBR post induction	HR post induction	HR post- surgery	OB return time	Recovery time
CG (n = 7)	88.86 ± 18.51	22 ± 5.53	46 ± 16.45	56.29 ± 13.9	649.9 ± 395.3	4569 ± 950.2
MG (n = 6)	107.03 ± 25.4	18.33 ± 7.1	47 ± 15.63	58.3 ± 18.8	1284 ± 855.8	6970 ± 2236

In both groups, there were no opercular beats at the end of surgery. This happened because the recirculating system used to maintain anesthesia simulates normal ventilation, thus fish did not need to move the opercula. During recovery, every fish was “walked” in the tank for 30 sec every 2 min, until it resumed spontaneous opercular beats. This time was recorded and did not show differences between groups ($P = 0.105$). However, 2 animals from MG had very low values, when compared with mean values (Fig. 2a) and this fact can have contributed to the lack of significant differences between groups. In mammals, opioids cause a dose-dependent respiratory depression that is primarily mediated by supraspinal μ opioid receptors (Kukanich & Wiese 2015), so it is feasible to suggest that methadone could also have some depressant effect on the Nile-tilapia respiratory center. Additionally, a synergistic action between propofol and methadone can have happened since, in mammals, propofol causes dose-dependent respiratory depression (Clarke *et al.*, 2014).

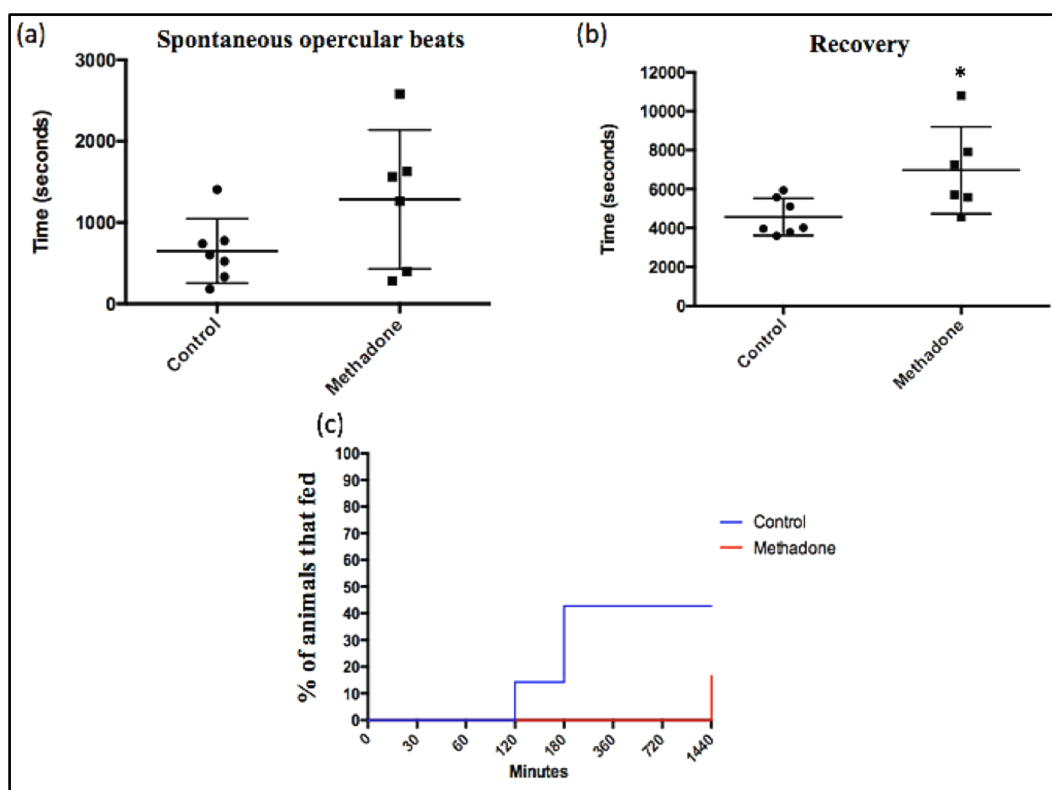


Figure 2. Dispersion graph of time to return to spontaneous opercular movement (a), recovery time (b) and a survival curve showing the percentage of animals that resumed feeding during a 24h period evaluation (c). * Statistical difference; unpaired *t* test ($P < 0.05$).

Recovery time was considered the time elapsed between the moment that the fish were moved to the recovery tank until they regained full equilibrium. Values for MG were significantly higher than in CG ($P = 0.024$) (Fig. 2b). There was a very weak negative correlation between this variable and surgical time in both groups (MG: $r = -0.189$; CG: $r = -0.272$). It follows that a possible sedative effect of methadone could have contributed to the result.

Oxygen dissolved in water may have an impact on the clearance of propofol from fish, as oxygen concentration delivered to the metabolizing tissue will be directly related with aquatic oxygen concentration (Barrionuevo, et al., 2010). The same authors considered oxygen concentrations of $7.5 \text{ mgO}_2 \text{ L}^{-1}$ as normoxic conditions, while concentrations of $4.3 \text{ mgO}_2 \text{ L}^{-1}$ as hypoxic conditions for different development stages of zebrafish. The water from recovery tank in this study was always aerated and oxygen concentrations measured always over $5 \text{ mgO}_2 \text{ L}^{-1}$. Even considering it a non-hypoxic concentration, the value

below optimal could have contributed to a slow recovery, as the clearance of propofol may have been affected.

Table II shows data of opercular beat rates, caudal fin beat rates and percentage of use of cover, obtained from the evaluation of the videos recorded during recovery.

Table II. Mean values and standard deviation of opercular beat rate (per minute), caudal fin beat rate (per minute), and percentage of use of cover in Nile tilapia (*Oreochromis niloticus*) anesthetized with propofol and treated or not with methadone, in different time spots, during 24 h.

Parameters	Group	1h	2h	3h	6h	12h	24h
OBR	CG	55.1 ± 12.7	44.5 ± 26.4	60.6 ± 16	71.1 ± 16.3	61.7 ± 18.3	53.7 ± 33.2
	MG	44.3 ± 35	49.3 ± 20.1	51.3 ± 17.6	62 ± 17.8	52.3 ± 31.5	45.6 ± 39.7
Caudal fin beats rate	CG	210.4 ± 108.8	121.3 ± 70.1	83.4 ± 82.8	77.5 ± 110	51.7 ± 85.3	17.3 ± 15
	MG	117.8 ± 121	82.3 ± 84	76.5 ± 55.1	35.8 ± 30.3	38.7 ± 60.8	48 ± 68.1
Use of cover (%)	CG	14.7	30.9	52.5	62.6	20.2	55.6
	MG	9.6	22.3	34.5	60.1	67.3	66.6

Swim activity in fish can be evaluated in different ways. It can be a percentage of movement in a period of time (Baker *et al.* 2013), a direct movement more than one head (Nordgreen *et al.* 2009) or body length (Reilly *et al.* 2008) or by caudal fin beat rate (Harms *et al.* 2005). The last one was chosen in the present study. In koi, these values remained unchanged when compared with basal ones, after animals had received butorphanol, showing that the drug has no effect over activity level in the species (Harms *et al.*, 2005). In contrast, morphine, also in koi, increased activity level in some moments and reduced it in others (Baker *et al.* 2013). Values in CG were greater than in MG at several moments during this study, but no statistical differences were noted. Therefore, at the dose used, methadone could have had a small effect on activity level in Nile tilapia.

Cover use was measured according to Reilly *et al.* (2008). In the present study, there were no changes in this measure when compared with basal values in both groups. This variable seems to show a species-specific response, as in koi and trout receiving the same noxious stimulus; the first one

did not change the cover use, while the second one increased it (Reilly *et al.* 2008). The same species had different results when an opioid was added to the protocol. Koi that received morphine or butorphanol (Baker *et al.* 2013), and rainbow trout that received morphine (Sneddon 2003), did not increase or reduce the cover use, even when stimulated with a noxious stimulus, suggesting that opioid may have had a behavioral effect on these animals.

Baker *et al.* (2013) demonstrated that food consumption is a reliable indicator of analgesia in koi. In the present study, food consumption was assessed by offering a floating pellet (same of standard diet) at the end of each video recording and each response was scored from 1 to 4 as described by Harms *et al.* (2005). The scores were attributed as follows: 1– pellet was offered and fish were visually oriented to it, eating it in less than 3 sec; 2– pellet was offered and fish were visually oriented to it, eating it after 3 sec but before 20 sec; 3– pellet was offered and fish were not visually oriented but consumed pellet between 20 sec and 3 min; and 4– fish did not consume pellet within 3 min, after which the pellet was removed. In rainbow trout that received morphine resumed feeding before the ones that did not (Sneddon 2003). In the present study, more animals (n=3) from CG feed when compared with animals from MG (n=1) within a 24-h period (Fig. 2c). The score attributed to all animals was 3. No statistical differences were noted; however, methadone seems to have a tendency to suppress the normal feeding behavior. One hypothesis for this is that methadone could have caused nausea and sedation, as these effects are described in mammals (Kukanich & Wiese 2015). However, such behavior was not observed when morphine was given to rainbow trout and koi (Sneddon, 2003; Baker, 2013), suggesting that these alterations may be species-specific or drug-specific.

Based on behavioral and physiologic parameters evaluated, it is impossible to affirm that 30 mg.kg⁻¹ of methadone cause analgesia in Nile tilapia, however, they suggest that the drug may have behavioral effects. Additionally, the small number of animals can have influenced the results. Other opioids showed variable results in different species of fish. Morphine did provide analgesia in rainbow trout (Sneddon, 2003), as did morphine and butorphanol in koi (Baker, 2013). However, morphine did not offer analgesia in goldfish (Nordgreen *et al.*, 2009). It has to be considered, though, that the dissimilarity

between the studies, especially of regarding species, drugs, doses, and type of stimulus, may be directly related to these factors.

New studies for establishing ideal doses of several types of analgesics for different species still need to be completed. The authors believe that fish do feel pain. However, the use of analgesic agents should be done carefully. Methadone had some behavioral effect in Nile tilapia, however, more studies are necessary for a better comprehension of possible analgesic effects of this drug, and to prove an effective dose.

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CAPÍTULO 4 – Artigo Científico⁵

Myocardial remodeling after endoscopy-guided heart apical amputation in Nile tilapia: a structural and functional analysis

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Abstract

Cardiovascular disease is a health problem worldwide spread. The need for understanding cardiac repair mechanism has increased the number of researches on cellular therapy field. In this context, teleost fish show as an experimental model, as they have morpho-functional and immunologic homology whit mammals. Nile tilapia (*Oreochromis niloticus*) has been used to study several diseases that affect humans. The present study aimed to develop a mechanically induce cardiac injury in Nile tilapia and characterize myocardial repair mechanisms by video surgical, special microscopy and electrocardiogram analysis. Data were recorded before cardiac injury and then one hour, seven, 14, 30, 60 and 90 days after cardiac injury. The ventricles of Nile tilapia, after lesion, did not showed any evidence of regeneration, progressing for a remodeling process, characterized by a thicker ventricle wall and epicardium, by cardiomyocytes hypertrophy, and changes in electrical activity. Changes in the interstitial collagen aggregation state, size and shape of cardiomyocytes nuclei, and in the supra-organization of nuclear chromatin of cardiomyocytes, as well as presence of ploydy, necrotic and apoptotic cells, were also observed. It was concluded that Nile tilapia seems to be a good experimental model organism for experimental cardiology studies, once its ventricle, after have its apex amputated, shows many of the alterations observed in hearts of humans cardiac patients. Additional advantages regarding the use of Nile tilapia in research include the size of the animals, that allows the use of cheaper techniques; and the practical way to keep a great number of animals in small spaces, with low mortality rates.

Keywords: cardiopathies; heart; regeneration; teleost; ventricle.

Introduction

Heart diseases, such as acute myocardial infarction (AMI), are serious conditions whose prevalence tend to increase as the population is aging and is more exposed to risk factors, including hypertension, smoking, and poor diet (da SILVA *et al.*, 2017). The World Health Organization has estimated that AMI is currently the leading cause of mortality worldwide (W.H.O. 2017). The AMI is characterized by the death of cardiomyocytes and the subsequent deposition of fibrin, which is replaced by scar tissue troughing a process of myocardial remodeling (HILL & OLSON 2008; XIE 2013; da SILVA *et al.*, 2017). Changes in left ventricular geometry due to myocardial remodeling are associated with the worst prognosis related to cardiac lesions, with a high prevalence of aortic rupture, arrhythmias, aneurysms formation, or heart failure (LAVIE *et al.*, 2008; LIEB *et al.*, 2014).

One of the major challenges of the science is to prevent the heart failure. The current options to prevent the heart failure consist in the control of the hyperactivation of the neuro-humoral system; in the treatment of the etiology, when possible; and in the prevention of sudden death, with an implantable cardioverter defibrillator; among others (GUO *et al.*, 2005; FERNANDES, SILVA, 2007; UYGUR, LEE, 2016). Such options increase patient survival but are far from ideal or definitive. Scientists and clinicians agree that the ideal to prevent heart failure would be to modulate the molecular, genetic, and cellular mechanisms of myocardial remodeling through biological therapies that include stem cell applications. Therefore, many laboratory studies employing model organisms for human heart diseases have been conducted in the scope (BRECKENRIDGE, 2010; HOUSER *et al.*, 2012; LIAO, HUANG, LIU, 2017).

Mammals until recently were a priority in research on cardiovascular disease; however, this scenario has changed. First, recombinant DNA technology and the creation of transgenic organisms led to a significant reduction in the number of animals used in research. Second, non-mammalian vertebrates are emerging as organism models for use in cardiovascular studies, since that some Cyprinidae teleosts and Urodele amphibians can regenerate

injured organs or limbs (BROCKES, 1997; POSS; WILSON; KEATING, 2002; NYE *et al.*, 2003; ALIBARDI, 2010).

Teleostei fish are widely used in studies on human cardiovascular diseases, among other reasons because the pathways that regulate the development and autonomous control of the heart are highly conserved across all vertebrates (LYONS, 1996; TAYLOR *et al.*, 2014). Currently, the zebrafish is the most studied Teleostei because it has a sequenced genome and is able to restore the morpho-functionality of its heart after an injury (POSS; WILSON; KEATING, 2002; WILKINSON; JOPLING; VAN EEDEN, 2014, KIKUCHI, 2015, da SILVA *et al.*, 2017). Studies on zebrafish have contributed to the understanding of molecular factors that theoretically can activate cardiac regeneration mechanisms in adult mammals, including humans. Zebrafish can provide information for potentiating the treatment of heart diseases. However, due to its high cardiac plasticity, it is not a model that mimics human cardiac dysfunction. Besides, the zebrafish body size (± 5 cm in length and mean weight of 0.19 g) may limit the use of this animal in the laboratory. Data on the physiology of the zebrafish heart, for example, vary widely due to the size of the animal's body and the lack of standardization of analytical methods (LIU *et al.*, 2016).

Adult Nile tilapias (Cichlidae, *Oreochromis niloticus*) weigh approximately 350 g and have the sequenced genome (GUYON *et al.*, 2012). They are capable of regenerating or repairing the cranial kidney, ovaries, caudal fin, and skeletal muscle (CAMARGO *et al.*, 2004). Besides, tilapias show physiological, immunological, and molecular homology with mammals and have been employed in many studies on the mechanisms of inflammation and sepsis (MARTINS *et al.*, 2008; PEREIRA *et al.*, 2010). More recently, tilapias have been used to study diseases related to lipid metabolism, which in humans represent risk factors for obesity, arteriosclerosis, diabetes, hepatic steatosis, and AMI (WRIGHT *et al.*, 1998; WRIGHT *et al.*, 1999; HAN *et al.*, 2011). It is plausible to think that Nile tilapias can be good as model organisms for studying the diseases of the human heart and its remodeling mechanisms.

The literature describes different animal models of induced cardiac injury; the most common are the model of surgical amputation of the heart apex, the myocardial cryoinjury model, and the injury model by genetic ablation of

cardiomyocytes (SINGH *et al.*, 2010; LAFLAMME; MURRY, 2011; DICHOVER; ZHANG; CHI, 2013; DICKOVER *et al.*, 2013). All of these models have some kind of technical limitation; in the case of surgical heart amputation, the risk of mortality and perioperative morbidity are high due to the size of the incisions needed to expose the pericardial cavity and exteriorize the heart. A substitute alternative to amputation of the apex of the heart with scissors is endoscopy-guided amputation. This method was successfully used by Marshall and co-authors (2017) to injure the heart of adult *Xenopus*; however, the viability of its execution, as well as its effects on myocardial microstructure and functionality, has never been evaluated in fish species.

The hypotheses of this study are that Nile tilapia can be used as a model organism for heart diseases, and the apex of the heart of this species can be safely injured through an endoscopy-guided amputation. Thus, the objective of the study was to evaluate some structural and functional parameters of the ventricular myocardium of Nile tilapias, before and at different times after an endoscopy-guided approach for heart apex amputation, besides presenting an endoscopic route to access the pericardial cavity and the heart.

Material and Methods

Animals and experimental design

The research was conducted after approval by the Commission on Ethics in the Use of Animals (Protocol No. 5222/15) of FCAV/Unesp. Procedures for fish handling and euthanasia followed the precepts of the German Animal Welfare Act (BGBI, IS 1206-1313). Nile tilapias of approximately 4 months old, male, with a mean weight of 200 g, and measuring between 15 and 22 cm in length, were selected for the study. Prior to inclusion, the fish were quarantined. Eighty-four individuals free of macroscopic lesions in eyes, mouth, tegument, gills, and fins were used.

Nile tilapias were grown in tanks with a capacity of 3500 L of water, with temperature ($25 \pm 2^{\circ}\text{C}$), pH (7.4–8.2), and oxygenation (7 ppm) within the comfort range for the species (LIM & WEBSTER, 2006). To reduce osmotic

stress, 0.6% non-iodized sea salt was added to the water. Feeding was provided once daily in the morning and consisted of pelleted feed containing 28% protein.

The tilapias were randomly distributed into seven groups ($n = 12$), with one control group ($n = 6$) and six experimental groups (formed of fish that had the apex of the amputated heart; $n = 12$ fish per group), which differed in the length of the follow-up until the euthanasia. The euthanasia times were 1 h, 7 days, 14 days, 30 days, 60 days, and 90 days post-amputation of the heart apex. The experimental protocol of this study is summarized in **Fig. 1**.

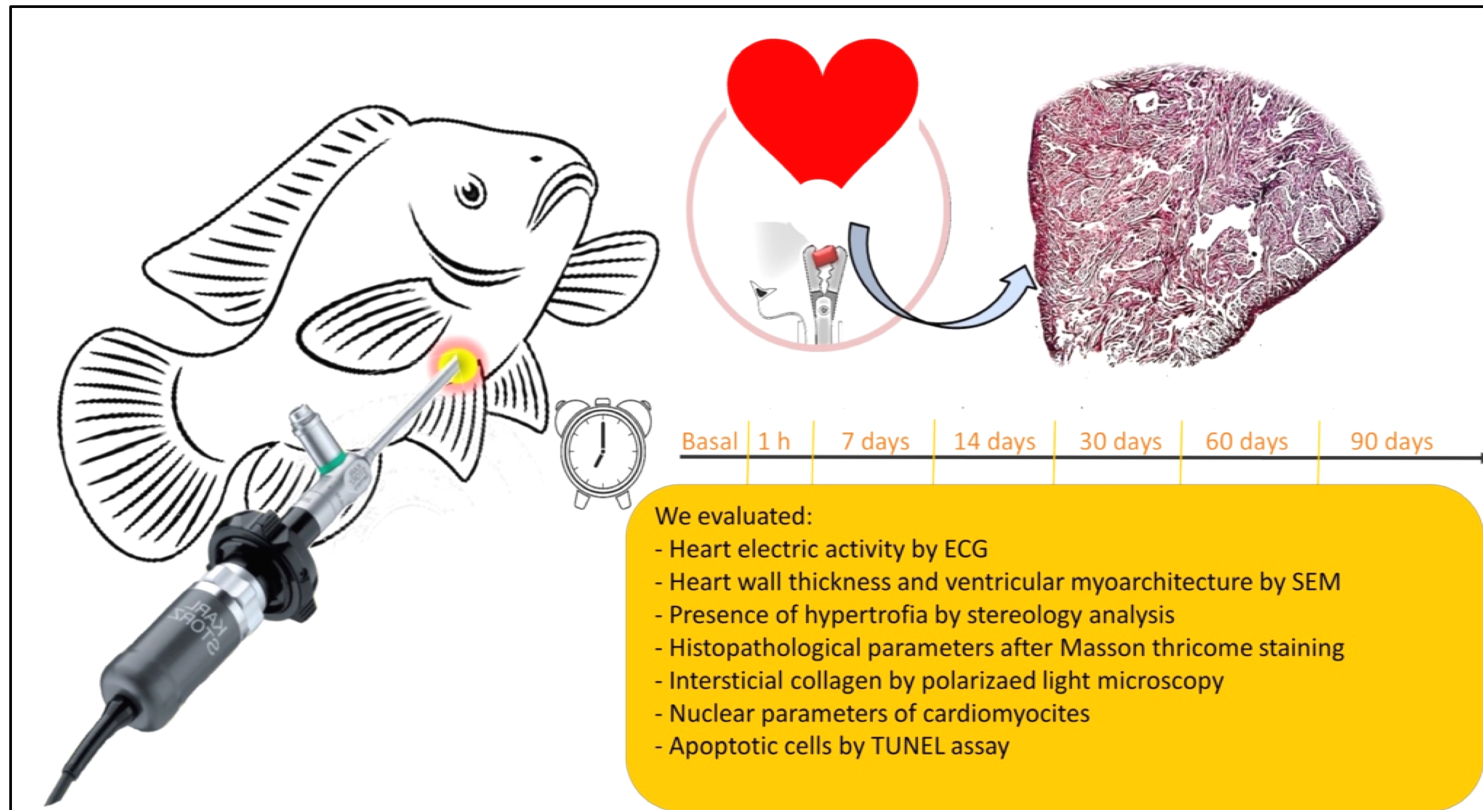


Figure 1. Experimental flowchart. Amputation of the heart apex of 72 Nile tilapias was performed with a biopsy forceps, through an endoscopic route. Functional aspects (electrocardiogram) of the heart were investigated at different moments. Fish were euthanized; the hearts were harvested at several time points and processed for structural evaluation by microscopy. Non-operated hearts from tilapias were used as the controls.

Endoscopy-guided heart apex resection

Twelve hours before the endoscopic procedure, the fish fasted. Immediately prior to endoscopy, the animals were anesthetized by immersion in a tank containing 5 mg/mL propofol (Cristália, Itapira, Brazil) (GRESSLER *et al.*, 2012; VALENTIM *et al.*, 2016) and then were weighed and evaluated by electrocardiogram (ECG) (baseline values). The anesthetic maintenance was done with a system of recirculation of aqueous anesthetic solution, through a normograde route through the operculum. All endoscopic procedures were performed with the animals out of the water, in right lateral decubitus on an acrylic surgical table positioned on top of the anesthesia tank. The animal's body was partially wrapped in cotton swab soaked in water to keep the skin moist.

The access point for endoscopy was created immediately below the operculum and above the pelvic fins. The scales were removed and then a sterile punch, measuring 4 mm in diameter and 7 mm in deep, was inserted through the skin until it reached the subcutaneous musculature, which was removed *en bloc*. A sterile swab was inserted for 1 min at the access point for cleaning and hemostasis of the site. Subsequently, a 4.2-mm endoscopic camera (Karl Storz, Tuttlingen, Germany) was inserted into the pericardial cavity to examine the coelom and locate the heart. A biopsy forceps, flexible, 5 Fr outer diameter, 3-mm oval cup jaw, 600-mm working length, ergonomic handle and autoclavable, was introduced through the working channel of the endoscope and guided towards the heart. The heart apex (6% of the heart) was amputated during a cardiac diastole. No fish died during the procedure. The illumination for endoscopy was made with fiber optic cable connected to a source of cold light emission (Karl Storz, Tuttlingen, Germany). All procedures were transmitted in real time to the screen of a Sony® monitor (Tokyo, Japan).

In the end, the access area for endoscopy was sutured with 3-0 Nylon wire (Shalom, São Luis de Montes, Brazil) using simple separated stitches.

Electrocardiographic (ECG) examination

All fish were examined before amputation of the heart apex and just before euthanasia. ECG traces (60 sec) in derivation DI were obtained using a computerized electrocardiograph (model TEB-ECGPC V6.2, LTDA, Brazil). The red (negative) and yellow (positive) electrodes were positioned, respectively, on the dorsal and left pectoral fins. The green and black electrodes were positioned in the caudal fin. Measured ECG variables were durations (ms) of PR, QRS, and QT intervals, amplitudes (mV) of R, P, and T waves, and heart rate (beats per minute, bpm). QT interval was corrected (QTc) using the Bazett formula.

Euthanasia, heart collection, and tissue preparation

Tilapias were anesthetized and weighed. A ventral incision was then made with the help of a blade 15 (Solidor, Joinville, Brazil) for exposure of the coelom and heart, which was evaluated for the formation of a clot, adherence, and fibrin. Then, a continuous intravenous bolus injection of 1 M KCl (Cristália, Itapira, Brazil) was performed by the caudal vein, to promote cardiac arrest in diastole and muscle relaxation.

After excision, the hearts were immediately washed in 0.9% ice-cold NaCl (Farmax, Divinópolis, MG) and then weighed. Three hearts were cut frontally, in anterior and posterior planes; while others three were transversally in the medioventricular portion, equidistant to the apex and base. Hearts from 3 individuals of each group were fixed using 2.5% glutaraldehyde (Merck, Darmstadt, Germany) for scanning electron microscopy (SEM). All other hearts (9 per group) were fixed by immersion in a 10% buffered formaldehyde solution (Dinâmica, São Paulo, Brazil), dehydrated in increasing ethanol solutions (Synth, São Paulo, Brazil), and included in paraplast without dimethyl sulfoxide (Merck, Darmstadt, Germany) for preparation of microtome sections (3 μ m and 7 μ m).

Scanning electron microscopy (SEM)

After fixation with 2.5% glutaraldehyde, hearts were washed in 0.1M sodium phosphate buffer, pH 7.4, post-fixed with 1% osmium tetroxide (Merck, Darmstadt, Germany), and dehydrated in increasing solutions of ethanol. Subsequently, they were placed in a critical-point dryer at 37°C, with a pressure of 1500 PSI, and sputtered with palladium gold. The ventricular myocardial architecture was evaluated using a JEOL SEM (Peabody, MA, USA) operating at 15 and 20 kV (magnification between 750× and 5,000×).

The wall thicknesses of the hearts were measured from the SEM images using the straight line tool available from the ImageJ software (<http://imagej.nih.gov/ij/>, National Institutes of Health, Bethesda, MD, USA). The spatial calibration of the image analysis system was done with a micrometer ruler for microscopy. Eight thickness measurements were taken from each heart and used to calculate an average.

Histopathological examinations and stereological parameters of myocardial structures

Paraffin-embedded heart sections (3 µm) were dewaxed in xylol (Synth, São Paulo, Brazil), hydrated in decreasing solutions of ethanol (Synth, São Paulo, Brazil) and in water, and stained with Hematoxylin-Eosin (Sigma-Aldrich, St. Louis, MO, USA) or Masson's trichrome (Sigma-Aldrich, St. Louis, MO, USA).

The samples were examined under a light microscope (Olympus BX53, Tokyo, Japan), equipped with Plan Neofluar objectives, optovar 1 to 2, and rotating stage. With the help of a high-resolution color video camera, the images were transmitted to a computer where they were digitized and analyzed using the ImageJ software. The ventricles of the injured hearts were evaluated for the presence of inflammatory response, vascularization, bleeding, necrosis, epicardial thickening, granulation tissue, and changes in interstitial connective tissue. Histopathological findings were scored using 4 grades: -, absent; +, minimum detection; ++, moderate; +++, intense. An examiner conducted all assessments blindly.

Stereological parameters of the myocardium were studied using a 100-point test system. Only the remote myocardium proximal to the amputation site was studied in the ventricles of injured hearts. The evaluation in the control group was random. The mean cross-sectional areas (A) of the cardiomyocytes (an indicator of hypertrophy) were estimated in square micrometers with the formula $A = VV/2 \times QA$, where Vv corresponds to the volume density of cardiomyocytes, and QA represents the number of nuclei within the microscopic field evaluated. Cells that touched the borders of the test system were not counted. The volume density (Vv) of the cardiomyocytes was calculated with the formula $Vv = PP/PT$, where PP corresponds to the number of points that touch the cardiac fibers and PT corresponds to the number of points that compose the test system (MANDARIM-DE-LACERDA, 2003; BEZNUSENKO *et al.*, 2005; MÜLFELD, NYENGAARD, MAYHEW, 2010).

Examination of interstitial collagen

After dewaxing and hydration, frontal/sagittal (7 µm) sections were stained with an acidic solution of ponceau SS (Sigma-Aldrich, St. Louis, MO, USA), pH 2.5 for 3 min (VIDAL; MELLO, 2005; ALDROVANI; GUARALDO; VIDAL, 2007). Collagen fibers were studied in the interstitium of the remote myocardium, post-amputation. In the control group, the evaluations of collagen were random, in ventricular regions equidistant and proximal to the heart apex.

The evaluations were performed under an advanced polarized light microscope (Olympus BX53-Pol, Tokyo, Japan), equipped with a 20/0.75 objective, interferential filter to obtain monochromatic light of 546 nm, a 100-W lamp, Köhler illumination, and Sénarmont compensator consisting of a quarter-wavelength retardation plate. The optical retardation (OR) associated with the brightness of the birefringence of the interstitial collagen fibers was measured in nanometers, with the long axis of the cardiac fibers oriented at 45 degrees to the plane of the polarized light (da SILVA *et al.*, 2006; ALDROVANI, GUARALDO; VIDAL, 2007). With the Sénarmont compensator, a destructive phase difference was introduced between the two polarized wavefronts

(ordinary and extraordinary rays) that passed through the sample. The phase difference angle was read and multiplied by 3.03 (da SILVA *et al.*, 2006).

Nuclear parameters of cardiomyocytes

After dewaxing and hydration, sections (7 μm) were submitted to the Feulgen reaction, which is the gold standard topochemical method for the detection of nuclear DNA (BIESTERFELD *et al.*, 2011). The acid hydrolysis of the Feulgen reaction was done in 4 M HCl (Vetec, Rio de Janeiro, Brazil), at room temperature of 25°C for 40 min (ALDROVANI *et al.*, 2017). To stop the hydrolysis, the material was rapidly washed with ice-cold 0.1 M HCl solution. Then the material was exposed for 40 min to a reactive Schiff base (Merck, Darmstadt, Germany), washed in sulfurous water (a mixture of distilled water, 10% sodium metabisulfite, and 1 M HCl; 18:1:1, vol/vol/vol), air dried, cleared twice in xylol (Synth, São Paulo, Brazil), and mounted with natural Canada balsam (Vetec, Rio de Janeiro, Brazil).

Feulgen-stained nuclei were analyzed under a light microscope (Olympus BX, Tokyo, Japan) equipped with a Neofluar Plan objective 40/0.75, optovar 2, a 100-W lamp, Köhler illumination, field diaphragm in its maximum aperture, and an interferential filter to obtain monochromatic light of 546 nm. With the help of a video camera, the nuclear images were transmitted from the microscope to a computer, where they were digitized and analyzed using the ImageJ software. The parameters of video image analysis studied were: nuclear area, in square micrometers; nuclear perimeter, in micrometers; optical density (OD), which reports on the state of chromatin/DNA compaction; standard deviation of the total densitometric values per nucleus (SDtd), which reports on the textural contrast between the more condensed and less condensed chromatin areas; and integrated optical density (IOD), which reports on Feulgen-DNA values related to nuclear ploidy (ALDROVANI *et al.*, 2017).

The ploidy levels of the cardiomyocyte nuclei were established from the relative frequency histograms of the IOD values. Smears from blood samples collected from Nile tilapia were also submitted to Feulgen reaction and evaluated by video image analysis; the red blood cells were used as diploid (2C) controls.

TUNEL assay

The TUNEL assay was used to detect chromatin fragmentation associated with apoptosis. The procedures were done following the manufacturer's recommendations (TUNEL POD Roche, Sigma-Aldrich, St. Louis, MO, USA). Heart sections treated with DNase I were used as positive controls. Negative controls were made by the exclusion of terminal deoxynucleotidyl transferase (TdT) from the reaction. Diaminobenzidine (DAB) (Dako Corporation, Carpinteria, CO, USA) was used as the chromogen. The apoptotic index (%) was estimated for 200 ventricular cardiomyocytes in each group, post-amputation. Samples from the control group were not studied.

Data analysis

Continuous variables were tested for statistical normality and evaluated using the Wilcoxon test, one-way ANOVA, and ANOVA for repeated measures with Tukey's post hoc test. Differences with $P < 0.05$ were considered significant. All calculations and comparisons were performed using Minitab (San Diego, CA, USA) and MedCalc (Mariakerke, Belgium) software programs.

Results

Endoscopy-guided heart apex amputation is associated with formation of clot, adherence, and fibrin, and leads to changes in the heart shape

The hearts collected 1 h after the endoscopic procedure for amputation of the heart apex showed extensive clot formation at the lesion site (**Fig. 2A**), whereas the hearts collected at days 7 (10% of the sample), 30 (44.4%), 60 (58.33 %), and 90 post-amputation presented adherences (**Fig. 2B**). Fibrin was observed at day 7 (**Fig. 2C**), and a significant change in the heart shape (dilated appearance) was observed at day 90 (**Fig. 2D**) post-amputation.

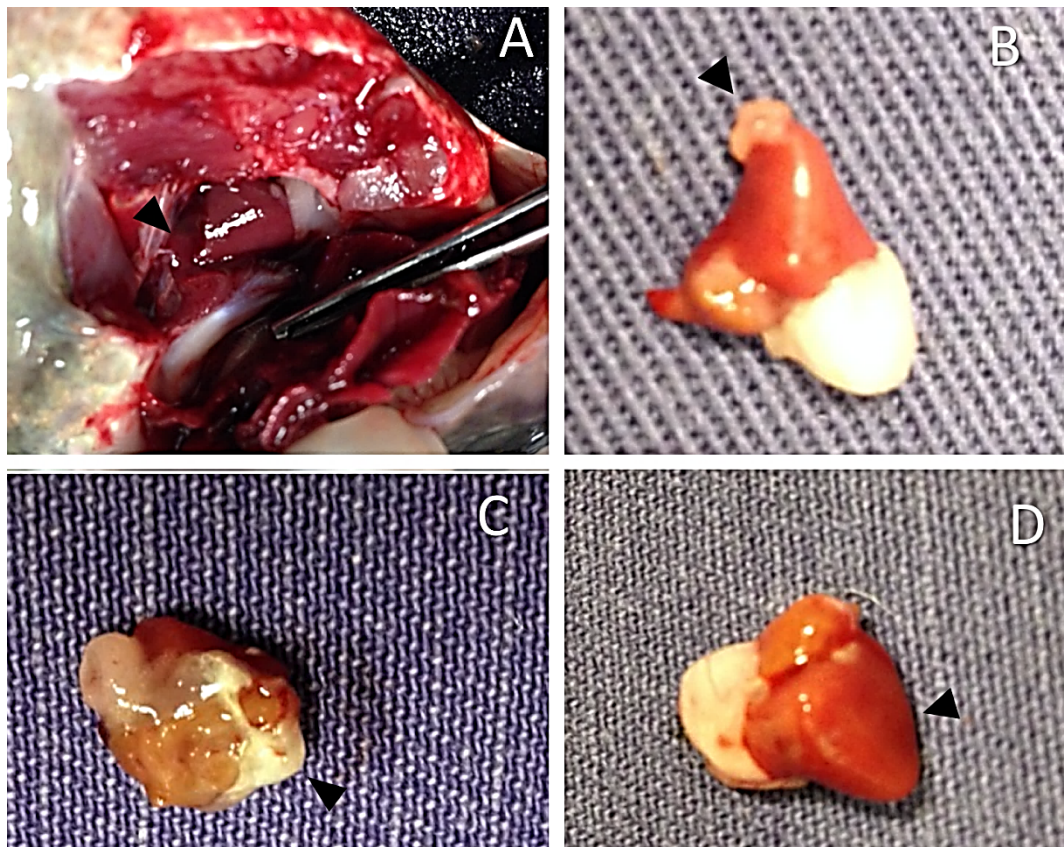


Figure 2. Macroscopic appearance of hearts collected at different times after amputation of the apex of the heart. (A) Clot formation in heart collected on day 7. (B) Adherence on day 60. (C) Fibrin on day 7. (D) Cardiac dilation on day 90.

Heart apex amputation altered ECG intervals and waves, but ST-segment morphology remained unchanged

Fig. 3A shows the positioning of ECG electrodes on the fins of the Nile tilapia. A representative ECG record of tilapia in the control group is shown in **Fig. 3B**. The ECG morphologies observed at different times after heart apex amputation differed from that of baseline ECG (before surgery). The most significant changes in ECG were observed at 90 days post-amputation and were characterized by prolongation of the PR, QRS and QT/QTc intervals and by elevation of R and P wave amplitudes (**Fig. 3C–I**) ($P < 0.05$ vs. controls). The ST-segment morphology remained unchanged throughout the follow-up time (**Fig. 3J**). Heart rates were altered (significant reduction) only on day 7 post-amputation. (**Fig. 3K**) ($P = 0.003$). The main ECG findings are summarized in **Fig. 3L**.

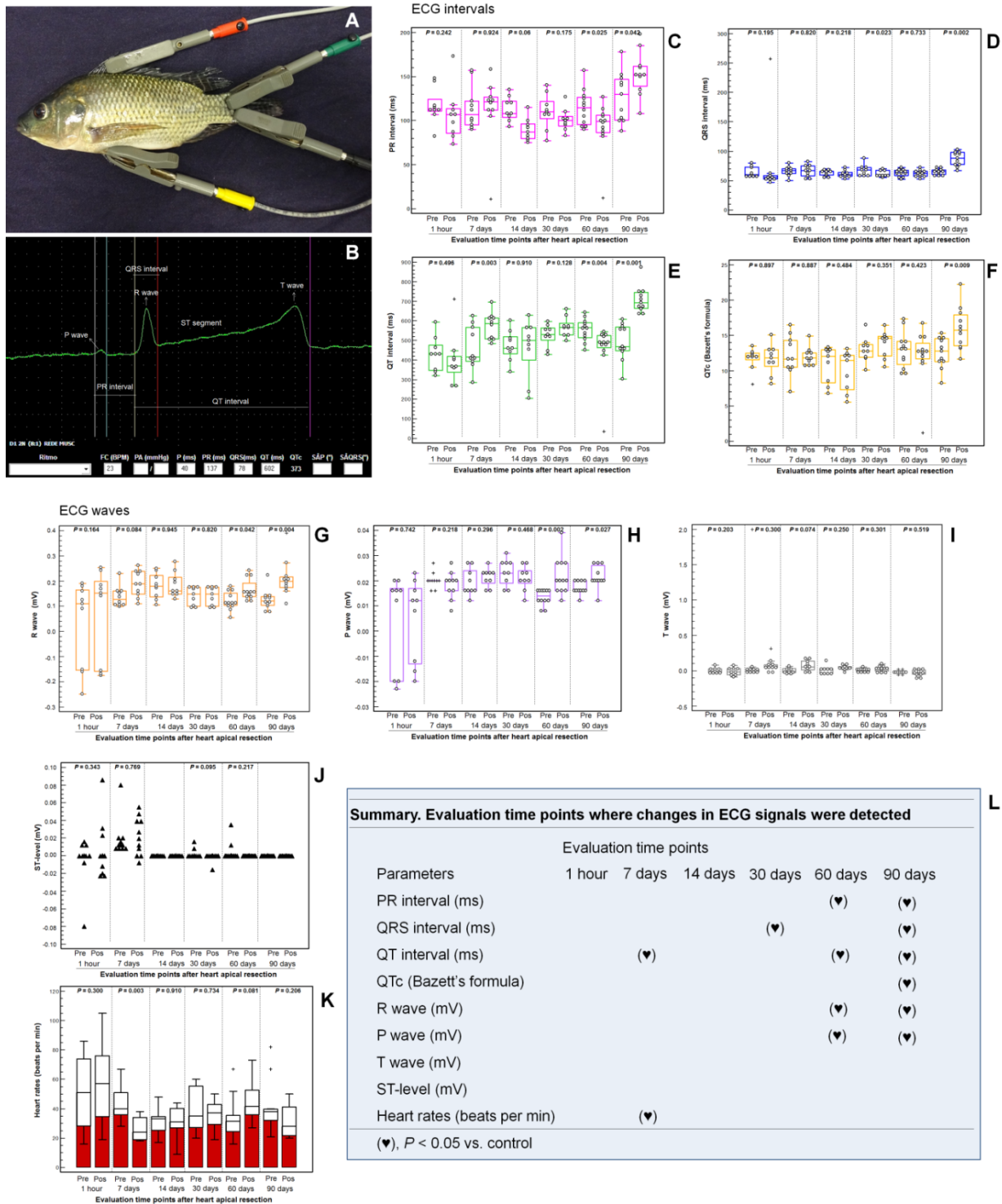


Figure 3. ECG parameters in Nile tilapia at several time points. (A) Electrodes were placed according to the base-apex plane. Negative (red), positive (yellow), and earth (green) electrodes were positioned to the dorsal, pectoral, and caudal fin, respectively. (B) Electrocardiographic trace obtained during the exam. (C) Duration of PR interval, (D) QRS duration, (E) QT and (F) QTc duration, before and after apex amputation. (G) R-wave amplitude, (H) P-wave amplitude, and (H) T-wave amplitude. (J) ST segment. (K) Heart rate variations through the experiment. (L) Summary of ECG parameters after heart apical amputation in Nile tilapias. Hearts correspond to statistical differences when compared to control group.

Heart apex amputation increased the thickness of the heart wall and altered ventricular myocardial architecture in Nile tilapia

The study of SEM images (Fig. 4A–D), magnification 750×) using ImageJ software revealed that the heart apex amputation resulted in a significant increase in the thickness of the heart wall (Fig. 4D). At first, ranging from day 14 to day 60 post-amputation, the wall thickness increased by approximately 18.75% vs. the basal ($P < 0.05$). At day 90 post-amputation, the wall thickness had increased by about 30% vs. the basal ($P < 0.05$). The Fig. 4A–C corresponds to SEM images showing the appearance of the heart in the main times in which changes in the wall thickness were observed.

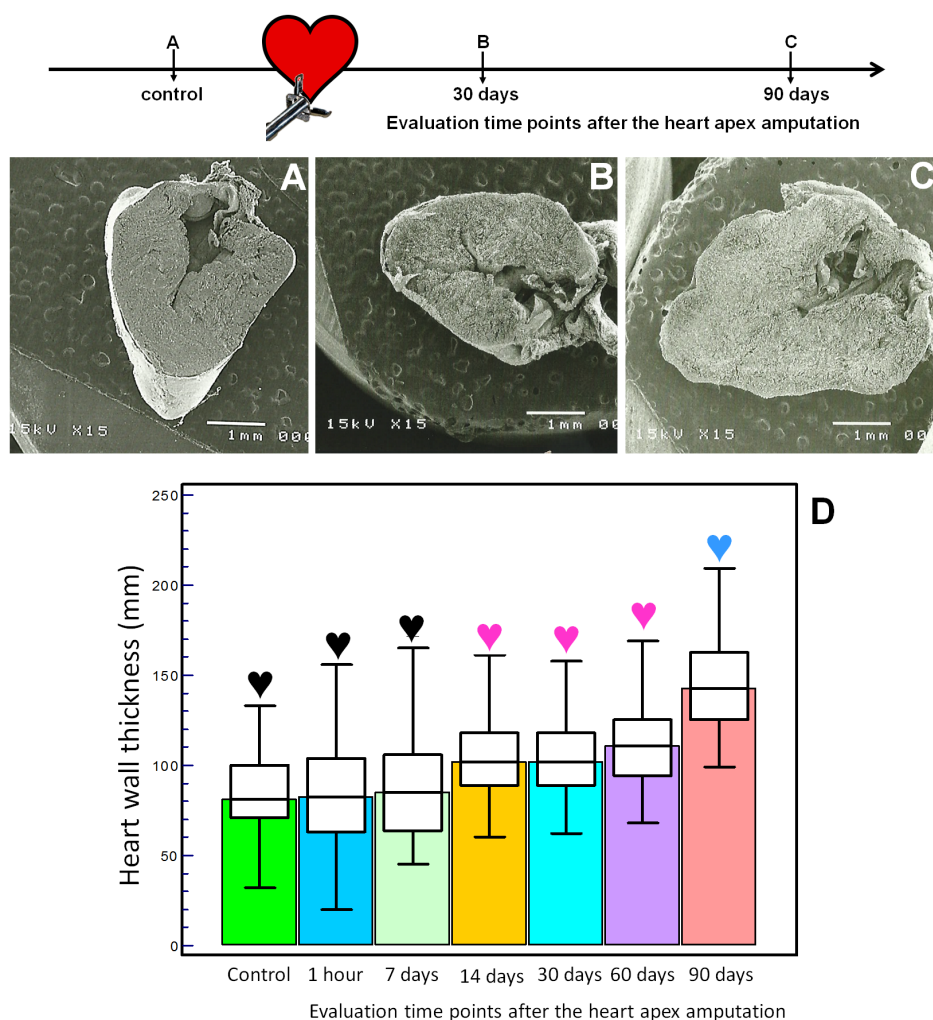


Figure 4. Results of scanning electron microscopy, of Nile tilapias hearts, sectioned sagittally for evaluation of heart wall thickness. (A) Normal aspect of a Nile tilapia's heart. (B) An aspect of a Nile tilapia's heart, 30 days after apex amputation. (C) An aspect of a Nile tilapia's heart, 90 days after apex amputation. (D) The graphic is showing an increase in heart wall thickness, over time, after apex amputation in Nile tilapias. Different hearts means statistical differences ($P < 0.05$).

SEM (magnification 5,000 $\times$) was not able to reveal details of the structure of cardiac fibers and the distribution of interstitial connective tissue, although the hearts were sectioned in the frontal/sagittal plane. The sarcomeres could not be visualized because many cardiomyocytes were cross-sectioned. Compared with the control heart, the injured hearts had ventricular cardiac fibers with a significantly increased cross-sectional diameter (**Fig. 5A–G**). The cardiac fibers were organized in bundles enveloped by a layer of connective tissue.

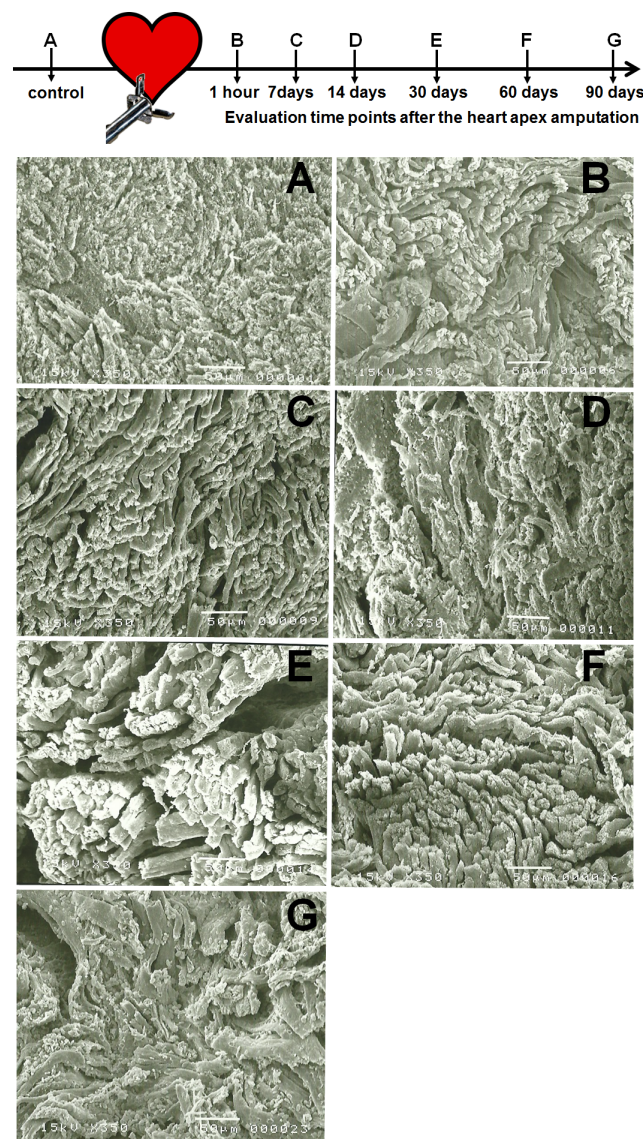


Figure 5. Hearts of Nile tilapias as seen by scanning electron microscopy. (A) Normal cardiomyocytes. Note a greater grouping of cardiac fibers. (B) Cardiomyocytes one hour after apex amputation. Note a disorganization of the cardiac fibers. (C–D) Cardiomyocytes at 7–14 days after apex amputation. Note that the disorder within fibers is still present. (E) Cardiomyocytes 30 days after apex amputation. Note an increase in cardiac fibers diameter. (F–G) Cardiomyocytes at 60–90 days after apex amputation. Note an improve in cardiac fibers organization.

Heart apex amputation caused deposition of connective tissue in a hypertrophic cardiomyocyte setting

Severity scores attributed to the main histopathological findings in the injured ventricles are presented in **Table 1**. Histopathological changes were more intense in the initial post-amputation periods (1h, 7 and 14 days) and were characterized by the presence of inflammatory cells, intense neovascularization, granulation tissue, bleeding, thickening of the epicardium, and necrotic cells (**Fig. 6 A–D**). At 7 days, the ventricles presented fibrin near the site of amputation. Deposition of connective tissue was observed in all samples from day 7 post-amputation, ranging from intense (in the first 30 days of evaluation) to moderate (in the final periods of evaluation) (**Fig. 6E**). At 90 days post-amputation, there was a highly organized connective tissue characteristic of fibrotic scarring (**Fig. 6F**).

Table 1. Severity scores of histopathological findings in the ventricular myocardium of Nile tilapias at different times after amputation of the heart apex

Hematoxylin-Eosin						
Samples	Inflammation	Necrosis	Vascularization	Granulation Tissue	Bleeding	Epicardium Thickening
1 hour	+++	-	+	-	++	+
7 days	+++	++	+++	+++	-	+++
14 days	+	+++	+	+	++	+
30 days	+	-	+	-	-	+
60 days	+	-	+	-	-	+
90 days	+	-	+	-	-	+
Masson's trichrome						
Connective Tissue						
1 hour	-					
7 days	+++					
14 days	+++					
30 days	++					
60 days	++					
90 days	++					

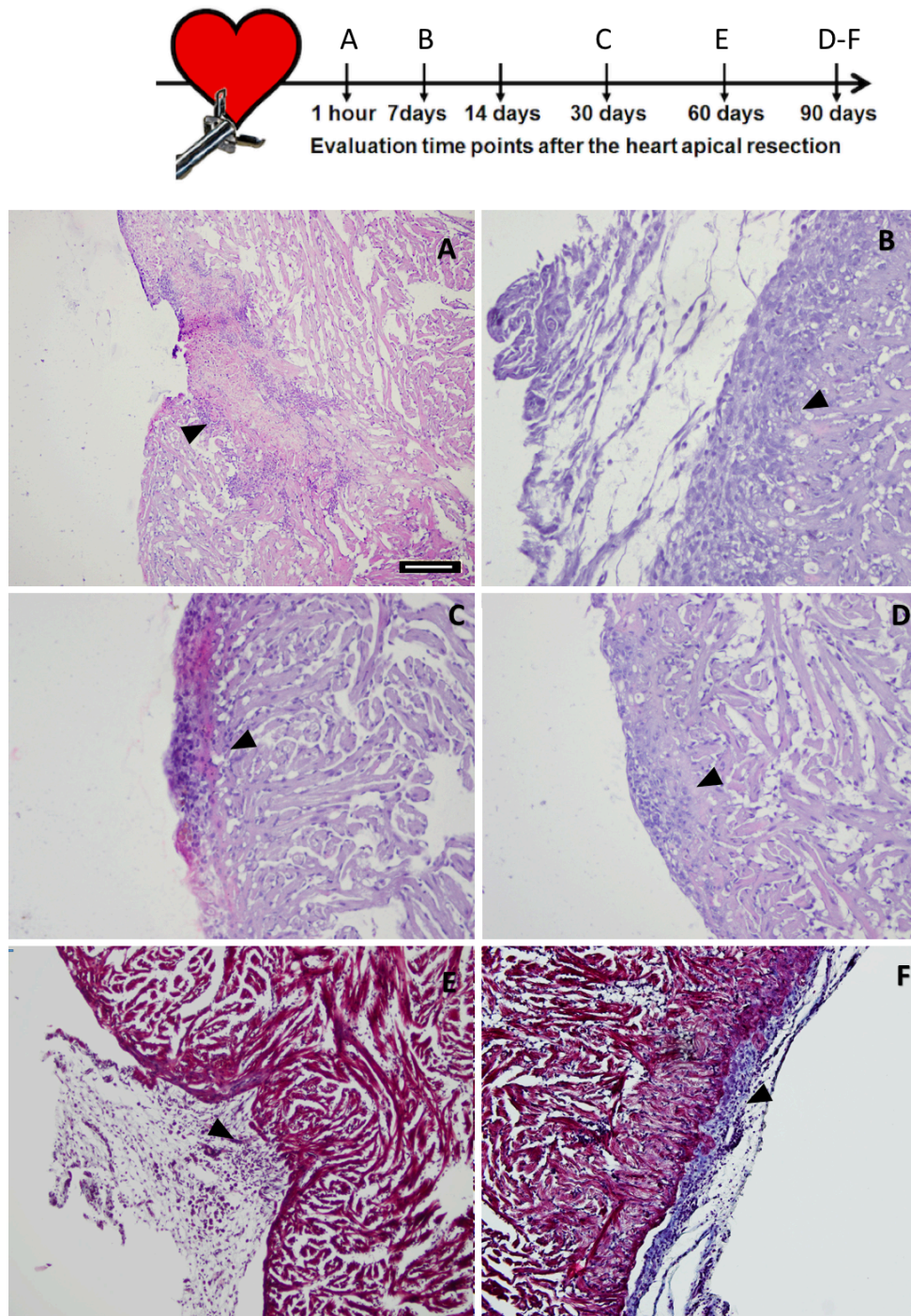


Figure 6. Images of Nile tilapia myocardium stained with hematoxylin-eosin (HE) or Masson's trichrome (MT), at different moments after apex amputation. (A) Remote myocardium stained with HE at 1 h after apex amputation. Note the intense inflammation area (arrow). (B) Remote myocardium stained with HE at 7 days after apex amputation. Note the fibroblasts hyperplasia and conjunctive tissue organization (arrow). (C) Remote myocardium stained with HE at 30 days after apex amputation. Note a necrosis area with pycnotic nuclei (arrow). (D) Remote myocardium stained with HE at 90 days after apex amputation. Note the connective tissue in a higher organization level. (E) Remote myocardium stained with MT at 60 days after apex amputation. Note an area with a moderate quantity of connective tissue (arrow). (F) Remote myocardium stained with MT at 90 days after apex amputation. Note connective tissue more organized (arrow).

Fig. **7A–G** shows the general myocardial microstructure appearance of the studied ventricles. The stereological examination of these ventricles showed that the cardiomyocytes proximal to the amputation site hypertrophied. A gradual increase in the cross-sectional areas of the cardiomyocytes was observed up to day 7 post-amputation (**Fig. 7H**).

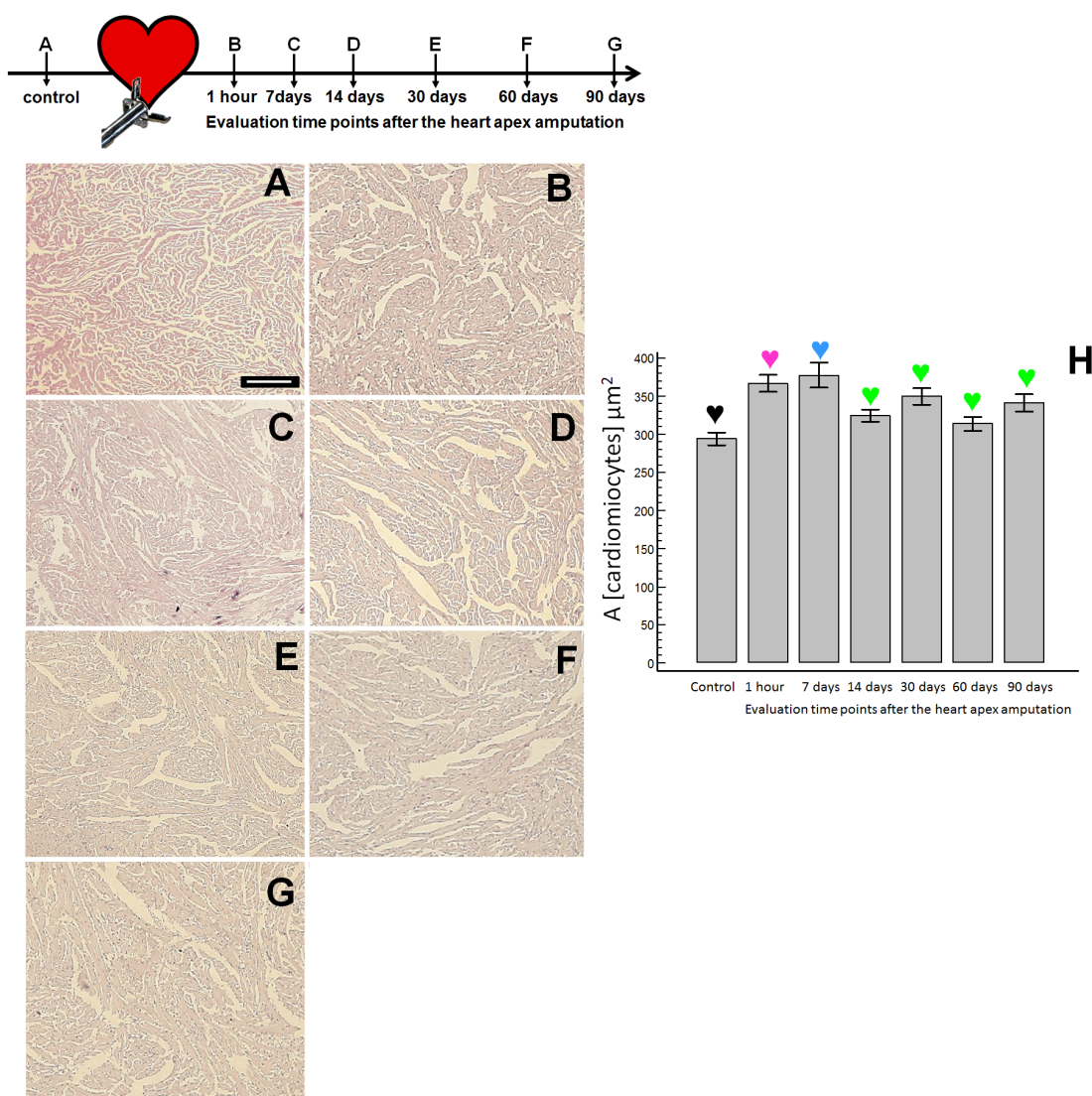


Figure 7. Photomicrographs of sagittal views from hearts of Nile tilapias showing an increase of cardiomyocytes areas throughout the evaluation moments, and a decrease in cellular interstitial area. (A) Control, (B) 1 h after apex amputation, (C) 7 days after apex amputation, (D) 14 days after apex amputation, (E) 30 days after apex amputation, (F) 60 days after apex amputation, and (G) 90 days after apex amputation. (H) Transversal areas quantified for cardiomyocytes from all research groups. Different hearts means statistical differences ($P < 0.05$).

Heart apex amputation caused interstitial collagen remodeling

Fig. 8A–G corresponds to images of ventricles stained with ponceau SS and visualized under polarized light microscopy. Interstitial collagen fibers are seen as thin, bright structures (golden), oriented parallel or perpendicular to the long axis (sagittal cut) of the thick cardiac fibers (red). Interstitial collagen fibers in the injured ventricles, except in the area of fibrotic scar formation, were less bright than the collagen fibers in the control ventricles.

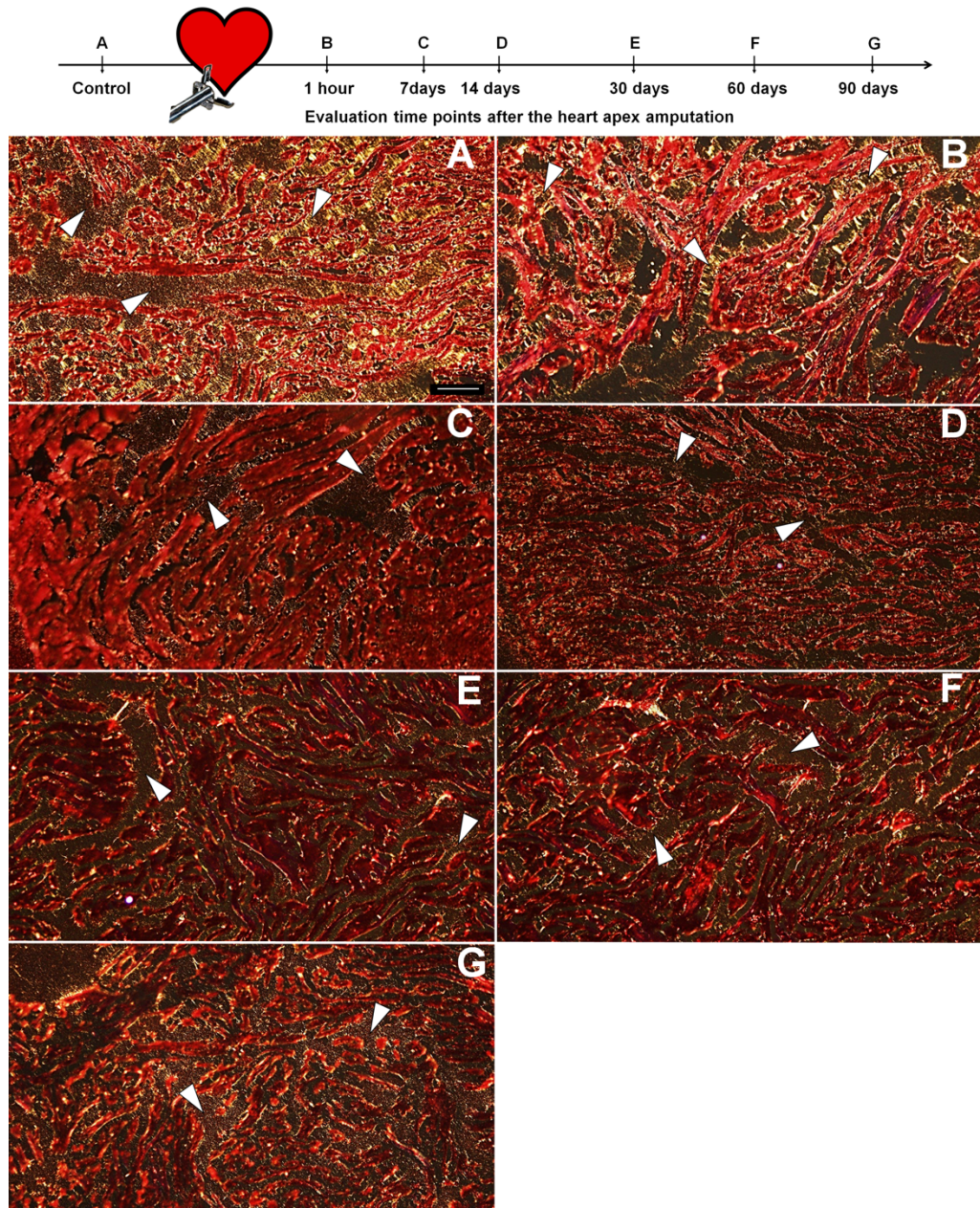


Figure 8. Images of Nile tilapia remote myocardium stained with ponceau SS and visualized under polarized light microscopy. Cardiomyocytes are seen in red, while collagen fibers in gold (arrow). (A) Cardiomyocytes before apex amputation. Note the smaller diameter of cardiac fibers when compared with operated groups, and a better organization of collagen fibers (arrows). (B) Remote myocardium at 1 h after apex amputation. Note cardiac fibers slightly

increased, and a disorganization of collagen fibers was beginning (arrows). (C) Remote myocardium at 7 days after apex amputation. Note a greater disorder of collagen fibers (arrows). (D) Remote myocardium at 14 days after apex amputation, with collagen fibers disorganization still visible (arrows). (E) Remote myocardium at 30 days after apex amputation with collagen fibers disorganization still visible (arrows). (F) Remote myocardium at 60 days after apex amputation with collagen fibers starting to organize (arrows). (G) Remote myocardium at 90 days after apex amputation, with collagen fibers in a more significant organization state (arrows).

The OR values associated with the birefringence brightness of the ventricular myocardial collagen fibers, before and after amputation of the heart apex are presented in **Table 2**. While the histopathological examination of the Masson's trichrome-stained samples revealed a matrix deposition at the amputation site, the qualitative examinations (visual inspection) and the OR measurements performed on the ponceau SS-stained samples revealed that the collagen fibers in the remote myocardium of the injured ventricles were unpacked and spatially disorganized.

Table 2. OPD values for collagen fibers from the remote myocardium of Nile tilapias, before and after heart apical amputation

Samples	OPD (nm)		
	Mean	± SD	Md
Control	1.934 ^a	0.540	1.818
Evaluation time points after the heart apical resection			
1 hour	1.816 ^b	0.536	1.795
7 days	1.366 ^c	0.460	1.212
14 days	1.521 ^d	0.315	1.515
30 days	1.597 ^e	0.372	1.582
60 days	1.727 ^f	0.521	1.690
90 days	1.714 ^f	0.425	1.716

Md, median; ± SD, ± standard deviation; nm, nanometers.

Measurements were made on a quantitative polarized light microscope equipped with 546-nm monochromatic light and Sénarmont compensator.

Differences were significant when $P < 0.05$ (ANOVA with Tukey post hoc test).

Different symbols represent the statistical differences.

Heart apex amputation altered the nuclear geometry, the state of chromatin compaction, and the DNA content of the cardiomyocytes

The results obtained for the video image analysis parameters that report on the nuclear geometry (area and perimeter), the state of chromatin compaction (OD and SDtd), and the DNA content (IOD) of the cardiomyocytes

are shown in **Table 3**. After amputation, the ventricles showed populations of cardiomyocytes with enlarged nuclei, with loose chromatin, with a reduced textural contrast between the areas of condensed chromatin and the less condensed chromatin, and with high DNA content.

Table 3. Results of video image analysis parameters that report on nuclear geometry, chromatin compaction state, and DNA content in cardiomyocytes before and after amputation of the heart apex

Nuclear parameters					
Samples	Area	Perimeter	OD	SDtd	IOD
Control	5.70 (4.53/7.58) ^a	9.81 (8.39/12/60) ^a	1.16 (1.09/1.24) ^a	0.16 (0.11/0.21) ^a	6.90 (5.51/8.61) ^{ad}
1 hour	7.43 (5.31/11.32) ^{bc}	12.13 (9.57/17.43) ^{be}	1.15 (1.09/1.22) ^a	0.14 (0.09/0.18) ^a	8.75 (6.39/13.75) ^b
7 days	6.04 (4.11/9.66) ^a	10.97 (8.50/15.22) ^{cd}	1.20 (1.12/1.27) ^b	0.14 (0.09/0.18) ^b	7.61 (5.04/11.40) ^{ac}
14 days	7.16 (5.23/10.29) ^b	11.62 (9.59/154.12) ^{bc}	1.06 (1.01/1.10) ^c	0.10 (0.08/0.13) ^c	7.64 (5.70/10.70) ^c
30 days	6.28 (4.45/9.04) ^a	11.15 (9.00/14.93) ^{cd}	1.12 (1.08/1.17) ^d	0.08 (0.06/0.11) ^d	7.00 (5.21/9.98) ^{ad}
60 days	5.94 (4.32/8.15) ^a	10.85 (8.75/14.01) ^d	1.11 (1.07/1.16) ^{de}	0.09 (0.07/0.12) ^e	6.80 (4.88/8.95) ^d
90 days	8.01 (5.87/12.54) ^c	12.48 (10.10/19.14) ^e	1.10 (1.05/1.15) ^e	0.09 (0.06/0.12) ^{de}	8.82 (6.74/13.87) ^b

OD, optical density; SDtd, standard deviation of the total densitometric values per nucleus; IOD, integrated optical density.

Data are presented as mean (range).

Differences were significant when $P < 0.05$ (ANOVA with Tukey post hoc test). Different symbols represent the statistical differences.

Relative frequency histograms of IOD values (**Fig. 9**) showed that the injured ventricles have a cardiomyocyte population presenting more than double DNA (IOD > 38 arbitrary units), compared to the diploid control (red blood cells), that is, they were tetraploids. A sort manipulation (Minitab software) of the IOD values allowed us to detect that the frequencies of tetraploid nuclei were 2.4% in 1 h, 1.2% at day 7, 1.4% at day 14, 0.2% at day 30, 0.8% at day 60, and 2.0% at day 90, post-amputation.

All of the study samples, including the diploid controls, had a nuclear population with very low IOD values (< 8 arbitrary units), corresponding to nuclei that lost DNA by cell death or during the acidic hydrolysis of the Feulgen reaction due to a low physicochemical stability.

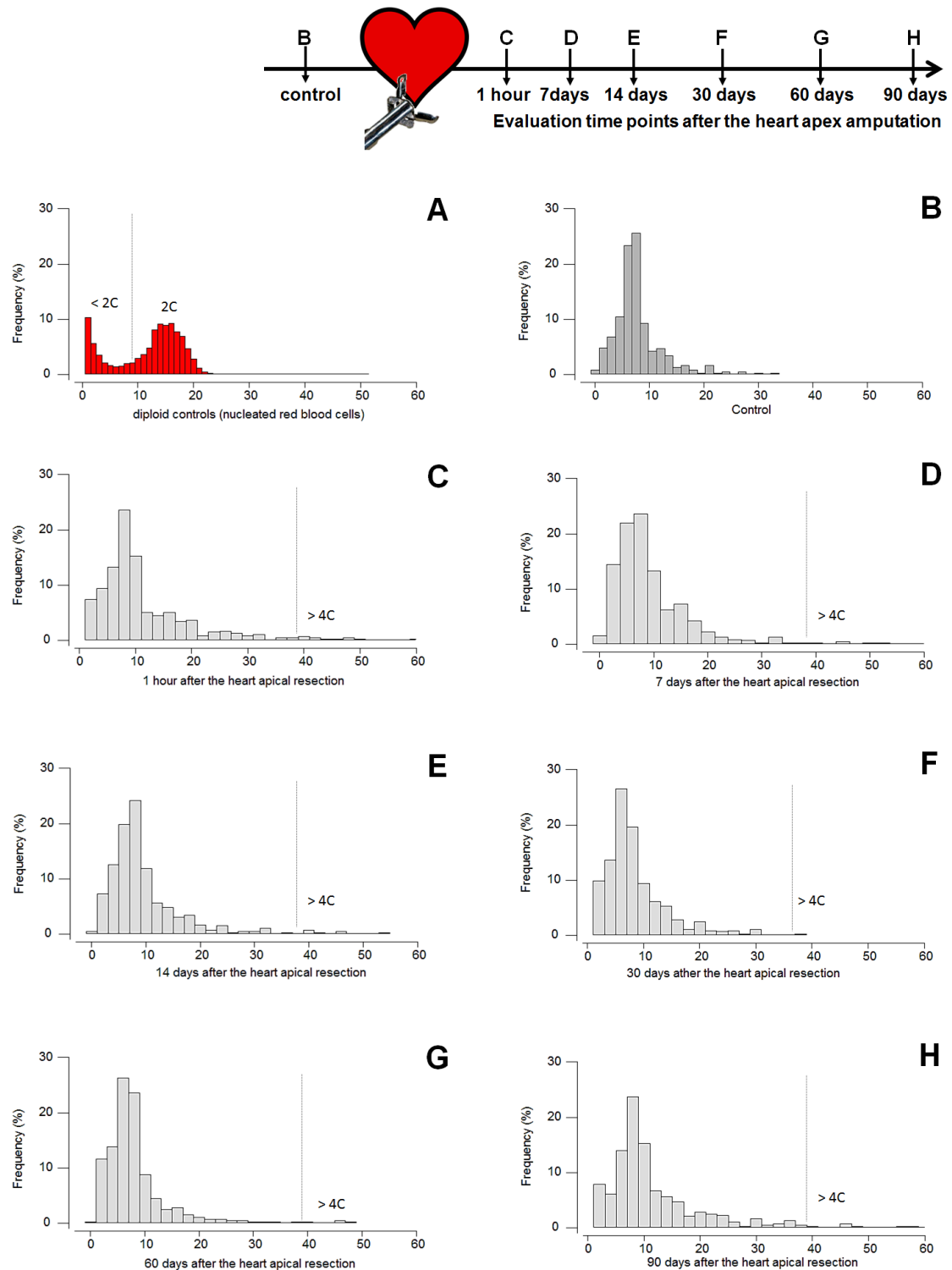


Figure 8. Frequency histograms of Feulgen-DNA (IOD) values, expressed in arbitrary units. (A) diploid controls, constituted by blood red cell nuclei of Nile tilapias. (B) Normal cardiomyocytes of a Nile tilapia. (C) Cardiomyocytes of Nile tilapias at one hour after apex amputation. (D) Cardiomyocytes of Nile tilapias at seven days after apex amputation. (E) Cardiomyocytes of Nile tilapias at 14 days after apex amputation. (F) Cardiomyocytes of Nile tilapias at 30 days after apex amputation. (G) Cardiomyocytes of Nile tilapias at 60 days after apex amputation. (H) Cardiomyocytes of Nile tilapias at 90 days after apex amputation.

Heart apex amputation caused cellular loss by apoptosis in the remote myocardium of Nile tilapia

Apoptotic cardiomyocytes were observed in the remote myocardium of injured ventricles (**Fig. 10**). Apoptosis was intense at day 7 post-amputation (**Fig. 10A, B, C**) and decreased in subsequent periods, almost disappearing at day 90 post-amputation (**Fig. 10D**).

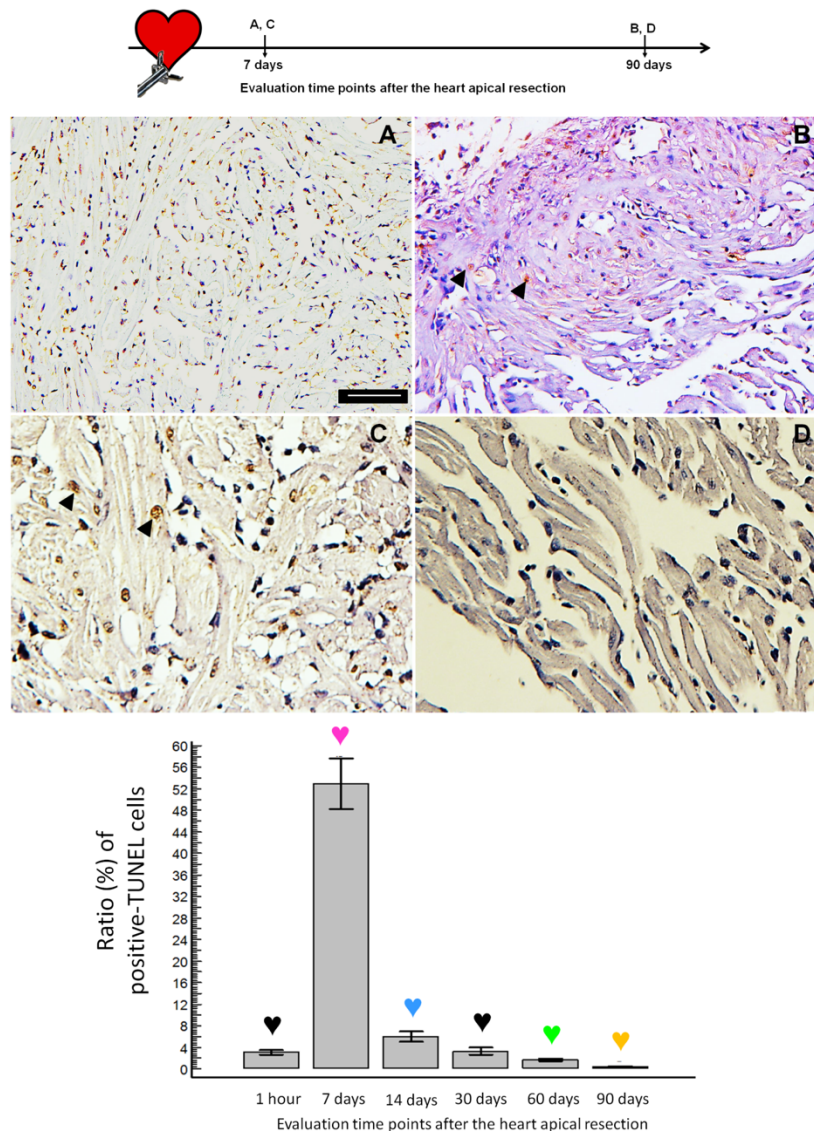


Figure 10. Results of the TUNEL assay for the detection of chromatin fragmentation associated with apoptosis. (A) Remote myocardium tested positively at TUNEL test at the seventh day of evaluation. (B) Remote myocardium at the 90th day of evaluation, with more discrete marking at TUNEL assay (arrow). (C) Image of the lamina stained by TUNEL assay at the seventh day of evaluation, evidencing apoptotic nuclei (arrows). (D) Image of the sample stained by TUNEL assay at the 90th day of evaluation, evidencing lack of marking. Index (%) of apoptotic cells responsive to TUNEL assay, in Nile tilapias myocardium, evaluated at different moments after heart apex amputation. Different hearts means statistical differences ($P < 0.05$).

Discussion

The heart of Nile tilapia is classified as type II, because it presents a pyramidal shape, with a small layer of peripheral compact myocardial tissue in the periphery and a vast central spongy myocardial tissue (MORRISON, 2007; GENTEN *et al.* 2009). The electrophysiology of the tilapias heart resembles that of the mammalian heart, despite anatomical differences between fish and mammalian heart chambers (LIU *et al.*, 2016).

In the present study, the anesthetized tilapias (considering the results of all groups) presented a heart rate average of 41 ± 17 bpm. In the control group, the range was 32 and 58 bpm. These findings show that in contrast to other small-sized species that have an accelerated heart rate, the tilapia has a low heart rate. Thus, Nile tilapias can be attractive models for studies on the basis of human pathological arrhythmias, which, despite the scientific criticism, are currently performed in mice that have a much higher heart rate (427-697 bpm) than humans (60-80 bpm) (LEONG *et al.*, 2010). Zebrafish, another model organism widely used in arrhythmia studies, has a mean heart rate of 118 ± 14 bpm (LIU *et al.*, 2016), but due to the body score it is difficult to conduct ECG tests in this species, and it is necessary to use specific electrodes and microvolts filters or place the ECG electrodes into the pericardial sac.

ECG is a common diagnostic tool for human cardiovascular diseases (LIU *et al.*, 2016). The ECG method used in this study is fast and non-invasive, and although ECG recording showed interference, probably caused by fish scales (LIU *et al.*, 2016), the exam provided reliable waves. We observed that the ECG morphology of tilapia is mimetic to that of humans.

ECG intervals and waves are extremely clinically relevant, as they indirectly report on the functional status of the heart and may indicate the presence of abnormalities (LIU *et al.*, 2016). Similarly to the ECG of the human heart, the ECG of the tilapia heart is composed of P wave, QRS complex, and T wave. The QT interval (measured from the beginning of the QRS complex to the end of the T wave) is considered the more important, as it results from the sum of the depolarization and the repolarization of the ventricular myocardium and informs about the electrical activity.

In the present study, tilapias evaluated by ECG 1 h after amputation of the apex of the heart presented a shortened QT interval, similar to that described in the literature for human hearts with ischemic lesions (POLLEHN, 2002; BRAUNWALD *et al.*, 2004). We believe that the shortening of the QT interval results from an increase in the heart rate to compensate for a possible heart ejection failure caused by the amputation of the heart apex, which together with the formation of a large clot at the amputation site, made myocardial contraction difficult. The fact that QTc (which is QT corrected for heart rate) has remained unchanged 1 h after amputation of the heart apex reinforces the idea that shortening the QT interval is associated with increased heart rate. Although no statistical difference in heart rate was observed between the baseline and 1 h post-amputation evaluations, the boxplot plot charts show that there was a tendency to increase the heart rate in the operated group. Our results differ from those reported for zebrafish that present shortening of the QTc interval in the short-term time (7 days) of the heart apex amputation (SUN *et al.*, 2009; LIU, *et al.*, 2016).

Seven days after amputation of the heart apex, the ECG exams showed a prolonged QT interval, indicating that at this time the heart ejection fraction deficit observed at 1 h was compensated or resolved. Replacement of clot by fibrin and collagen, as seen in the histopathological examination, may also have contributed to the prolongation of the QT interval. Late, that is, 90 days post-amputation, both QT interval and QTc were prolonged, and QRS duration was also increased, indicating a pathological alteration in the heart. It is possible that the scar tissue deposition and myocardial remodeling, secondary to amputation of the heart apex, altered the depolarization of the injured ventricle. The increased duration of the QRS suggests that there were changes in the tissues conducting electrical waves of depolarization.

In this study, the PR interval remained unchanged (vs. basal) in the short-term time (up to day 7) of the heart apex amputation; this finding is similar to that described for zebrafish (SUN *et al.*, 2009) and suggests that there were no changes in atrial depolarization. Interestingly, at days 14 and 60 post-amputation, there was a significant shortening of the PR interval, which may be related to inflammatory processes. Inflammation can delay the propagation of

the electrical pulse of the heart by modifying the duration of the PR interval (ACAR *et al.* 2014)

The most important alteration in atrial depolarization was detected 90 days after the heart apex amputation and was characterized by a prolongation of the PR interval, suggesting a chronicification of the cardiac injury. Changes in the duration of the PR interval do not occur in the zebrafish because, after injury, the myocardium of this species evolves to a regeneration process, which does not modify the ventricular geometry and the conduction of electrical stimuli (POSS *et al.*, 2002; *et al.*, 2009; LIU *et al.*, 2016).

No sign of regeneration was observed in the tilapias of this study. Thus, we investigated signs of hypertrophy, which is an adaptive response to cardiac stress that can lead to heart failure (HILL & OLSON, 2008; XIE *et al.*, 2013). One of the more used methods for studying hypertrophy is stereology, which has been used since the early 1960s (MOUTON, 2005).

To determine whether amputation of the heart apex led to hypertrophy, we measured the cross-sectional area and the volume density of the remaining cardiomyocytes in the injured ventricle. Previously, SOETIKNO *et al.* (2012) also used these stereological parameters to evaluate hypertrophy in the myocardium of diabetic rats. Our results showed that the heart apex amputation leads to a significant increase in the cross-sectional area of the cardiomyocytes, indicating that hypertrophy has occurred and that apparently it was responsible for the increased heart wall thickness detected by SEM.

In the setting of post-amputation hypertrophy, the examinations of the Masson's trichrome-stained samples revealed deposition of collagen-rich connective tissue in the injured ventricle. In addition, the polarized light microscopic analysis of the ponceau SS-stained samples revealed that the collagen fibers in the remote myocardium (not in the lesion area) accumulated changes in their ordered aggregational states, seen as a reduction in the birefringence brightness and in the OR values. Changes in the organization of collagen contribute to myocardial remodeling and, in the present study, were more significant in the initial post-amputation periods. From day 60 post-amputation, an organizational restructuring of the collagen was observed, with OR values very close to those observed in the control. Under conditions that may progress to myocardial failure, it is common to find transient or permanent

changes in interstitial collagen as a result of changes in fibroblast functionality or an increase in the synthesis of metalloproteinases due to oxidative stress (SIWID *et al.*, 2011). Collagen unpacking reduces the resistance of the extracellular matrix to the tensile forces and has been considered one of the factors responsible for the changes seen in myocardial architecture and function of hearts with idiopathic heart disease (WEBER, 1989).

In rats, the pathologic cardiac hypertrophy is associated with reactivation of the cardiac gene program (AANCHAL *et al.*, 2013). Also, previous studies have implicated the SWI/SNF chromatin remodeling enzymes as regulators of the fetal cardiac gene program in surgical models of heart hypertrophy (BARRY *et al.*, 2008; AANCHAL *et al.*, 2013). Therefore, the present study evaluated nuclear geometry, chromatin compaction, and DNA content in ventricular cardiomyocytes after the heart apex amputation. The methodology used in this study to evaluate the nuclei and chromatin of cardiomyocytes is well established in the literature and has contributed to the understanding of changes related to modifications in cell metabolism, gene transcription, epigenetic enzyme activity, and nuclear ploidy (MELLO & VIDAL, 2017).

In this study, amputation of the heart apex was associated with chromatin decompaction ($< OD$ values vs. controls) in cardiomyocytes. Chromatin decompaction is a permissive event to gene transcription and which may be associated with the activation of fetal genes in the myocardial hypertrophic environment of the tilapia. In the presence of hypertrophy, the chromatin in cardiomyocytes of many species remodels to allow silenced genes after birth to restore their expression, including genes encoding atrial natriuretic peptide, brain natriuretic peptide, and fetal isoforms of various contractile proteins and cardiac ion channels (FREY & OLSEN, 2003; BARRY *et al.*, 2008; AANCHAL *et al.*, 2013). The chromatin decompaction that precedes the alterations in gene expression is critical for the promotion of molecular changes that result in myocardial remodeling (FREY & OLSEN, 2003; EI-OSTA, 2011; AANCHAL *et al.*, 2013). The fact that tilapia accumulated changes in cardiomyocyte chromatin after amputation of the heart apex enabled this fish species as a model organism to study the genetic and epigenetic basis of cardiac hypertrophy, as well as to test the effects of epigenetic drugs in the control of the progression of hypertrophy.

Cardiomyocytes studied at different times after amputation of the heart apex had enlarged nuclei and high IOD values (Feulgen-DNA content). This nuclear phenotype is associated with cellular polyploidization and has been observed in hypertrophic hearts of human and rats; appearing to be an adaptive response of cardiomyocytes to changes occurring in the size and shape of the heart (GERDES & ZIMMER, 1994; AHUJA *et al.*, 2007).

Analysis of the frequency histograms of the IOD values confirmed the existence of cell populations with increased ploidy (i.e., tetraploids) in the injured ventricles. The increased ploidy appears to be a mechanism by which a cell directs its energy into protein production, to maintain or increase the metabolic activity. Polyploid cells have been observed in various tissues under a variety of pathological conditions, including cancer and degenerative diseases (PANDIT *et al.*, 2013). Polyploidization results in increased cell size and in genetic diversity, which can provide a structural adaptation to chronic lesions and stress (PANDIT *et al.*, 2013); however, the role of tetraploid cells in hypertrophic hearts is not fully understood. Also, it is not known whether polyploidization represents an advantage or disadvantage for cardiomyocytes (ANATSKAYA & VINOGRADOV, 2007).

In cardiomyocytes, a possible function of polyploidization would be to increase the production of proteins, such as sarcomere proteins. In support of this, there is evidence that polyploid smooth muscle cells increase both mRNA and protein productions (RASLOVA *et al.*, 2003; JONES & RAVID, 2004), raising the transcriptional rate of a gene pool associated with a crucial cellular function (PANDIT *et al.*, 2013). Besides, the contractile function of the heart of cyclin G1-null rats (cyclin G1 is involved in the polyploidization process) is much lower than that observed in infarcted hearts of normal rats, suggesting that polyploidization may contribute to the maintenance of cardiac contractility (LIU *et al.*, 2010).

With respect to the death of cardiomyocytes, which is also involved in the genesis of human heart diseases (Whelan *et al.*, 2010), the fish models of cardiac injury in the present study presented both apoptotic cells and necrotic cells. Both necrotic and apoptotic cells were observed in greater amounts in the initial periods (14 days) post-amputation and were not replaced by viable cardiomyocytes, that is, there was no myocardial regeneration.

All our results show that the heart of Nile tilapia, unlike the heart of zebrafish, does not regenerate after injury. Studies with teleosts without scarce and the only fish whose literature affirms not to regenerate the heart is Medaka (*Oryzias latipes*) (ITO *et al.*, 2014).

In conclusion, Nile tilapias are good model organisms for heart disease. The apex of the heart of this species can be safely injured through endoscopy-guided amputation. Studies in tilapia can help in understanding the genetic, epigenetic, and molecular mechanisms of cardiac remodeling, as well as assist in the elucidation of pathways associated with loss of cardiac regenerative process. In addition, the use of fish in researches facilitates the experimental logistics, since it is possible to house much fish in a small space, facilitating the accomplishment of high-performance experiments (SUN *et al.*, 2009).

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