UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

PETRA KARLA BÖCKELMANN

"ANÁLISE HISTOQUÍMICA, ULTRA-ESTRUTURAL E MORFOMÉTRICA DO EFEITO DE DROGAS ANTI-INFLAMATÓRIAS NÃO ESTERÓIDES (NAPROXENO E INDOMETACINA) SOB A REGENERAÇÃO DA NADADEIRA CAUDAL DE TELEÓSTEO, *Cyprinus carpio* (CARPA)"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) PETRA KARLA BÖCKELMANN e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Histologia.

Orientadora: Profa. Dra. Ivanira José Bechara

Campinas, 2008

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

B349a

Böckelmann, Petra Karla
Análise histoquímica, ultra-estrutural e morfométrica do efeito de drogras anti-inflamatórias não esteróides (naproxeno e indometacina) sob a regeneração da nadadeira caudal de teleósteo, *Cyprinus carpio* (carpa) / Petra Karla Böckelmann. – Campinas, SP: [s.n.], 2008.

Orientadora: Ivanira José Bechara.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Regeneração (Biologia). 2. Nadadeira. 3. Teleósteos. 4. Naproxeno. 5. Indometacina. I. Bechara, Ivanira José. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

(rcdt/ib)

Título em inglês: Histochemical, ultra-structural and morphometric analysis of the effect of nonsteroidal anti-infammatory drugs (naproxen and indomethacin) under the tail fin regeneration of teleostm *Cyprinus carpio* (carp).
Palavras-chave em inglês: Regeneration (Biology); Fin; Teleosts; Naproxen; Indomethacin.
Área de concentração: Histologia.
Titulação: Doutora em Biologia Celular e Estrutural.
Banca examinadora: Ivanira José Bechara, Maria Inês Borella, Maria Tercília Vilela de Azeredo-Oliveira, Maeli Dal Pai Silva, Edson Rosa Pimentel.
Data da defesa: 24/06/2008.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 24 de junho de 2008

BANCA EXAMINADORA

Profa. Dra. Ivanira José Bechara (Orientadora) _____
Assinatura

Profa. Dra. Maria Tercilia Vilela de Azeredo Oliveira _____
Assinatura

Profa. Dra. Maeli Dal Pai Silva _____
Assinatura

Profa. Dra. Maria Inês Borella _____
Assinatura

Prof. Dr. Edson Rosa Pimentel _____
Assinatura

Profa. Dra. Patrícia Pasquali Parise Maltempi _____
Assinatura

Profa. Dra. Maria Alice da Cruz Höfling _____
Assinatura

Profa. Dra. Lucia Elvira Alvares _____
Assinatura

AGRADECIMENTOS

Agradeço, primeiramente, a minha orientadora, Profa. Dra. Ivanira José Bechara, por todos esses anos de orientação, convívio, carinho e respeito. Cada dia que passa tenho ainda mais certeza de que nenhuma outra pessoa poderia ter me orientado de forma tão coerente ao meu jeito de ser. Agradeço também a todos os funcionários do Departamento de Histologia e Embriologia e, também a todos os funcionários da secretaria de Pós-Graduação, principalmente, um agradecimento todo especial enviado com um abraço bem forte a nossa Liliam.

Há tantas pessoas para agradecer que como tenho medo de esquecer alguém, agradecerei sem nomes: Muito Obrigada por todos esses anos, pelo companheirismo, carinho, atenção, amor, enfim... POR TUDO!!!! Cada um de vocês tem um lugar no meu coração PARA SEMPRE!

E, por fim, mas com a maior importância, agradeço a minha família por SEMPRE estar comigo em todos os momentos. Agradeço especialmente aos meus docinhos mais doces: Giulia e Caio, que sem perceber me tornaram uma pessoa MUITO MELHOR. Amo muito minha família e tenho muito orgulho de fazer parte dela. Sou realmente uma pessoa de MUITA SORTE !!!!!!!!!!!!!!!!!!!!!!

OBRIGADA

RESUMO

As nadadeiras caudais de teleósteos, quando parcialmente amputadas, passam por um rápido processo de regeneração chamado de regeneração epimórfica, caracterizado pela formação de uma massa de células indiferenciadas, diferenciação dessas células, síntese e deposição de matriz extracelular e restauração morfológica.

A regeneração da nadadeira é extremamente sensível a fatores físicos e químicos externos, tais como variações na temperatura, intensidade da luz, ação de alguns agentes contaminantes ambientais e ação de algumas drogas que podem interferir na capacidade regenerativa das nadadeiras dos peixes teleósteos.

Existem relatos na literatura, que drogas anti-inflamatórias não esteróides, podem interferir de alguma forma na restauração tecidual de diversos organismos, uma vez que inibem a ação da enzima ciclooxigenase e, conseqüentemente, a conversão do ácido araquidônico em prostaglandina, elementos que desempenham funções importantes na proteção celular, crescimento, angiogênese e produção de matriz extracelular.

Em vista disso, este trabalho teve como objetivo avaliar os efeitos de drogas anti-inflamatórias não esteróides, naproxeno e indometacina, durante o processo regenerativo da nadadeira caudal de peixe teleósteo *Cyprinus carpio* (carpa). Para isso, foram montados experimentos com cinco grupos: grupo formado com peixes que serviram como controle, grupo formado com peixes que entraram em contato com o naproxeno, na dose de 15,6 mg/L, e três grupos formados por peixes que tiveram contato com a indometacina nas doses 10, 20 e 30 mg/L cada. Os peixes foram anestesiados e suas nadadeiras caudais foram amputadas transversalmente e, após a amputação os peixes foram divididos entre os cinco grupos e permaneceram nos aquários até que a regeneração ocorresse.

Os animais foram anestesiados, sacrificados e as nadadeiras em regeneração foram excisadas e fixadas em intervalos de 1, 2, 4, 5, 6, 8, 10 e 12 dias após a amputação. As amostras foram processadas para permitir uma análise histoquímica, ultra-estrutural e morfométrica das possíveis alterações no processo regenerativo da nadadeira caudal de teleósteo em contato com as drogas em questão.

Os grupos tratados com o naproxeno e a indometacina utilizada na dose de 10 mg/L apresentaram o processo de regeneração de forma semelhante ao grupo controle, ou seja, não afetaram a formação da capa epidermal, a formação do blastema, a diferenciação das células blastemais, bem como a síntese, deposição, organização e mineralização dos componentes da matriz lepidotriquial e a síntese das actinotriquias durante o processo regenerativo da nadadeira caudal. No entanto, os peixes tratados com a indometacina nas doses de 20 e 30 mg/L apresentaram um atraso no processo de regeneração da lepidotriquia e da actinotriquia quando comparados com os peixes do grupo controle.

Estudos mais detalhados sobre os mecanismos de ação das drogas anti-inflamatórias não esteróides e a ação dessas drogas sob a expressão ou a inibição da expressão de alguns genes envolvidos no processo de regeneração da nadadeira caudal de teleósteo talvez possam responder a razão das diferenças de efeitos entre essas duas drogas.

ABSTRACT

The fins of teleosts, when partially amputated, they pass for a quick regenerative process called epimorphic regeneration, characterized by the formation of a mass of undifferentiated cells, by the differentiation of these cells, by the synthesis and the deposition of the extracellular matrix and morphological restoration.

The regeneration of the fin is extremely sensitive to external physical and chemical factors such as temperature variations, light intensity, the action of some environmental contaminants and the action of some drugs that can interfere in the regenerative capacity of teleost fins.

There are some studies that show that nonsteroids anti-inflammatory drugs can interfere somehow in the tissue restoration of many organisms, as they inhibit the action of ciclooxygenase enzyme and, consequently, the conversion of arachidonic acid in prostaglandins, elements that execute important roles in cell protection, growth, angiogenesis and in the production of extracellular matrix.

In this sense, this study aimed to evaluate the effects of nonsteroid anti-inflammatory drugs, naproxen and indomethacin, during the regenerative process of the teleost tail fin *Cyprinus carpio* (carp). Therefore, experiments were undertaken in five groups: the control group fish, group of fish in touch with naproxen in doses of 15.6 mg/L, and three groups of fish in contact with indomethacin in doses of 10, 20 and 30 mg/L each. The fish were anesthetized and their fins transversally amputated and, after amputations the fish were divided among the five groups described above and were left in the aquaria until the occurrence of regeneration.

The animals were anesthetized, sacrificed and the regenerating fins excised and fixed in intervals of 1, 2, 4, 5, 6, 8, 10 and 12 days after amputation. The samples were processed in order to permit a histochemical, ultra-structural and morphometric analysis of the possible alterations

in the regenerative process of the tail fin of the teleosts in contact with the drugs mentioned above.

The group treated with naproxen and indomethacin in a 10 mg/L dose showed a regenerative process similar to the control group, thus, it did not affect the formation of the epidermal layer, the formation of blastema, and the differentiation of blastemal cells, as well as its synthesis, deposition, organization and mineralization of the lepidotrichial matrix and the synthesis of actinotrichia during the process of regeneration of the tail fin. However, the fish treated with indomethacin in doses of 20 and 30 mg/L presented a delay in the regenerative process of the lepidotrichia and actinotrichia when compared to the control group fish.

Detailed studies about the mechanisms of nonsteroids anti-inflammatory drugs action and the action of these drugs under the expression or inhibition of expression of some genes involved in the teleost tail fin regenerative process could explain more precisely the reason of the differences of effect between these two drugs.

SUMÁRIO

INTRODUÇÃO

1. Modelo Biológico

Por muitos anos os anfíbios foram os organismos escolhidos para o estudo da regeneração em vertebrados. São organismos capazes de regenerar membros, cauda, olhos, nervo óptico, cordão espinhal e uma parte do coração (Brockes et al, 2001; Brockes & Kumar, 2002).

No entanto, o peixe teleósteo também regenera várias estruturas (nadadeira, nervo óptico, coração, cordão espinhal e escamas) (Broussonet, 1786; Morgan, 1901; Bereiter-Hahn & Zylberberg, 1993; Bernhardt et al., 1996, Becker et al., 1997; Poss et al.,2002b), e tem sido freqüentemente utilizado como modelo biológico, não somente para o acompanhamento ambiental da poluição aquática, mas como uma alternativa experimental de vertebrados para estudos comparativos e detalhados do mecanismo de ação de carcinógenos e outras substâncias (Dawe, 1982; Bailey et al., 1984).

Estes animais oferecem algumas vantagens como modelo biológico, por exemplo:

a) Apresentam grande número de descendentes a partir de uma mesma desova;

b) Possibilitam a obtenção de um grande número de exemplares aparentados e a baixo custo, permitindo assim ampliar a amostragem;

c) A dieta pode ser cuidadosamente controlada assim que começam a aceitar ração;

d) Várias vias de administração química podem ser empregadas, como pelo acréscimo à ração, por injeções, ou pela adição à própria água do tanque;

e) A simples estrutura das nadadeiras e a rápida velocidade de regeneração apresentada por elas tornam essas estruturas um modelo biológico adequado para o estudo *in vivo* do crescimento dos tecidos conjuntivos e, também, para o estudo do efeito de substâncias que possam, por ventura, interferir com esse processo regenerativo;

f) Possibilidade de avanços nas descobertas de genes que podem ser aplicados para estudos de mecanismos moleculares.

Em certos tipos de estudos moleculares, os peixes têm oferecido vantagens sobre os roedores, o tradicional modelo biológico, como por exemplo: viabilidade de obtenção de clones genéticos totalmente homozigóticos (Streisinger et al., 1981), a utilização de embriões como modelo, o que permite testar em alta escala diminutas quantidades de substâncias químicas (Hendricks et al., 1980) e, ainda, uma óbvia importância para estudos de toxicologia aquática (Zodrow & Tanguay, 2003).

2. Peixe Teleósteo: A carpa

A carpa (*Cyprinus carpio*) é um peixe ósseo, de água doce, caracterizada por apresentar nadadeiras com raios (Orr, 1986). É integrante da família dos Ciprinídeos e possui escamas grandes, nadadeira dorsal longa e duas barbelas ou bigodes de cada lado da boca conhecidos como barbilhões. Não têm dentes no interior da boca, mas sim no começo da garganta (Azevedo & Frota-Pessoa, 1981).

É um peixe originário da Ásia e foi introduzido em todos os continentes pela piscicultura de consumo. É cultivado tanto nas áreas temperadas como nas áreas subtropicais e tropicais. No Brasil, a carpa foi introduzida por volta de 1904 (Azevedo & Frota-Pessoa, 1981).

É um peixe de crescimento rápido. Sua comida natural é zooplâncton, quando juvenil, e animais de fundo, como minhocas e larvas de insetos, quando adulta. Consome e utiliza bem todos os materiais comestíveis, como a ração apropriada para peixes, como alimento complementar junto da dieta natural. Ela se propaga com bom êxito em águas paradas onde não há outros peixes, especialmente carnívoros (Woynarovich, 1988). Possui uma capacidade excepcional para regenerar várias estruturas, tais como todos os tipos de nadadeiras, cordão espinhal e nervo óptico (Nechiporuk & Keating, 2002).

3. A estrutura da nadadeira dos Peixes Teleósteos

A estrutura das nadadeiras dos peixes teleósteos, de interesse para as pesquisas desde o século retrasado (Krukenberg, 1885; Harrison, 1893), mostra que não há diferenças fundamentais entre as nadadeiras de um mesmo peixe, ou seja, entre as nadadeiras dorsal, peitoral, pélvica, anal e caudal **(Figura 1)**.

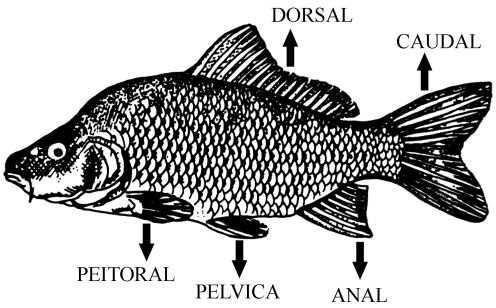


Figura 1: Carpa indicando a localização de suas nadadeiras (Dorsal, Peitoral, Pélvica, Anal e Caudal) (Azevedo & Frota-Pessoa, 1981).

A nadadeira caudal de peixe teleósteo é composta de múltiplos raios esqueléticos e articulados, denominados lepidotriquias, cobertos pela pele (Montes et al., 1982; Becerra et al., 1983; Santamaría & Becerra, 1991; Géraudie & Singer, 1992). Esses raios originam-se da base da nadadeira e se estendem distalmente, ramificando-se dicotomicamente em direção à margem (Arita, 1971), exceto os raios laterais da nadadeira caudal que não sofrem bifurcação (Akimenko et al., 2003) **(Figura 2)**.

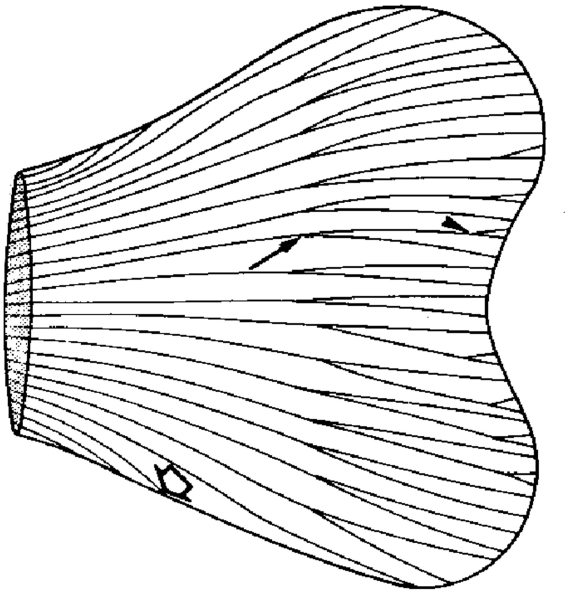


Figura 2: Nadadeira caudal de um peixe teleósteo mostrando os raios lepidotriquiais. A seta grossa indica os raios laterais que não sofrem bifurcação, a seta fina indica o início da bifurcação dos raios e a cabeça de seta mostra os raios ramificando-se em direção à margem.

Cada lepidotriquia é formada por um par de estruturas alongadas e curvas dispostas bilateralmente: os demirraios (ou hemirraios), que são subdivididos longitudinalmente em hemissegmentos lepidotriquiais (ou segmentos lepidotriquiais) que estão conectados entre si através de ligamentos, o que possibilita o movimento articulado da nadadeira (Arita, 1971; Becerra et al., 1983). O crescimento da nadadeira em comprimento ao longo da vida do peixe é feito através da adição distal dos hemissegmentos de cada raio (Haas, 1962) **(Figura 3)**.

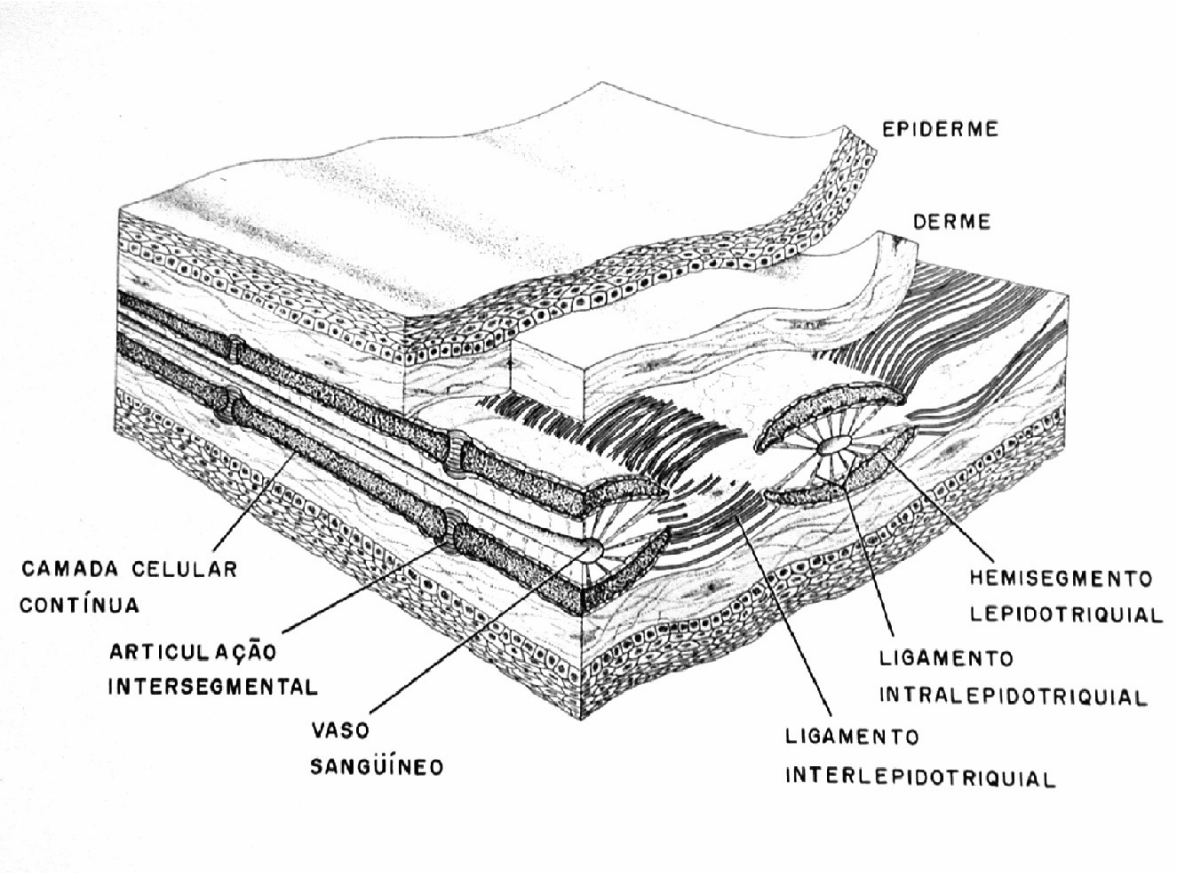


Figura 3: Reconstrução tridimensional da estrutura geral da nadadeira caudal de teleósteo (Becerra et al., 1983).

Os hemissegmentos lepidotriquiais são recobertos por uma camada de células, denominadas escleroblastos, e internamente são preenchidos por uma matriz extracelular contendo fibrilas de colágeno do tipo II envolvidas por uma substância fundamental mineralizada que contém condroitim sulfato (Montes et al., 1982; Marí-Beffa et al., 1996). Sua matriz é rica em glicosaminoglicanos sulfatados, muitos dos quais formam proteoglicanos, e o colágeno abundante interage fortemente com os glicosaminoglicanos e esta interação aumenta progressivamente com a maturação tecidual. A matriz lepidotriquial ainda apresenta, em sua composição, glicoproteínas estruturais ricas em resíduos de manose que têm como função unir os elementos da matriz extracelular entre si e com receptores celulares específicos (Santamaría et

al., 1992). Esses hemissegmentos lepidotriquiais são mantidos no lugar através de ligamentos de colágeno (Becerra et al., 1983).

Estendendo-se da porção distal de cada lepidotriquia em direção à margem da nadadeira encontra-se um aglomerado de espículas pequenas, fusiformes, rígidas e delgadas chamadas actinotriquias, que sustentam a borda da nadadeira (Arita, 1971). A actinotriquia é formada por macrofibrilas hiperpolimerizadas de elastoidina, uma proteína de características semelhante ao colágeno (Krukenberg, 1885) e, diferente da lepidotriquia, a actinotriquia não é mineralizada (Géraudie, 1977) **(Figura 4)**.

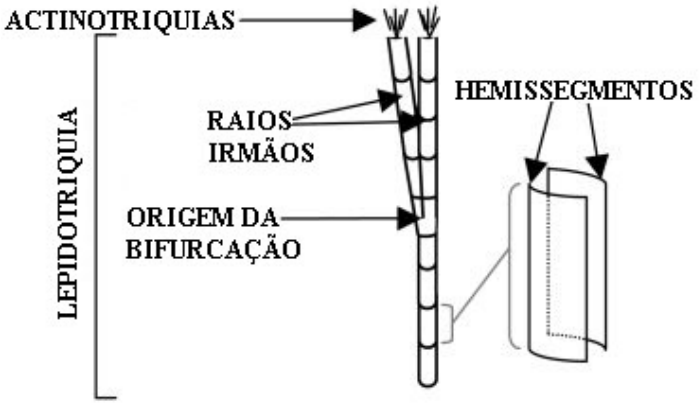


Figura 4: Representação esquemática das unidades esqueléticas da nadadeira caudal de teleósteo (Akimenko et al., 2003).

A vascularização da nadadeira é garantida pelos capilares arteriais presentes entre dois hemissegmentos bilaterais, ou seja, presentes no espaço intra-raio, e pelos capilares venosos que estão localizados fora do raio, em um compartimento presente entre os demirraios chamado de espaço inter-lepidotriquial. Axônios são encontrados, organizados em feixes, tanto no espaço intra-raio como no espaço inter-lepidotriquial e, tendões são observados conectando cada hemissegmento mais proximal dos raios a músculos estriados abdutores e adutores, os quais estão

restritos a base da nadadeira. A contração desses músculos é responsável pelo preciso controle do movimento da nadadeira durante o nado (Akimenko et al., 2003).

Além desses componentes, células pigmentares e fibroblastos estão dispostos no espaço intra-raio tão bem como no espaço inter-lepidotriquial (Johnson & Weston, 1995). Ou seja, tanto a lepidotriquia como a actinotriquia estão envolvidas por um tecido conjuntivo, que contém vasos sanguíneos, nervos, células pigmentares e fibroblastos, e por uma epiderme multiestratificada (Becerra et al., 1983).

4. A regeneração da nadadeira dos Peixes Teleósteos

Quando parcialmente amputadas, as nadadeiras de teleósteos passam por um processo de regeneração chamado de regeneração epimórfica, caracterizado pelas seguintes fases: cicatrização a partir da formação de uma capa epidermal multiestratificada, formação de uma massa de células mesenquimais multipotentes, o blastema, diferenciação dessas células, síntese e deposição de matriz extracelular, crescimento e restauração morfológica (Goss & Stagg, 1957; Santamaría & Becerra, 1991; Géraudie & Singer, 1992; Santos-Ruiz et al., 2002; Akimenko et al., 2003).

Após a amputação, o tecido conjuntivo presente dentro do raio é selado por um coágulo com numerosas células sanguíneas, principalmente leucócitos, e enquanto isso, células da epiderme lateral migram em direção ao plano de amputação para fechar a ferida. Um dia depois da amputação, a ferida já se encontra completamente fechada a partir da formação de uma capa epidermal apical que é separada do tecido conjuntivo adjacente por uma membrana basal. Durante os dois ou três dias seguintes, o tecido conjuntivo presente entre os dois hemissegmentos localizados logo abaixo da capa epidermal sofre uma desorganização e uma desdiferenciação e, células indiferenciadas migram distalmente em direção à capa epidermal, com a finalidade de

formar o blastema, que é invadido por terminações nervosas (Goss & Stagg, 1957; Poleo et al., 2001; Santos-Ruiz et al., 2002).

Durante a formação do blastema, a camada epidermal basal, composta de células cubóides, se dispõe de maneira adjacente ao tecido blastemal, sendo separadas apenas pela presença da membrana basal (Poss et al., 2003).

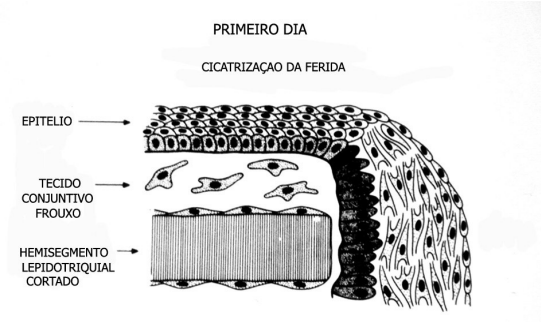
As células que migraram em direção à capa epidermal se proliferam e se acumulam para formar o blastema e algumas dessas células blastemais, de morfologia homogênea, começam sua diferenciação aderindo à membrana basal da epiderme e iniciam a síntese das lepidotriquias. Essas células diferenciadas são conhecidas como escleroblastos. Quando o hemissegmento lepidotriquial em contato com a membrana basal se encontra evidente, alguns escleroblastos migram ao longo da margem lepidotriquial entrando em contato com a membrana basal, habitando um espaço subepidermal, contribuindo, assim, para o crescimento do hemissegmento através da deposição de matriz na outra face da lepidotriquia. Por volta do quinto dia, inicia-se a segmentação e, aproximadamente no sétimo dia, os raios já se bifurcaram. O padrão de segmentação e ramificação varia nos diferentes animais (Kemp & Park, 1970; Santamaría & Becerra, 1991).

O blastema é formado, então, por três regiões: uma área distal em contato direto com a capa apical e que é formado por células que não estão sofrendo proliferação, uma área proximal com células em proliferação e, uma região mais proximal ao local da amputação onde a proliferação é menos intensa e onde há novas células diferenciadas que iniciam a síntese tanto dos componentes da matriz extracelular do raio como dos componentes que fazem parte do tecido conjuntivo intra-raio (Nechiporuk & Keating, 2002; Santos-Ruiz et al., 2002). Como a principal função do blastema é suprir as células necessitadas, durante a regeneração o número de células a serem supridas depende da quantia de tecido perdido. Além disso, a taxa de crescimento do

blastema é correlacionada com a configuração da nadadeira, ou seja, nas nadadeiras caudais, a região do blastema presente no raio marginal da nadadeira cresce mais rápido do que o blastema presente nos raios centrais (Géraudie & Birraux, 2003; Nakatani et al., 2007).

As actinotriquias aparecem pela primeira vez na matriz do tecido conjuntivo adjacente à epiderme, aproximadamente no quinto e sexto dia de regeneração, encontrando-se rodeadas parcial ou totalmente por células semelhantes à fibroblastos. A abundância de retículo endoplasmático granular encontrado nessas células indica uma síntese ativa de proteína enquanto a actinotriquia se desenvolve (Kemp & Park, 1970). O tamanho e a distribuição final do ramalhete de actinotriquias são alcançados quase no décimo dia, mantendo-se em posição distal durante todo o processo (Marí-Beffa et al., 1989; Becerra et al., 1996).

A regeneração total da nadadeira é completada aproximadamente dentro de três semanas (Marí-Beffa et al., 1996; Akimenko et al., 2003) **(Figura 5)**.


PRIMEIRO DIA
CICATRIZAÇAO DA FERIDA
EPITELIO
TECIDO CONJUNTIVO FROUXO
HEMISEGMENTO LEPIDOTRIQUIAL CORTADO


SEGUNDO DIA
ESTABELECIMENTO DE UMA CAPA EPIDERMICA APICAL
E FORMAÇAO INICIAL DO BLASTEMA
EPITELIO
TECIDO CONJUNTIVO FROUXO
HEMISEGMENTO LEPIDOTRIQUIAL CORTADO
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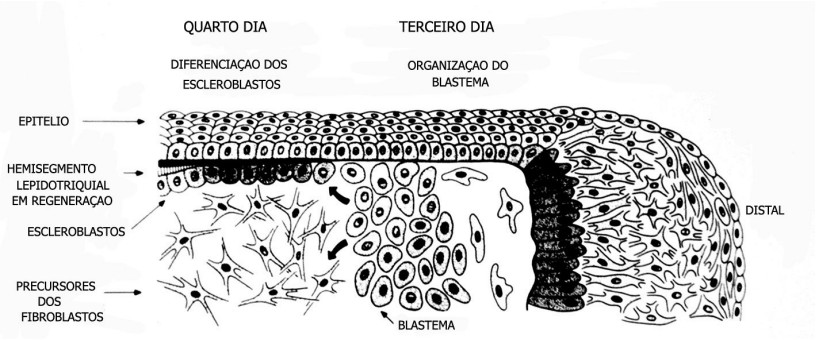
QUARTO DIA
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ORGANIZAÇAO DO BLASTEMA
EPITELIO
HEMISEGMENTO LEPIDOTRIQUIAL EM REGENERAÇAO
ESCLEROBLASTOS
PRECURSORES DOS FIBROBLASTOS
BLASTEMA
DISTAL

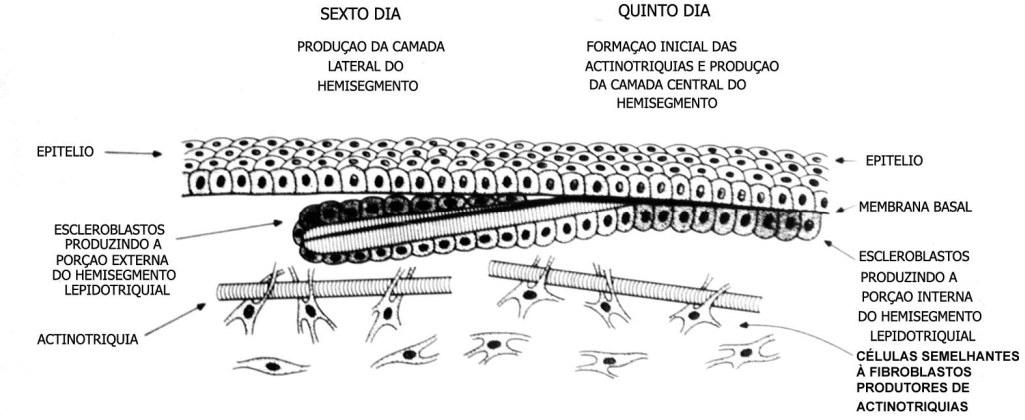

SEXTO DIA
PRODUÇAO DA CAMADA LATERAL DO HEMISEGMENTO
QUINTO DIA
FORMAÇAO INICIAL DAS ACTINOTRIQUIAS E PRODUÇAO DA CAMADA CENTRAL DO HEMISEGMENTO
EPITELIO
ESCLEROBLASTOS PRODUZINDO A PORÇAO EXTERNA DO HEMISEGMENTO LEPIDOTRIQUIAL
ACTINOTRIQUIA
EPITELIO
MEMBRANA BASAL
ESCLEROBLASTOS PRODUZINDO A PORÇAO INTERNA DO HEMISEGMENTO LEPIDOTRIQUIAL
CÉLULAS SEMELHANTES À FIBROBLASTOS PRODUTORES DE ACTINOTRIQUIAS

Figura 5: Diagrama ilustrando a seqüência de eventos envolvidos na regeneração dos raios da nadadeira de teleósteos. De acordo com Becerra et al. (1996), a regeneração ocorre logo após a excisão parcial da nadadeira. Os tecidos, epitelial e conjuntivo, estão envolvidos neste processo, onde o epitélio cobre a extremidade ferida e o tecido conjuntivo subjacente é modificado para restaurar os elementos esqueléticos que estão faltando: lepidotriquia e actinotriquia.

5. Genes envolvidos no Processo de Regeneração

Atualmente, a nadadeira caudal de teleósteo constitui um importante modelo para o estudo das bases moleculares envolvidas na regeneração tecidual. Há uma cascata de genes, que codificam moléculas sinalizadoras e fatores de transcrição, que são induzidos após a amputação ou a injúria da nadadeira, levando à restauração das estruturas perdidas, incluindo aquelas responsáveis pela cicatrização da ferida, ou formação da capa epidermal, formação do blastema, diferenciação das células blastemais, crescimento tecidual e restauração morfológica.

Já no primeiro dia de regeneração, as células da epiderme migram para a região amputada para cobrir rapidamente a ferida. Um dos primeiros marcadores moleculares que aparece logo nas primeiras horas após a amputação, e que é mantido durante todo o processo, é a expressão da β-*catenina* nas células epidermais que estão cobrindo o local da amputação, formando a capa epidermal (Poss et al., 2000a). Pressupõe-se que o aumento da expressão desse gene está relacionado com a função em manter a interação célula-célula para facilitar a migração das células epidermais e para realizar a manutenção da epiderme (Poss et al., 2003).

Suspeita-se que a capa epidermal madura seja a fonte de fatores de crescimento que estimulam a formação e a manutenção da função do blastema em regeneração, uma vez que na ausência da capa epidermal não há regeneração (Goss, 1991). Um dos fatores que pode estimular

a formação do blastema é o gene Wnt5, que é detectado na capa epidermal, principalmente na camada basal da epiderme, nos últimos estágios da regeneração (Poss et al., 2000a).

Poss et al. (2000a) observaram que *lef1,* um membro da via de sinalização Wnt, é expresso na capa epidermal recém formada da nadadeira em regeneração e é mantido na camada basal epidermal durante a formação do blastema. Durante o crescimento do regenerado a expressão de *lef1* é fortemente induzida nas células epidermais adjacentes aos escleroblastos recém alinhados, enquanto que a sua expressão na epiderme adjacente a áreas onde o raio já está maduro ou adjacente a células blastemais proliferativas é baixa ou até mesmo não detectável. Estes resultados sugerem que a expressão de *lef1* está relacionada com o alinhamento dos escleroblastos.

Recentes trabalhos têm mostrado que o blastema é dividido em três compartimentos durante o crescimento da nadadeira em regeneração (Nechiporuk & Keating, 2002): blastema distal com células essencialmente não proliferativas e que expressam *msxb* (genes da família homeobox presentes em vertebrados relacionados com seqüências de "*Drosophila* muscle segment homeobox" - *msh*), um gene que é induzido durante a formação do blastema e que pode funcionar como um ativador da desdiferenciação celular no conjuntivo subjacente a capa epidermal (Akimenko et al., 1995; Odelberg et al., 2000; Poss et al., 2000b), blastema proximal com células altamente proliferativas que expressam *msxc* (Nakatani et al., 2007) e *mps1* (regulador de ciclo celular), este último um gene que é induzido e requerido para estabelecer ou manter a intensa proliferação nesta região e, uma região mais próxima ao local da amputação contendo células que não expressam *msxb* ou *mps1* e onde a proliferação é menos intensa e onde ocorre a diferenciação das células blastemais em escleroblastos e o alinhamento dessas células (Poss et al., 2002a) **(Figura 6)**.

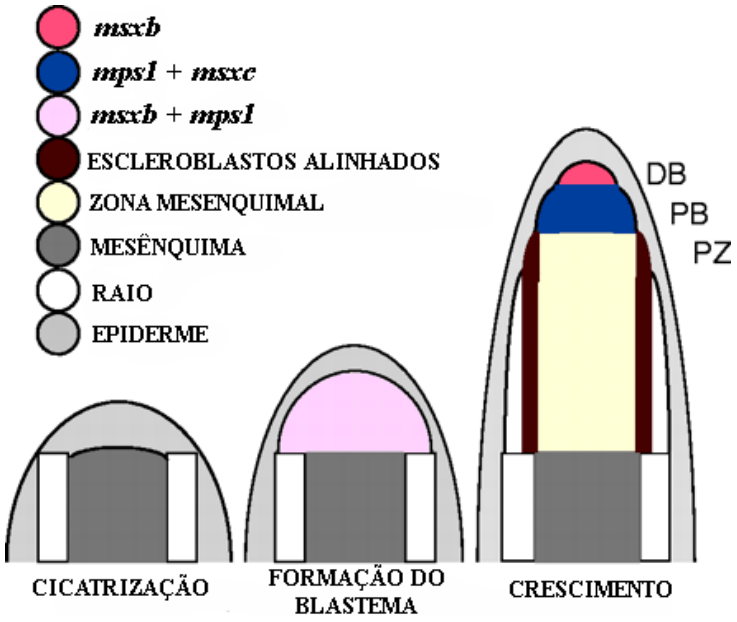


Figura 6: Modelo celular e molecular para a regeneração da nadadeira. Durante o crescimento, o blastema distal (DB) é definido pelo *msxb* (rosa), o blastema proximal (PB) pelo *mps1* e *msxc* (azul) e a zona de restauração (PZ) é definida pelos escleroblastos recém-alinhados (marrom) (Poss et al., 2002a).

Akimenko et al. (1995) estudaram a expressão de quatro genes homeobox da família *msx*: *msxA, msxB, msxC* e *msxD* durante a regeneração da nadadeira e observaram que a expressão desses genes é fortemente reinduzida durante o processo regenerativo. O nível de expressão desses genes aumenta muito durante a formação do blastema e, em seguida, cai progressivamente e desaparece quando a nadadeira volta ao seu tamanho normal. Esses autores observaram também que as células do blastema expressam *msxB* e *msxC*, e que as células da epiderme que reveste a nadadeira expressam *msxA* e *msxD* , no entanto não houve expressão detectável desses quatro genes na região da nadadeira que já sofreu diferenciação.

Nechiporuk & Keating (2002) analisaram a expressão do gene *msxb* nas células blastemais e perceberam que no início do processo de regeneração todas as células do blastema em proliferação expressaram *msxb* e que esta expressão, durante a fase do crescimento

regenerativo, se tornou restrita a um pequeno número de células não proliferativas localizadas na região distal do blastema. Os autores sugerem que a função dessas células blastemais distais não proliferativas e que expressam *msxb* é reprimir a diferenciação através da redução da proliferação celular providenciando, assim, uma direção no crescimento da nadadeira em regeneração.

A via de sinalização Fgf (*Fator de Crescimento dos Fibroblastos*) pode estar diretamente envolvida na indução e manutenção da expressão de *msxb*, como também na proliferação do blastema. Recentes estudos demonstraram que *fgfr1* é expresso nas células blastemais durante a formação do blastema, mas esta expressão se limita à região do blastema mais distal durante o crescimento. Até um breve tratamento com SU5402, um inibidor específico do gene *fgfr1*, anula a expressão de *msxb,* bloqueia a proliferação das células blastemais e inibe a formação do blastema (Poss *et al.,* 2000b), implicando que a expressão de *msxb* é mantida via sinalização Fgf (Nechiporuk & Keating, 2002).

Outro fator de crescimento expresso na capa epidermal é o gene *shh*, que é responsável pela diferenciação, alinhamento e proliferação dos escleroblastos (Poss et al., 2003). Igualmente, durante a regeneração da nadadeira, os genes *hoxa11b* e *hoxa13b* são induzidos em todas as células blastemais não diferenciadas localizadas entre a capa epidermal e o nível de amputação, enquanto que as células do blastema presente entre os raios não expressaram nenhum desses genes durante o estabelecimento do blastema e seu crescimento. Depois, durante a regeneração da lepidotriquia, esses dois genes são expressos nos escleroblastos, e o gene *hoxa13b* parece participar da restauração do raio durante toda a regeneração da nadadeira (Géraudie & Birraux, 2003).

Laforest et al. (1998) analisaram a expressão de genes envolvidos na via de sinalização SHH (*Sonic Hedgehog*) e observaram que *shh*, *ptc1* e *bmp2* são expressos, durante a regeneração da nadadeira, de forma coordenada sugerindo uma função destes genes na interação epiderme-

blastema que leva à síntese e à restauração do raio. *Shh* é expresso exclusivamente nas células da camada basal do compartimento epitelial localizadas na região em contato com que os escleroblastos e *ptc1* e *bmp2* são detectados nas mesmas células que *shh* é expresso, mas também em escleroblastos. Os autores sugerem que a expressão de *shh* pode estar envolvida na indução da diferenciação dos escleroblastos ou na sinalização para os escleroblastos recém-diferenciados iniciarem a síntese da matriz lepidotriquial. Além disso, o ácido retinóico pode regular diretamente a expressão do gene *shh* durante a regeneração da nadadeira, uma vez que o tratamento com o ácido retinóico causou uma inibição da expressão desse gene perturbando altamente o desenvolvimento dos raios da nadadeira. No final do tratamento, a síntese dos raios foi reiniciada e coincidiu com a reativação da expressão de *shh* (Laforest et al., 1998).

Resultados similares foram encontrados por Quint et al. (2002) que investigaram os efeitos da ciclopamina, um alcalóide que bloqueia o sinal de transdução de hedgehog (hh), sob a expressão dos genes sinalizadores *shh* e observaram que houve uma redução seguida de inibição na regeneração da nadadeira, bem como a formação de pouca ou nenhuma actinotriquia e um acúmulo distal de células pigmentares. Esses efeitos foram acompanhados pela redução na proliferação celular dentro do blastema e uma diminuição no tamanho blastemal. As mudanças morfológicas foram acompanhadas pela expansão seguida da redução da expressão de *shh* e pela rápida extinção da expressão de *ptc1*. Além disso, os autores observaram que os raios tratados com ciclopamina raramente se bifurcam, o que pode ser devido à ruptura da expressão de *shh*, já que a bifurcação dos raios (menos dos raios laterais que não sofrem bifurcação) é sinalizada pela duplicação do domínio único de *shh* e a expressão de *ptc1* nestes dois domínios.

Tabela 1. Expressão dos Genes durante os vários estágios da regeneração da nadadeira caudal em teleósteo (zebrafish) (Poss et al., 2003)

Domínio	Gene e Referência°
Cicatrização da ferida Capa epidermal	*apoE*[1]; β-*catenin*[2]; *lef1*[2]; *wnt5*[2]
Formação do blastema Capa epidermal Camada basal da epiderme Formação do blastema Diferenciação dos escleroblastos	*apoE; β-catenin; msxa*[3]; *msxd*[3] *apoE; bmp2b*[4]; *lef1; shh*[4]; *ptc1*[4]; *wnt5* *bmp4*[5]; *eve1*[6]; *evx1*[7]; *evx2*[6]; *fgfr1*[8]; *hoxd11*[5]; *hoxd12*[5]; *msxb*[38]; *msxc*[38], *RAR-*γ[9] *bmp2b; ptc1; evx1*
Crescimento regenerativo Capa epidermal Camada basal da epiderme Blastema Escleroblastos	*apoE; β-catenin; msxa; msxd; wfgf*[8] *apoE; bmp2b; lef1; fgfr1; ptc1; shh; wnt3a*[2]; *wnt5* *bmp4; eve1; evx2; fgfr1; hoxd11; hoxd12; lef1; msxb; msxc; RAR-*γ *bmp2b; evx1; ptc1*

°**References: [1] Monnot et al.,(1999); [2] Poss et al.,(2000a); [3] Akimenko et al.,(1995); [4] Laforest et al.,(1998); [5] Geraudie et al.,(1998); [6] Brulfert et al.,(1998); [7] Borday et al.,(2001); [8] Poss et al.,(2000b); [9] White et al.,(1994).**

6. Fatores que influenciam a Regeneração

O crescimento durante a regeneração depende de fatores externos e internos (Buser & Blanc, 1949). Dentre os fatores internos (bióticos), a atividade da tireóide e da hipófise parece regular a velocidade de crescimento na regeneração (Grassé, 1958). Outro fator interno é a necessidade de um suprimento nervoso adequado para que ocorra a regeneração. Secção nervosa na faixa peitoral em teleósteos no momento da amputação da nadadeira peitoral impede a formação do blastema. Quando esta secção é feita após a formação do blastema, a desnervação bloqueia o crescimento do blastema e altera a síntese de macromoléculas (Géraudie & Singer, 1979a).

Dentre os fatores externos (abióticos), a temperatura tem sido citada como sendo importante para alguns peixes (Johnson & Weston, 1995). Estes sofrem uma diminuição na velocidade de crescimento durante o inverno, que tem um efeito marcante na regeneração da nadadeira. Existe uma temperatura crítica (de 18ºC), abaixo da qual a taxa de regeneração diminui muito. No entanto, de acordo com Nechiporuk & Keating (2002) a velocidade de regeneração aumenta na temperatura de 33°C. Uma evidência disso é que o blastema é formado 24 horas após a excisão. Além da temperatura, a luz também pode influenciar a taxa de regeneração. Segundo Buser & Blanc (1949), a regeneração ocorreu em baixas temperaturas quando o aquário foi fortemente iluminado. Entretanto, em temperaturas elevadas a regeneração ocorreu mesmo na escuridão total. Portanto, se a ação da temperatura é primordial, a da iluminação é apenas subsidiária à osteogênese. Pode-se precisar que é mais a intensidade do que a duração da luz que intervém neste caso (Bertin, 1958).

A regeneração da nadadeira é extremamente sensível à ação de agentes físicos e químicos externos. O metil-mercúrio, o óleo combustível, o TCDD (2,3,7,8-Tetraclorodibenzeno-p-dioxina) e outros possíveis agentes contaminantes ambientais retardam ou até inibem a regeneração (Fingerman, 1980; Zodrow & Tanguay, 2003), enquanto que o cádmio e o zinco interatuam antagonicamente com os anteriores, estimulando o crescimento (Weis & Weis, 1980). Certas drogas como, por exemplo, o beta-aminopropionitrilo, a penicilamina, a dexametasona, e o ácido acetilsalicílico inibem a síntese de colágeno e, conseqüentemente, a regeneração (Bechara et al., 2000).

7. Drogas Anti-inflamatórias Não Esteróides

Drogas anti-inflamatórias não esteróides (AINEs) são comumente prescritas para condições doloridas e inflamatórias, tais como injúrias traumáticas, artrites e dismenorréia (Davies et al., 1984; Dimer et al., 1996; Goodman, 2000; Urban, 2000).

O modo primário da ação dessas AINEs é a inibição da atividade da enzima ciclooxigenase (COX), a qual é responsável pela conversão do ácido araquidônico em prostaglandinas, substância essencial ao processo inflamatório (Samuelsson, 1974; Goodman & Gilman, 1985).

Há três isoformas da ciclooxigenase: a enzima ciclooxigenase-1 (COX-1), considerada uma enzima constitutiva, expressa em praticamente todos os tecidos, e que é responsável pela produção de prostaglandinas envolvidas nas funções fisiológicas basais, tais como citoproteção gástrica e vascular e homeostase renal; a enzima ciclooxigenase-2 (COX-2) que quando expressa induzidamente produz prostaglandinas envolvidas em condições patológicas, tais como nos momentos de dano tecidual e inflamação e, auxilia na diferenciação celular, mitogênese e na transdução de sinais especializados; e, a enzima ciclooxigenase-3 (COX-3), recentemente descrita como uma variante da COX-1 e que é extremamente abundante no córtex cerebral e no coração (Jouzeau et al., 1997; Willoughby & Tomlinson, 1999; Paquette & Willians, 2000; Steinmeyer, 2000; Chandrasekharan et al., 2002; Goodman et al., 2002;).

Existem AINEs convencionais ou não específicas, que são inibidoras das duas isoformas da enzima ciclooxigenase (COX-1 e 2), e as específicas, que inibem apenas a ação da enzima ciclooxigenase 2 (COX-2) (Vane & Dotting, 1997; Lipsky et al. , 2000; FitzGerald & Patrono, 2001).

A inibição direta da enzima ciclooxigenase (COX) bloqueia a produção de prostaglandinas em sítios inflamatórios, reduzindo o inchaço, a dor e a febre (Vane & Dotting, 1995). No entanto, COX em vários órgãos quando inibida, causa efeitos colaterais indesejáveis como dano gastrointestinal (Garcia Rodriguez, 1997), falha renal (Clive & Stoff, 1984) e inibição da agregação plaquetária (Freed et al., 1994), o que limita o uso a longo prazo dessas drogas anti-inflamatórias não esteróides.

8. Naproxeno

O naproxeno é uma droga anti-inflamatória não esteróide e não específica derivada do ácido propiônico, que apresenta três principais tipos de efeito: efeito antipirético (redução da temperatura elevada), efeito analgésico (redução de certos tipos de dor) e efeito anti-inflamatório (modificação da reação inflamatória) moderado. Em geral, todos estes efeitos estão relacionados à ação primária da droga – inibição da ciclooxigenase do ácido araquidônico e, portanto, inibição da produção de prostaglandinas e tromboxanos (Rang & Dale, 1993), importantes mediadores de processos inflamatórios (Vane, 1971).

É uma droga comumente usada no tratamento clínico de doenças articulares degenerativas (Ratcliffe et al., 1993) como a osteoartrite, a artrite reumatóide, gota e reumatismo das partes moles (Rang & Dale, 1993), reduzindo a inflamação e proporcionando um alívio sintomático, agindo como anti-inflamatório e analgésico (Glazer, 1993). Também é indicado para doenças infecciosas, para usos ginecológicos, usos cirúrgicos e traumáticos, enxaqueca e dor de cabeça e para doenças periarticulares e musculoesqueléticas.

O naproxeno é um sólido cristalino branco, inodoro, muito solúvel em água. No homem, o naproxeno é rápido e completamente absorvido no tubo gastrointestinal após administração oral e, juntamente com seus metabólitos, são essencialmente excretados por via renal (Goodman & Gilman, 1983).

Quando o naproxeno é usado em doenças articulares, o que geralmente exige doses muito grandes e uso prolongado, há elevada incidência de efeitos colaterais, principalmente no trato gastrointestinal, mas também no fígado, rim, baço, sangue e medula óssea (Rang & Dale, 1993).

De acordo com relatos na literatura, o naproxeno quando administrado pode ou não exercer um efeito antiproliferativo. Segundo Srinivas et al. (1994), a síntese de colágeno do tipo II em cultura de condrossarcomas de ratos é inibida na administração de alta concentração de

naproxeno (50 μg/mL ou mais), mas não age na síntese de glicosaminoglicanos, contrastando com efeitos inibitórios registrados em outras culturas de condrócitos. Já segundo Ratcliffe et al. (1993), a administração oral de naproxeno na dose de 40-50 μg/mL em cães não afetou a composição da cartilagem articular, ou seja, não houve mudança na taxa de água, colágeno e proteoglicano. No entanto, oprimiu o catabolismo do proteoglicano, diminuiu a atividade da metaloproteinase, sugerindo a capacidade desta droga em modular a atividade metabólica em tecidos com osteoartrite.

Arumugham & Bose (1981) observaram que o naproxeno administrado na dose de 25 mg/kg em ratos albinos com artrite acelera a síntese do colágeno e a conversão do colágeno solúvel para o insolúvel, acompanhado pela inibição do catabolismo do colágeno, porém, inibe a biossíntese de glicosaminoglicanos sulfatados em ratos albinos normais (Arumugham & Bose, 1982).

Solheim et al. (1986) estudaram o efeito do naproxeno na síntese e mineralização do colágeno em fêmur de ratos e, na dose de 20 mg/kg/12h, apenas a deposição mineral foi reduzida. No processo de cicatrização de feridas, o naproxeno, administrado em ratos na dose de 10 mg/kg^{-1}, diminuiu significantemente a deposição de colágeno (Muscará et al., 2000).

O naproxeno na dose de 100 μg/mL inibiu potencialmente a proliferação de células de tendão humano (tendão de patela e tendão de flexor digital) e inibiu a síntese de glicosaminoglicanas *in vitro*, indicando que essa dose farmacológica tem um efeito deletério no metabolismo da matriz do tendão e nos processos reparadores (Riley et al., 2001).

Smith et al. (1995) utilizaram naproxeno na dose de 10, 30 e 90 μg/mL em condrócitos de cartilagem articular de humano adulto *in vitro* e constataram que a droga, nestas concentrações, não afeta a síntese de colágeno. Já em culturas secundárias de condrócitos de coelhos, o

naproxeno inibiu a síntese de glicosaminoglicanos em altas, mas não em baixas concentrações (Bjelle & Eronen, 1991).

De acordo com Sadowski & Steinmeyer (2001), o naproxeno não só age contra os sintomas das doenças articulares, como também interfere na atividade e na expressão da metaloproteinase. Os autores observaram que condrócitos da cartilagem articular bovina, quando incubados com naproxeno na concentração de 10 μM, sofreram uma redução significativa na atividade da metaloproteinase-1 (MMP-1:colagenase, responsável pela quebra do colágeno tipo II) e/ou na expressão do RNA mensageiro de MMP-1. No entanto, não reduziu o efeito da atividade da metaloproteinase-3 (MMP-3:estromelisina, responsável pela degradação da agrecana) e nem interferiu na expressão do mRNA e nem na síntese da proteína MMP-3, além disso,o naproxeno não inverteu os efeitos inibitórios de interleucina-1 (IL-1) na expressão de TIMP-1 (inibidor tecidual de metaloproteinase), aumentando a expressão e atividade das MMPs na presença de IL-1, destruindo a cartilagem articular durante a osteoartrite e a artrite reumatóide.

Aybar et al. (2004) observaram o efeito do naproxeno em células fibroblásticas, *in vitro*. Essas células foram empregadas sob dois materiais de barreira de regeneração óssea guiada e verificaram que a droga teve um papel na inibição da viabilidade celular, no número de células e na síntese dessas células fibroblásticas. No entanto, o naproxeno não teve um efeito significante na síntese de glicosaminoglicanos e de proteínas pelos condrócitos em cultura de cartilagem articular normal de cão (Brandt & Albrecht, 1990). Igualmente, não prejudicou a cicatrização e nem as propriedades mecânicas quando utilizado após transecção do ligamento colateral medial do joelho de ratos (Hanson et al., 2005).

Goodman et al. (2002) analisaram o efeito do naproxeno sob o crescimento ósseo e a diferenciação tecidual em uma câmara de titânio implantada na tíbia de coelho e constataram que a droga reprimiu a formação óssea.

Estudos experimentais utilizando naproxeno durante a regeneração do músculo gastrocnêmio de ratos (Thorsson et al., 1998) mostraram que a proliferação de células satélites e fibroblastos não foi afetada pelo tratamento e que não houve diferença significativa na produção de miotubos e capilares entre animais controle e tratados. Entretanto, Mendias et al. (2004) estudaram a ação do naproxeno em cultura de células satélites musculares esqueléticas isoladas de ratos e constataram que a droga, na dose utilizada, diminuiu a diferenciação e a fusão dessas células e, conseqüentemente, afetou o processo de regeneração muscular.

Böckelmann & Bechara (2004) estudaram o efeito do naproxeno, na dose de 10,4 mg/L, na matriz da lepidotriquia da nadadeira caudal da carpa e não observaram alterações na síntese, deposição, organização e mineralização dos componentes da matriz extracelular. Esses mesmos autores verificaram anteriormente que a dose de 20,8 mg/L do naproxeno foi letal para todos os peixes.

9. Indometacina

A indometacina é uma droga anti-inflamatória não esteróide e não específica que, como o naproxeno, apresenta três tipos de efeito: efeito analgésico, efeito antipirético e efeito anti-inflamatório (Goodman & Gilman, 1983). É um inibidor potente da ciclooxigenase e, portanto, um potente bloqueador da síntese de prostaglandinas (Vane, 1971). Também inibe a motilidade dos leucócitos polimorfonucleares e tem a capacidade de deprimir a biossíntese dos glicosaminoglicanos (Goodman & Gilman, 1983).

A indometacina é absorvida rápida e completamente pelo trato gastrointestinal depois da administração oral. Ela se liga às proteínas plasmáticas numa percentagem de 90% e também se liga amplamente aos tecidos, depois é convertida principalmente em metabólitos inativos. Alguns destes metabólitos são detectáveis no plasma, e metabólitos livres e conjugados são eliminados pela urina, bile e fezes (Goodman & Gilman, 1983).

É uma droga utilizada no tratamento de doenças como a osteoartrite, a artrite reumatóide (Sadowski & Steinmeyer, 2001), gota aguda, cancêr, na prevenção de doenças cardiovasculares e de doenças ginecológicas (Pai et al., 2001) e também utilizada no alívio da dor após danos ou cirurgia em tendões e ligamentos (Riley et al., 2001).

Quando utilizada em doses altas e/ou em tempo prolongado, a indometacina pode apresentar efeitos colaterais tais como: anorexia, náusea, dor abdominal, diarréia, cefaléia, sonolência e vertigem (Goodman & Gilman, 1983). Porém, seu efeito mais adverso está relacionado com danos e ulcerações na mucosa gastroduodenal (Mohajer et al., 2002), freqüentemente associadas com perfuração e sangramento gastrointestinal (Pai et al., 2001).

Estudos *in vivo* mostram que a indometacina e outras drogas anti-inflamatórias não esteróides interferem com a cicatrização de feridas teciduais (pele e osso, por exemplo) (Haws et al., 1996; Quirinia & Viidik, 1997) e de úlceras gástricas (Tarnawski et al., 1991; Schmassmann, 1998).

Indometacina, nas doses 0,25 e 0,5 mM, quando administrada, *in vitro*, em um modelo de monocamada celular de epitélio gástrico injuriado de rato, inibiu a re-epitelialização da ferida gástrica. Isto ocorreu devido a um desarranjo dos componentes do citoesqueleto, prejudicando, dessa forma, a migração celular (Pai et al., 2001).

Arumugham & Bose (1981) observaram que a indometacina administrada na dose de 6 mg/kg em ratos albinos com artrite acelerou a maturação do colágeno acompanhado pela redução do catabolismo do colágeno solúvel, não alterando a síntese colagênica, porém, inibiu a biossíntese de glicosaminoglicanos sulfatados em ratos albinos normais (Arumugham & Bose, 1982).

A indometacina na concentração de 20 μg/mL inibiu a proliferação de células de tendão humano (tendão de patela e tendão de flexor digital) e inibiu a síntese de glicosaminoglicanos *in vitro*, prejudicando o metabolismo da matriz do tendão e os processos reparadores (Riley et al., 2001).

Collier & Ghosh (1991) observaram que a indometacina na concentração de 0,1μg/mL, quando administrada em cultura de condrócitos de cartilagem articular de coelho, inibe a secreção de proteoglicanos.

Guez et al. (2001) administraram indometacina na dose de 7,5 mg/kg^{-1} em um modelo de remodelação óssea em ratos e verificaram que a droga prejudicou todas as etapas da remodelação óssea (reabsorção e formação óssea). De forma semelhante, locais onde houve extração de molares em ratos e tratados com indometacina na dose de 7,5 mg/kg, apresentaram uma inibição na reabsorção óssea e uma queda no número total de osteoclastos (Leroux & Saffar, 1993).

No entanto, indometacina na dose de 12,5 mg/kg não afetou a atividade do processo de remodelação óssea nos ossos corticais e trabeculares de coelhos adultos (Keller et al., 1990).

Forslund el al. (2003) testaram indometacina na restauração do tendão de Aquiles de ratos e a droga, administrada diariamente nas doses de 1,5, 3 e 5 mg/kg, reduziu a espessura do tendão em regeneração após 14 dias de tratamento, mas não interferiu na resistência e rigidez deste órgão.

Cohen et al. (2006) observaram que a indometacina, quando administrada na dose de 3 mg/kg em ratos, inibiu a cicatrização no local da inserção tendão-osso presente no punho desses animais. Os autores sugerem que essa inibição pode ser devido a algum dano na diferenciação dos osteoblastos inibindo, conseqüentemente, a formação e a maturação do colágeno, e essa

interferência provavelmente é causada pela inibição da produção de prostaglandina por meio do uso da indometacina.

Azoubel et al. (2007) investigaram o efeito da indometacina no tratamento de periodontite experimental em ratos e observaram que os animais tratados diariamente com 5 mg/kg de indometacina apresentaram uma redução na infiltração de células inflamatórias, bem como na perda de osso alveolar e na destruição das fibras de colágeno. No entanto, o uso da indometacina provocou vários danos na mucosa gástrica e intestinal desses animais, sendo provavelmente o fator responsável pela perda de peso corpóreo e a alta mortalidade observada neste estudo.

JUSTIFICATIVA

Alguns vertebrados basais, tais como anfíbios urodelos e peixes teleósteos, possuem uma alta habilidade de regeneração em uma variedade de tecidos e órgãos, enquanto os mamíferos têm uma capacidade regenerativa limitada a alguns casos, como a regeneração do chifre de cervos, a cartilagem da orelha de coelhos, tão bem como a regeneração do fígado, sangue, pele e a ponta dos dedos em infantes (Goss, 1983; Nechiporuk & Keating, 2002; Hata et al., 2007; Yoshizato, 2007). Devido a isso, a evolução dos vertebrados é vista como sendo um processo onde os animais foram perdendo gradualmente a sua habilidade em regenerar partes perdidas ou danificadas. Esta mudança na capacidade regenerativa durante o curso da evolução está intimamente ligada com a conservação ou não de muitos genes e com a expressão e função desses entre as espécies de vertebrados (Nakatani et al., 2007). Portanto, um estudo do mecanismo da regeneração em urodelos e teleósteos pode fornecer uma base de conhecimento essencial para possíveis terapias na regeneração de tecidos e órgãos em mamíferos, incluindo humanos (Masaki & Ide, 2007).

A nadadeira caudal dos peixes teleósteos é um órgão que possui uma estrutura relativamente simples e simétrica com limitados tipos celulares. Além disso, é um órgão de fácil acesso para manipulação e possíveis danos na sua estrutura não comprometem a sobrevivência do animal. Em vista disto, a nadadeira caudal representa um modelo simples de sistema em regeneração que pode ser útil para ilustrar os princípios biológicos envolvidos na regeneração, e, também, para o estudo do efeito de substâncias que possam, por ventura, interferir nesse processo regenerativo.

Sabe-se que a regeneração da nadadeira de peixe teleósteo é extremamente sensível a fatores físicos e químicos externos, tais como variações na temperatura (Johnson & Weston, 1995; Nechiporuk & Keating, 2002), intensidade da luz (Buser & Blanc, 1949), ação de alguns

agentes contaminantes ambientais, como o TCDD (Fingerman, 1980; Zodrow & Tanguay, 2003) e ação de algumas drogas que inibem a síntese de colágeno e a regeneração (Bechara et al., 2000 e 2003).

Além disso, há relatos na literatura (Srinivas et al., 1994; Muscará et al., 2000; Pai et al., 2001; Cohen et al., 2006), que as drogas anti-inflamatórias não esteróides, naproxeno e indometacina, podem interferir de alguma maneira na restauração tecidual de diversos organismos, uma vez que inibem a ação da enzima ciclooxigenase e, conseqüentemente, a conversão do ácido araquidônico em prostaglandina, elementos que desempenham funções importantes na proteção celular, crescimento, angiogênese e produção de matriz extracelular (Savla et al., 2001). Verificou-se, também, que a aspirina, uma droga anti-inflamatória não esteróide, inibiu a regeneração dos elementos esqueléticos e das nadadeiras caudais da carpa como um todo (Bechara et al., 2000; Bechara et al., 2003).

Em vista de todas as considerações acima, o estudo do processo regenerativo da nadadeira, juntamente com a utilização das drogas naproxeno e indometacina traz, não somente uma contribuição para uma melhor compreensão dos processos regenerativos dos tecidos conjuntivos, como também, uma contribuição para o conhecimento dos mecanismos de ação das drogas anti-inflamatórias não esteróides, tão intensamente utilizadas no tratamento de inúmeras doenças.

OBJETIVOS

O objetivo geral deste trabalho foi o de avaliar os efeitos de drogas anti-inflamatórias não esteróides, naproxeno e indometacina, sobre o processo de regeneração das nadadeiras caudais das carpas. Para isso os objetivos específicos foram:

- Análise das possíveis alterações no processo regenerativo da nadadeira caudal de teleósteo, levando em conta a síntese, deposição e organização dos componentes da matriz extracelular lepidotriquial, a síntese da actinotriquia, bem como a regeneração da epiderme e do tecido conjuntivo presente na estrutura da nadadeira. As possíveis alterações foram avaliadas pelos métodos histoquímico e ultra-estrutural;

- Análise da área em regeneração da nadadeira como um todo, incluindo lepidotriquia, actinotriquia, epiderme e tecido conjuntivo e, análise da espessura da lepidotriquia em regeneração. Para isso foram feitas análises morfométricas.

ARTIGO SUBMETIDO À REVISTA "MICROSCOPY RESEARCH AND TECHNIQUE"

"Influence of Indomethacin on regenerative process of the tail fin of Teleost"

Petra Karla Böckelmann *; Ivanira José Bechara *

* Department of Histology and Embryology, Institute of Biology, State University of Campinas (UNICAMP), Campinas (SP), Brazil.

Mailing address: Prof. Dr. Ivanira José Bechara, Laboratório de Histofisiologia e Histopatologia Experimental em Animais Ectotérmicos, Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, SP, Brasil. Tel: (55) (19) 3521-6245, Fax: (55) (19) 3289-3124, E-mail: ibechara@unicamp.br.

Running title: Regeneration of the tail fin and indomethacin

Key Words: nonsteroidal anti-inflammatory drug, regeneration, lepidotrichia, actinotrichia, fish

ABSTRACT

When partially amputated or severely injured, teleost fins suffer a regenerative process called epimorphic regeneration characterized by the following stages: the formation of a multistratificated epidermal layer, the disorganization and distal migration of multipotent mesechymal cells, the proliferation of these cells in order to form the blastema, continuous proliferation of distal blastema to facilitate the growth, and differentiation of the proximal blastema in order to restore its lost structure. The regeneration suffered by the fin is extremely sensitive to the action of some drugs that can interfere in its structure restoration. For this reason, and also based on papers relating that indomethacin can interfere somehow in the tissue restoration of many different organisms, the aim of this work is to evaluate the possible effects of this drug in three different doses in the regeneration of the teleost fish tail fin, taking into consideration the synthesis, the deposition and organization of lepidotrichial matrix components, the restoration of actinotrichia, as well as the fin area itself. Therefore, histochemical, ultra-structural and morphometric analysis were done and it was observed that indomethacin in doses of 20 mg/L and 30 mg/L caused a delay in the regenerative process of the dermal skeleton (lepidotrichia and actinotrichia) of the tail fins. These doses could have interfered, momentaneously, in the process of blastemal cell differentiation in the cells responsible for the synthesis and deposition of actinotrichia and lepidotrichia or, probably by interfering of signaling necessary for the recent differentiated cells to synthese the components of the dermal skeleton.

INTRODUCTION

The tail fin of teleost is composed of structural units named rays or lepidotrichia surrounded by a multilayered epidermis (Goodrich, 1904). Each lepidotrichium consists of a pair of concave hemirays formed by multiple segments joined end to end by ligaments (Becerra et al., 1983). The space between two hemirays is filled with connective tissue and contains nerves, blood vessels, pigment cells and fibroblasts. Each ray ends distally with a row of small, rigid, fusiform spicules, known as actinotrichia (Marí-Beffa et al., 1989).

The lepidotrichia are filled with extracellular matrix containing type II collagen fibrils of different orientations surrounded by a mineralized fundamental substance rich in chondroitin sulfate (Montes et al., 1982). The actinotrichia are formed from hyperpolymerized macrofibrils of elastoidin, a protein with characteristics similar to those of collagen (Krukenberg, 1885).

When partially amputated or severely injured, fins are able to complete self-restoration through a process of epimorphic regeneration (Géraudie and Singer, 1992). Fin regeneration is characterized by: formation of a multilayered wound epidermis, disorganization and distal migration of mesenchymal cells proximal to the amputation plane, proliferation of these mesenchymal cells to form the regeneration blastema, continued distal blastemal proliferation to facilitate outgrowth and proximal blastemal differentiation to replace missing structures (Goss and Stagg, 1957; Johnson and Weston, 1995; Poss et al., 2000a, b; Santamaria and Becerra, 1991).

It is known that the regeneration of teleost fin is extremely sensitive to physical and chemical external factors, such as temperature variation (Johnson and Weston, 1995; Nechiporuk and Keating, 2002), light intensity (Buser and Blanc, 1949), the action of some environmental contaminants, such as TCDD (2,3,7,8-Tetrachlorodibenzo-*p*-dioxin) (Fingerman, 1980; Zodrow and Tanguay, 2003) and the action of some drugs that inhibit the synthesis of collagen and the regeneration (Bechara et al., 2003; 2000).

Indomethacin is a nonsteroidal anti-inflammatory drug (NSAID) commonly used in the treatment of diseases such as osteoarthritis, rheumatic arthritis (Sadowski and Steinmeyer, 2001), gout, cancer and in the prevention of cardiovascular diseases and gynaecologic diseases (Pai et al., 2001) and also used to alleviate the pain after damages or surgery in the tendons and ligaments (Riley et al., 2001). It is currently thought that NSAIDs inhibit enzyme cyclooxigenase and thus prostaglandin synthesis, substances integral to the inflammatory process.

In vivo studies have shown that indomethacin and others NSAIDs interfere with healing of tissue wounds (e.g. skin and bone) (Haws et al., 1996; Quirinia and Viidik, 1997) and gastric ulcers (Schmassmann et al., 1998; Tarnawski et al., 1991).

Indomethacin used, *in vitro*, in wounded gastric epithelial cell monolayer model of rat inhibited the re-epithelialization of wounded gastric (Pai et al., 2001). Collier and Ghosh (1991) observed that the same drug administered in rabbit articular chondrocyte cultures, inhibited proteoglycan secretion.

Guez et al. (2001) analysed the action of indomethacin in a model of bone remodelling in rats and showed that the drug affected at various stages of this remodelling sequence, as both bone resorption and formation.

Various studies have shown the influence of indomethacin in the synthesis of the extracellular matrix of connective tissue (Arumugham and Bose, 1982; Riley et al., 2001). Thus, the objective of the present study was to observe the possible effects of this drug on tail fin regeneration of the teleost fish, considering the synthesis, deposition and organization of the lepidotrichial extracellular matrix components, the actinotrichia regeneration and the total area of regenerating fins.

MATERIALS AND METHODS

Four glass aquaria, each containing 10 litres of water, were prepared at the beginning of the experiment. The water was clean and dechlorinated, the temperature maintained at 26ºC, the pH at 8.0 and aeration was constant. Indomethacin (Merck Sharp and Dohme, USA) was dissolved in the water of 3 of the aquaria, where each aquarium received a different concentration, i.e., 10 mg/L, 20 mg/L and 30 mg/L, and in the other aquarium, containing only water, served as the control.

One hundred and sixty carp alevins, *Cyprinus carpio*, obtained from a fish farm, weighing on average 1.4 g and measuring on average 4.7 cm in length, were used in this experiment.

The fishes were divided aleatoryly into 4 glass aquaria (n=40), were anaesthetized with benzocaine (SYNTH, Brazil) (1:10000) and their tail fins were amputated transversally (in the dorso-ventral direction) at a distance of 3 mm from the tail muscle peduncle using a sharp razor, according to Becerra et al. (1996). The fish were left in the aquaria until the occurrence of regeneration. Half the water in each aquarium was replaced daily with fresh clean dechlorinated water and half the doses of indomethacin, was added to the experimental aquaria until the end of the experiment to maintain the initial levels.

The animals were then anaesthetized, sacrificed and the regenerating fins excised and fixed at intervals of 1, 2, 4, 5, 6, 8, 10 and 12 days after amputation, using 5 specimens for each time interval.

For each time interval, fragments of regenerating fins from control and indomethacin treated fish were processed for light microscopy and for transmission electron microscopy.

The collected samples for light microscopy were fixed in Bouin's solution for 6 hours, embedded in paraffin and sectioned in 6 μm thick. Longitudinal and transversal sections were stained with Picrosirius-Hematoxylin and observed and photographed under microscopes (Nikon Eclipse E800, Japan) using conventional light and polarized light.

The regenerating fins, of fishes of four aquaria, collected on the intervals of 4, 6, 8 and 10 days after amputation and processed for conventional light microscopy had theirs images analyzed using an image analyzer (Image Pro-Plus, version 4.1.12, USA). For each fish, obtained of control and treated aquarium, 25 longitudinal fin sections were randomly selected. In addition, the areas of regenerating fins and the widths of regenerating lepidotrichia were measured with a 2x objective lens.

The results were compared statistically trough the mean of the areas and trough the mean of the widths of each time interval (mean ± S.E.M) using one-way analysis of variance (ANOVA) followed by Tukey's mean comparison test. The level of significance was set at 5% ($p<0.05$). The statistical analyses were done using the statistical program INSTAT v 2.01 (GraphPad, San Diego, CA. USA).

For transmission electron microscopy, small fragments of regenerating fins were fixed in Karnovsky for 4 hours at 4°C, washed with a 0.1 M phosphate buffer solution (pH 7.4) containing 7.5% saccharose and subsequently postfixed with 1% osmium solution in 0.2 M phosphate buffer for 1 hour at 4°C. The samples were then washed with glycosated saline, dehydrated with increasing acetone concentrations, pre-embedded in a mixture of acetone and epon (1:1) for 3 hours and embedded in pure epon for 24 hours. The tissue fragments included, longitudinal and transversally to the rays, were polymerized in an incubator at 60ºC for 48 hours. Semi-fine sections (1 μm) were obtained using a LEICA ultramicrotome (Germany) and stained in hot Toluidine Blue. Further ultra-fine sections (60-70 nm) were subsequently obtained, contrasted with uranyl acetate and lead citrate, and examined and micrographed in a transmission electron microscope (LEO 906, Germany).

RESULTS

The control group fish, which were not in touch with the drug (indomethacin) and the fish treated with a dose of 10 mg/L of indomethacin, presented a similar regenerative process of the tail fin.

One day after partial amputation of the tail fin, epidermal cells migrated and completely covered the cut edge, achieving its complete formation on the second day of regeneration (Fig.1A, 1B).

On the fourth day of the regenerative process, it was observed blastema formation, a mass of cells of homogeneous aspects right underneath the regenerative epidermis (Fig.2A, 2B). Some

blastema cells formed a row of cells, one next to the other, and immediately beneath the epidermis, in strong association with the basal layer in both sides of the fin. These cells known as scleroblasts are responsible for the synthesis and deposition of the lepidotrichial extracellular matrix in the region turned to the basal layer, and therefore between the row of scleroblasts and the basal layer of the epidermis.

With six days of regeneration, scleroblasts migrated to the other side of the hemisegment of the regenerating lepidotrichia and were interposed between the epidermis and the hemisegment, maintaining the disposition of a single layer of cells involving, this time, both sides of the lepidotrichial hemisegment and started to secrete extracellular matrix to the hemisegment direction. This action of the scleroblasts is responsible for the growth in width of the hemisegment through appositional growth (central lepidotrichia layers are older that the further external ones) (not showed).

Due to the deposition of extracellular matrix on the hemisegment side, two light regions (less electron dense) were observed, one turned to the basal layer and the other to the hemisegment and they corresponded to the extracellular matrix newly synthesized by the scleroblasts, where collagen fibrils are easily observed, because there was no deposition of calcium salts. There was also a dark central region (more electron dense) called nucleation center, that showed a mineralized fundamental substance that obscured the collagen fibrils, indicating that this region was under a more advanced level of regeneration than both the lighter areas surrounding it (Fig.3A, 3B).

Actinotrichia was initially observed around the fourth day after the excision of the fin (four days of regeneration), next to the connective tissue cells, similar to fibroblasts, indicating that probably they were the cells involved in the synthesis of elastoidin. They initiated their regeneration in the fin distal region, inside the connective tissue matrix, in adjacent position to the epidermis, laid in bilateral rows (Fig.5A, 5B).

On days 8, 10 and 12 of regeneration, the fin was thoroughly grown, that is, the epidermis, the connective tissue, the actinotrichia and the lepidotrichia were completing their regenerative process (Fig.4A, 4B and Fig.6A, 6B).

Similarly to the control group and to the group treated with a dose of 10 mg/L of indomethacin, the treated group with doses of 20 mg/L and 30 mg/L presented complete epidermal cap formation (Fig.1C, 1D), blastema formation (Fig.2C, 2D) and regeneration of the

connective tissue laid in between one lepidotrichia and other and in between one lepidotrichial hemisegment and other. However, the fish that belonged to these groups presented a delay in the dermal skeleton regenerative process (lepidotrichia and actinotrichia) of the tail fins (Fig.3C, 3D; Fig. 4C, 4D; Fig. 5C, 5D and Fig. 6C, 6D).

Both lepidotrichia and actinotrichia initiated their regenerative process around the eighth day after amputation (8 days of regeneration), showing a delay of approximately 4 days in their regeneration. The migration of scleroblasts to the other side of the hemisegment of the regenerating lepidotrichia, responsible for the growth in width of the hemisegment, initiated only on the tenth day after amputation and the nucleation center formation, that is, the beginning of calcium salts deposition in the lepidotriquial matrix, happened around the twelfth day of regeneration. This delay was observed, quantitative, through morphometric analysis done in the widths of lepidotrichia regenerating (Fig. 7).

Although there was a delay in the regeneration of the tail fin dermal skeleton of the fish treated with doses of 20 and 30 mg/L of indomethacin, the morphometric analysis done compared the mean of the areas of regenerating fins of the control group fish as well as the treated fish with 3 different doses of indomethacin when compared did not indicate any significant alteration ($p>0.05$) (Fig.8).

DISCUSSION

Some basal vertebrates, such as urodelic amphibians and the teleost fish, have a high ability to regenerate in a great variety of tissues and organs, while mammals have a limited capacity. This difference is understood as part of a process where the animals along evolution started loosing gradually their ability to regenerate lost or wounded parts (Nakatini et al., 2007).

Teleost tail fins are organs that present a relatively simple and symmetric structure with limited cell types. Furthermore, it is an organ of easy access for manipulation and, possible wounds in its structure do not compete to the survival of the animal. Therefore, the tail fin represents a simple model of the system in regeneration that can be useful to illustrate the biological principles involved in the regeneration, and, also, for the study of the effect of substances that can, perhaps, interfere in this regenerative process.

Indomethacin is a nonsteroid and nonspecific anti-inflammatory drug that causes three kinds of effects: analgesic, antipyretic and anti-inflammatory. According to some studies (Azoubel et al., 2007; Cohen et al., 2006; Collier and Ghosh, 1991; Forslund et al., 2003; Guez et

al., 2001; Leroux and Saffar, 1993; Pai et al., 2001; Riley et al., 2001) indomethacin can interfere somehow in the restoration of the tissue of many organisms, since it inhibits the action of the ciclooxygenase enzyme and consequently the conversion of arachidonic acid into prostaglandin, elements that perform important functions in cell protection, growth, angiogenesis and production of extracellular matrix (Savla et al., 2001).

In this sense, the aim of this study is to evaluate the possible effects of indomethacin over the regeneration of the teleost tail fin. Therefore, histochemical, ultra-structural and morphometrical analysis were done and it was shown that the fish treated with a dose of 10mg/L of indomethacin, did not showed alteration of morphology on the regenerative process of their tail fins, thus, this dose did not affect the formation of the epidermal cap, connective tissue, blastema, blastemal cells differentiation and formation of actinotrichia, as well as synthesis, deposition, organization and mineralization of the components of the lepidotrichial matrix. Similarly, the fish treated with doses of 20mg/L and 30mg/L showed a complete formation of epidermal cap, blastema formation and a regeneration of the connective tissue matrix presented between two lepidotrichial hemisegments and between two rays. However, these fish presented a delay in the process of dermal skeleton regeneration (lepidotrichia and actinotrichia) of the tail fins.

During the fin regeneration, a large number of genes, signaling molecules and transcription factors, are expressed with the finality of to restore the loss and damaged parts. One of these genes is the gene *msxb,* express in nonproliferative cells of blastema distal. Is suggested that the function of these cells is to suppress the differentiation through the reduction of cell proliferation providing therefore a new direction in the growth of the regenerative fin (Nechiporuk and Keating, 2002).

Another gene involved in regeneration of the fin is *lef1*, which appears in the recently formed epidermal cap of the regenerative fin and it is kept in the epidermal basal layer during blastema formation. These results suggest that *lef1* expression is related to the alignment of the scleroblasts (Poss et al., 2000a).

Laforest et al. (1998) analyzed the expression of genes involved in the pathway of signalling SHH (*Sonic Hedgehog)* and observed that *shh, ptc* and *bmp2* are expressed during the fin regeneration in a coordinated way, suggesting the function of these genes in the interaction epidermis-blastema, which leads to the synthesis and to the restoration of the ray. *Shh* is

expressed exclusively in cells of the basal layer of the epithelial compartment, located in the region in contact with the scleroblasts. Therefore, the researchers suggest that the expression of this gene can be involved in the induction of scleroblasts differentiation or in the signalling to the recently formed scleroblasts to start the lepidotrichial matrix synthesis.

Quint et al. (2002) investigated the effects of cyclopamine under the expression of signalizing genes *shh* and noticed that there was a reduction followed by inhibition in the fin regeneration, as well as the formation of little or non actinotrichia and a distal accumulation of pigment cells. This data suggest that a delay or even an inhibition in the fin regenerative process is strongly linked to an alteration in the expression of these genes.

Therefore we believe that probably doses of 20mg/L and 30mg/L did not interfere with the expression of the genes required to the formation of the epidermal cap and the blastema, but could have interfered, momentaneously, in the process of blastemal cells differentiation on the cells responsible for the synthesis and deposition of actinotrichia and lepidotrichia, or even in the necessary signalization to differentiated cells to trigger the synthesis of the components of the dermal skeleton.

Although there was a delay in the regenerative dermal skeleton of the tail fin of the fish treated with doses of 20 and 30mg/L of indomethacin, the morphometric analysis done in the fish treated with the 3 different doses of indomethacin, did not indicate any significant alteration in relation to the total area of the fin in regeneration. This occurred because in the fish treated with doses of 20 e 30mg/L, the region that should have been filled with dermal skeleton in regeneration was filled by the connective tissue in regeneration, not alternating the total area of the fin.

In the present study, we showed that a lower concentration of indomethacin, such as 10 mg/L, did not affect the total area of the regenerated zone neither the eskeletics elements of carp tail fins. However, when the dose was increased to 20 and 30mg/L, there was a delay in the process of dermal skeleton regeneration (lepidotrichia and actinotrichia) of the tail fins, indicating a possible effect of indomethacin dose dependency of in this regeneration.

The above studies suggest that there is presence of various genes in the expression of this regeneration and that although the tail fin is a good model to study the toxicity of drugs and for the study of regenerative processes, much more has to be done in order to understand the mechanism involved in the process.

Detailed studies about the mechanisms of nonsteroid anti-inflammatory drug action and the action of these drugs under the expression or inhibition of expression of some genes involved in the teleost tail fin regenerative process could explain more precisely the influency in indomethacin in the regenerative process.

ACKNOWLEDGMENTS: The authors are grateful to CAPES/DS and to the UNICAMP Teaching and Research Support Foundation (FAEP/UNICAMP) for their financial support of this research. The authors also wish to thank Sr. Baltazar Pereira de Paula and Sra. Cleusa de Oliveira Franco for technical assistance, M.Sc. Sr. Patrick Vianna Garcia for morphometric assistance and the “Recanto dos Peixes” Fishing Club for providing the fish.

FIGURE LEGENDS

Figure 1. Longitudinal section through the distal end of a regenerating tail fin of a fish 24 hours after amputation (1 day of regeneration) stained with picrosirius-hematoxylin. Observe that the regenerating epidermis (E) has already fully covered the amputated region of the tail fin, forming the epidermal cap. Note the loose connective tissue (C) between the old lepidotrichial hemisegments (stars). The arrows indicate the site of amputation.

1.A. Regenerating tail fin of a fish of control group. Bar= 33µm.

1.B. Regenerating tail fin of a fish of group treated with indomethacin in the dose of 10 mg/L. Bar= 23µm.

1.C. Regenerating tail fin of a fish of group treated with indomethacin in the dose of 20 mg/L. Bar=23µm.

1.D. Regenerating tail fin of a fish of group treated with indomethacin in the dose of 30 mg/L. Bar= 26µm.

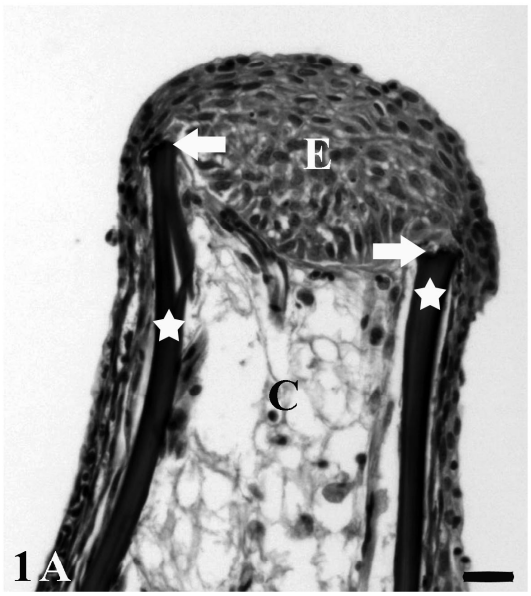
Figure 2. Longitudinal section of the tail fin of a fish after 4 days of regeneration (4 D.R.) stained with picrosirius-hematoxylin. Observe the epidermal cap (E), the blastema (B) and the lepidotrichial hemisegment (stars). The arrows indicate the site of amputation. Note basal epidermal layer composed of cuboidal cells, adjacent to blastemal tissue (arrowheads).

2.A. Regenerating tail fin of a fish of control group. Bar=43µm.

2.B. Regenerating tail fin of a fish of group treated with indomethacin in the dose of 10 mg/L. Bar=37µm.

2.C. Regenerating tail fin of a fish of group treated with indomethacin in the dose of 20 mg/L. Bar= 44µm.

2.D. Regenerating tail fin of a fish of group treated with indomethacin in the dose of 30 mg/L. Bar= 35µm.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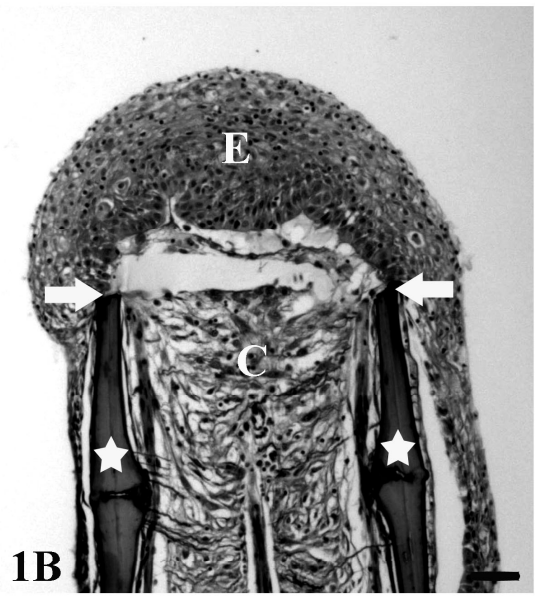
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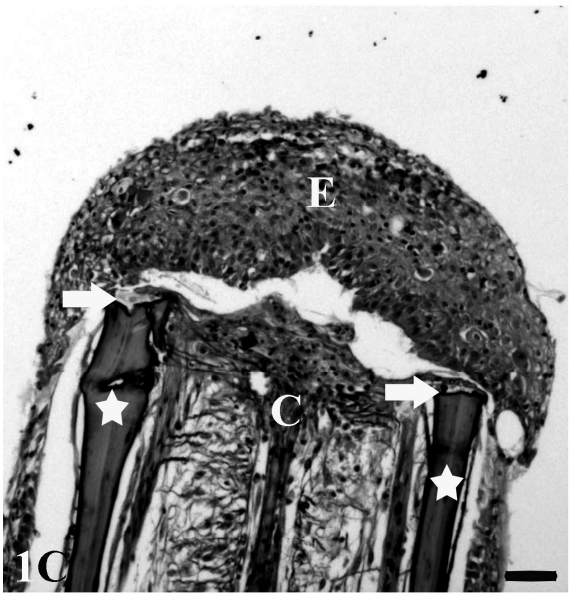
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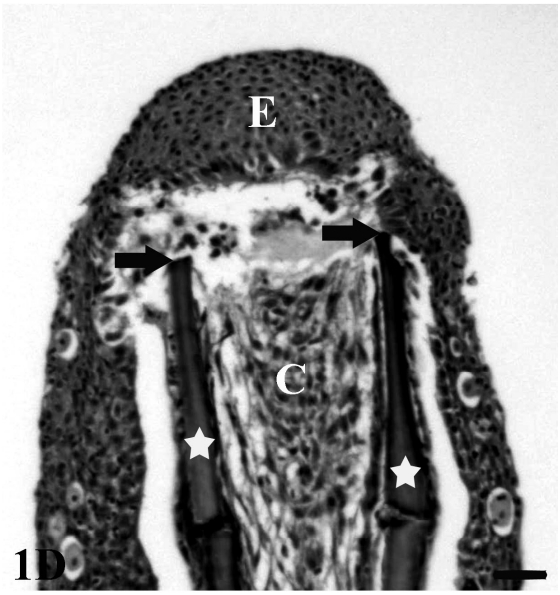
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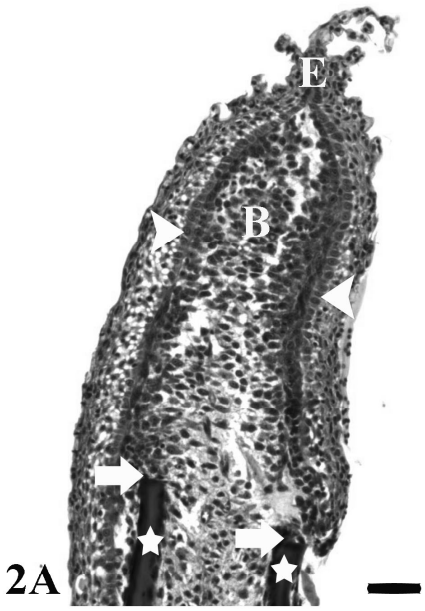
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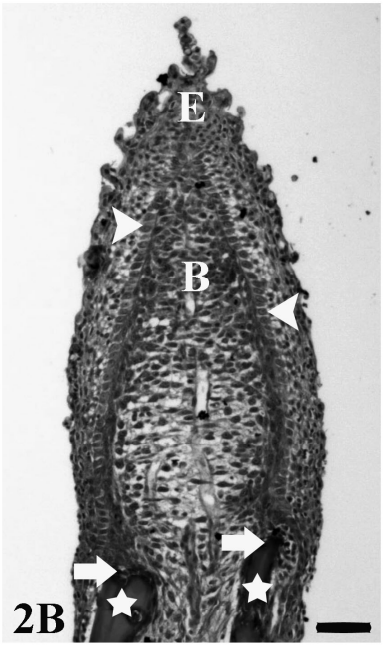
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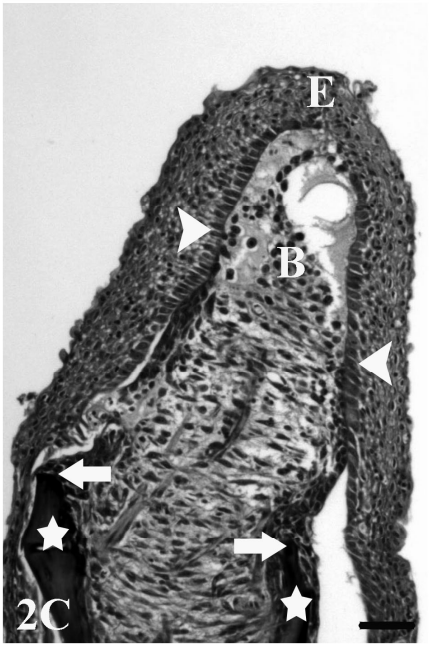
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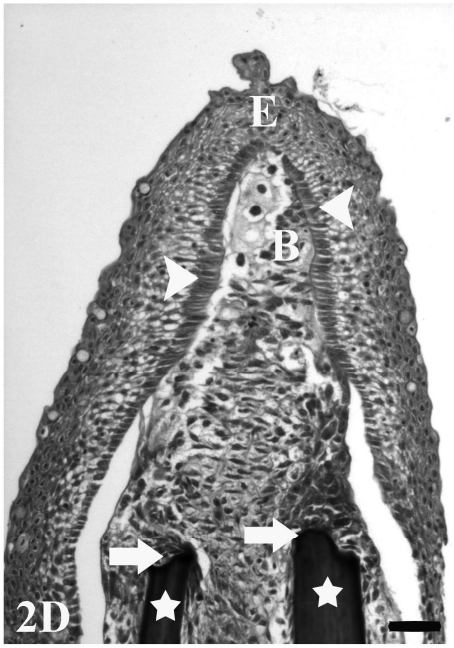
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Figure 3. Electron micrograph of a longitudinal section of a regenerating lepidotrichia after 8 days of regeneration. Observe the scleroblasts (asterisk). Note in lepidotrichial matrix, two light regions, less electron-dense (l) and a central dark region (nucleation center), more electron-dense (m). The arrow indicates the collagen fibrils arranged in various directions.

3.A. Regenerating lepidotrichia of a fish of control group. Bar= 1.2µm.

3.B. Regenerating lepidotrichia of a fish of group treated with indomethacin in the dose of 10 mg/L. Bar= 0.8µm.

3.C. Regenerating lepidotrichia of a fish of group treated with indomethacin in the dose of 20 mg/L. Observe the epidermis (E), the regenerating lepidotrichial hemisegment (L) with the collagen fibrils arranged in various directions (arrow) and the scleroblast (asterisk). Note the absence of nucleation center. Compare with **Figure 3.A**. Bar= 0.6µm.

3.D. Regenerating lepidotrichia of a fish of group treated with indomethacin in the dose of 30 mg/L. Observe the same structures that **Figure 3.C.** Bar= 0.4µm.

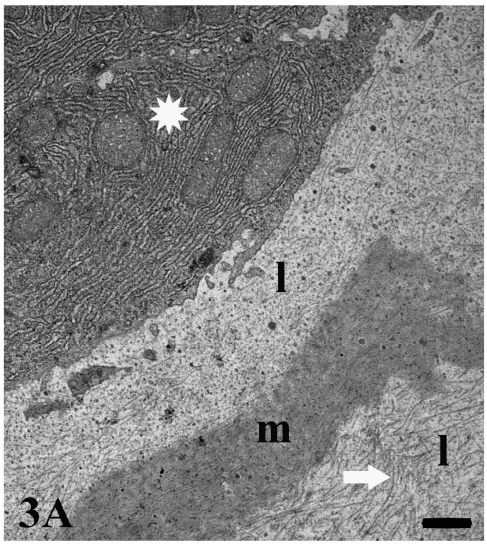
Figure 4. Longitudinal section of the tail fin of a fish after 10 days of regeneration stained with picrosirius-hematoxylin and observed under polarized light. Observe the old lepidotrichium (stars) and the regenerating lepidotrichium (L). Note the brightness of the collagen molecules present in the lepidotrichial matrix against a dark background. The arrows indicate the site of amputation.

4.A. Tail fin of a fish of control group. Bar= 38µm.

4.B. Tail fin of a fish of group treated with indomethacin in the dose of 10 mg/L. Bar= 36µm.

4.C. Tail fin of a fish of group treated with indomethacin in the dose of 20 mg/L. Observe the same structures that **Figure 4.A**. and note the delay in the regenerating lepidotrichium. Bar= 37µm.

4.D. Tail fin of a fish of group treated with indomethacin in the dose of 30 mg/L. Observe the same structures that **Figure 4.A**. and note the delay in the regenerating lepidotrichium. Bar=36µm.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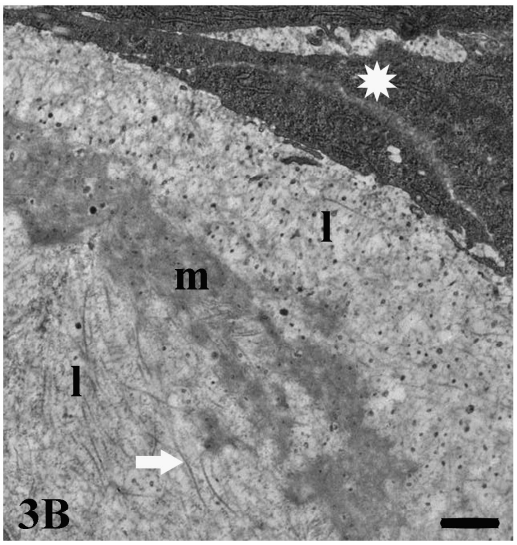
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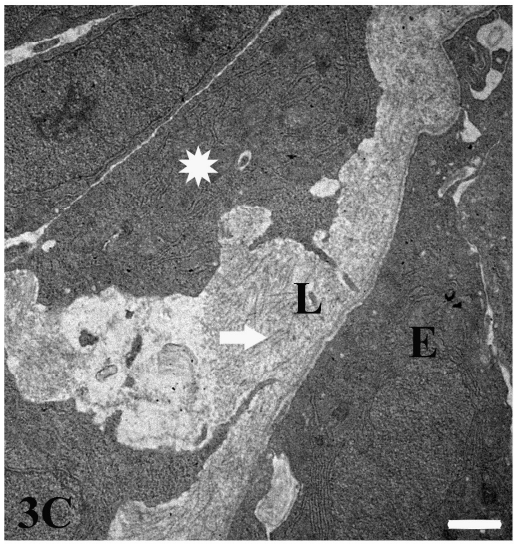
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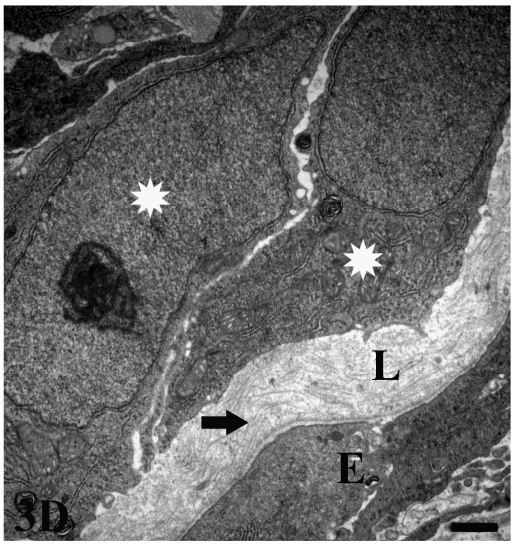
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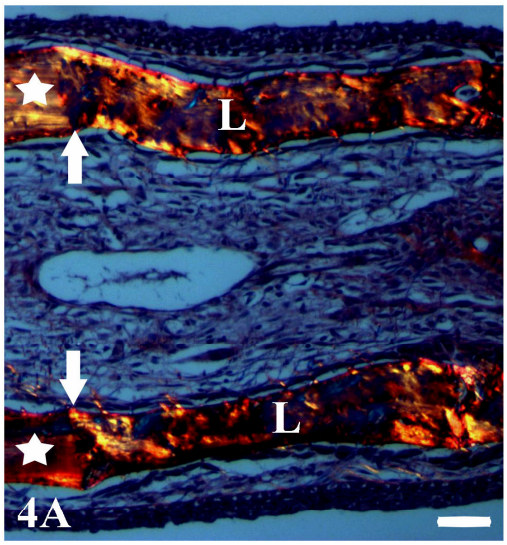
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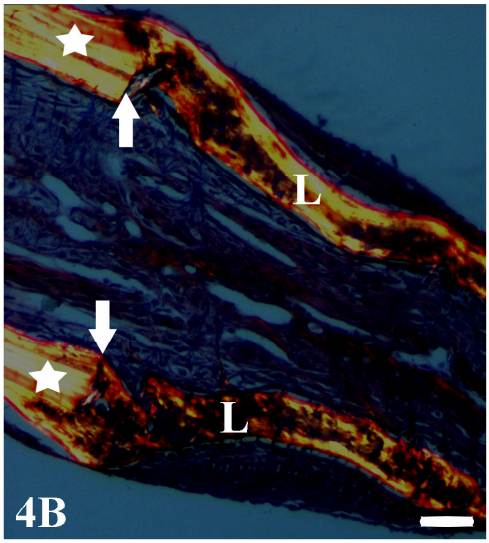
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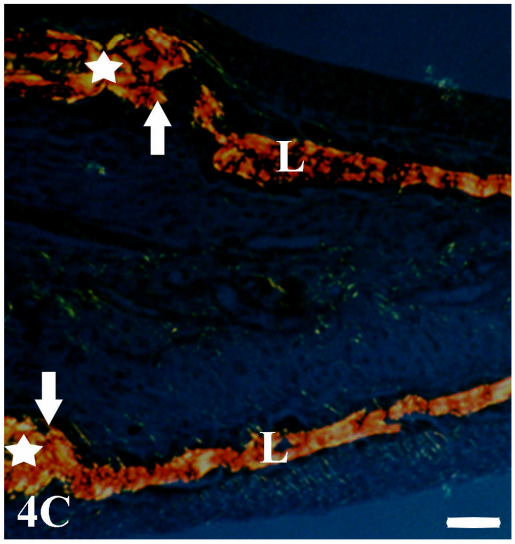
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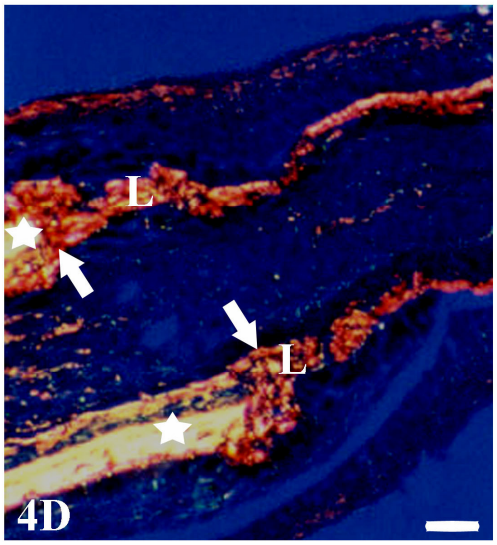
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Figure 5. Electron micrograph of a transversal section of the tail fin of a fish after 5 days of regeneration. Observe the regenerating actinotrichia (A) surrounded by cytoplasmic processes (arrows) of connective tissue cells, similar to fibroblasts (F). Observe the basal lamina of the epidermis (arrowhead).

5.A. Tail fin of a fish of control group. Bar: 1.8µm.

5.B. Tail fin of a fish of group treated with indomethacin in the dose of 10 mg/L. Bar= 2.0µm.

5.C. Tail fin of a fish of group treated with indomethacin in the dose of 20 mg/L. Observe the epidermis (E), the basal lamina of the epidermis (arrowhead), the connective tissue cells, similar to fibroblasts (F) and note the absence of regenerating actinotrichia. Bar: 1.8µm.

5.D. Tail fin of a fish of group treated with indomethacin in the dose of 30 mg/L. Observe the same structures that **Figure 5.C.** Bar= 1.5µm.

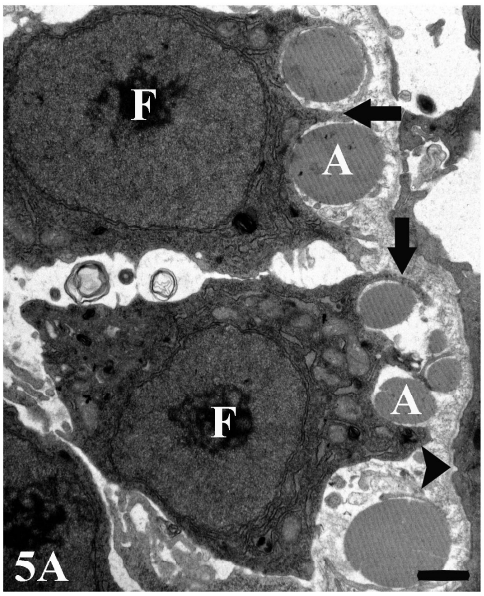
Figure 6. Transversal section of the regenerating tail fin of a fish after 8 days of regeneration stained with picrosirius-hematoxylin. Observe the epidermis (E), the connective tissue (C) and the regenerating actinotrichia (arrows).

6.A. Tail fin of a fish of control group. Bar: 28µm.

6.B. Tail fin of a fish of group treated with indomethacin in the dose of 10 mg/L. Bar= 26µm.

6.C. Tail fin of a fish of group treated with indomethacin in the dose of 20 mg/L. Observe the same structures that **Figure 6.A**. and note the delay in the regenerating actinotrichium. Bar= 35µm.

6.D. Tail fin of a fish of group treated with indomethacin in the dose of 30 mg/L. Observe the same structures that **Figure 6.A**. and note the delay in the regenerating actinotrichium. Bar= 36µm.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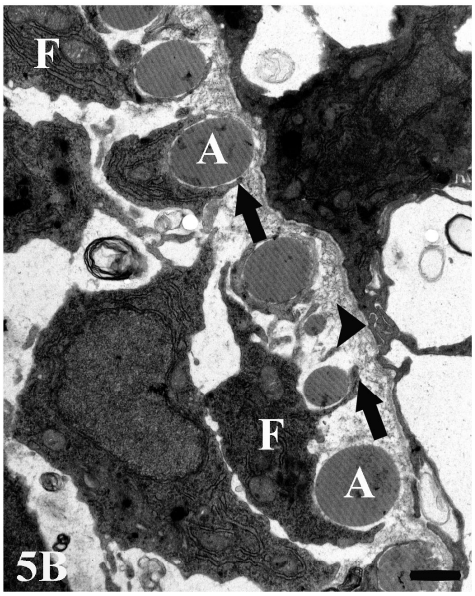
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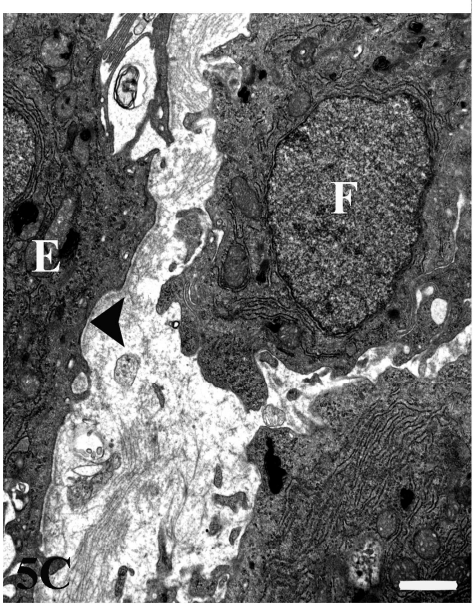
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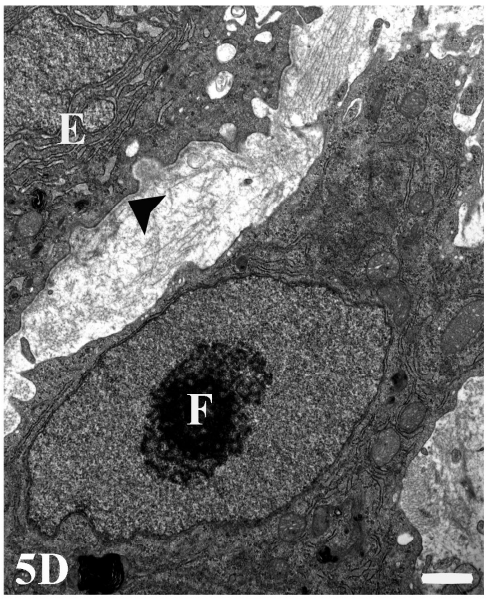
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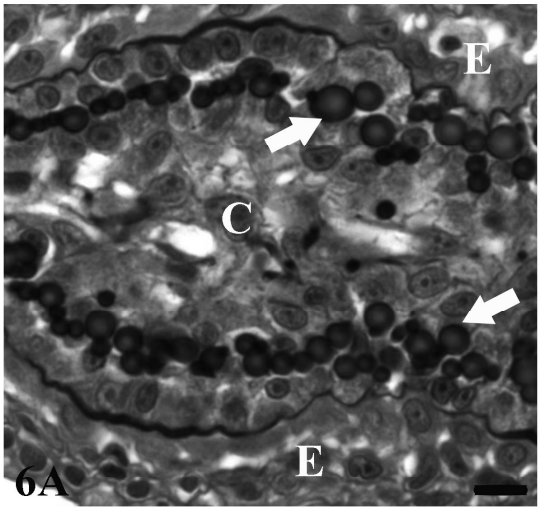
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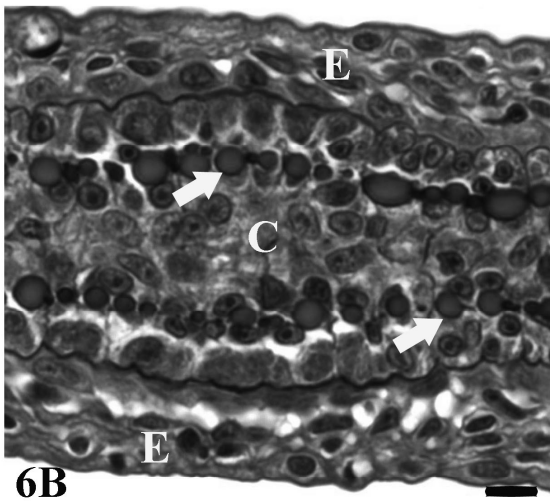
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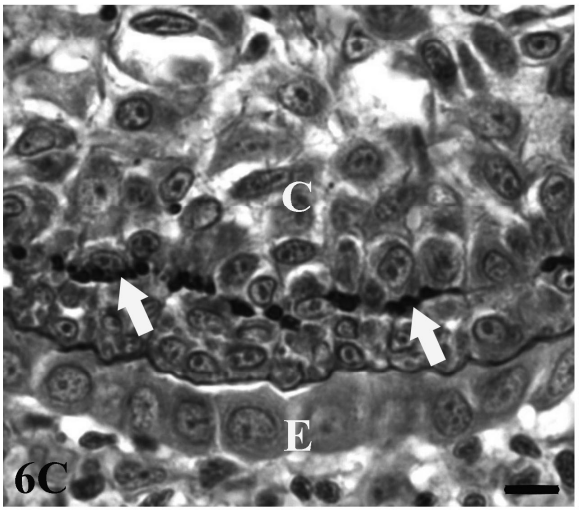
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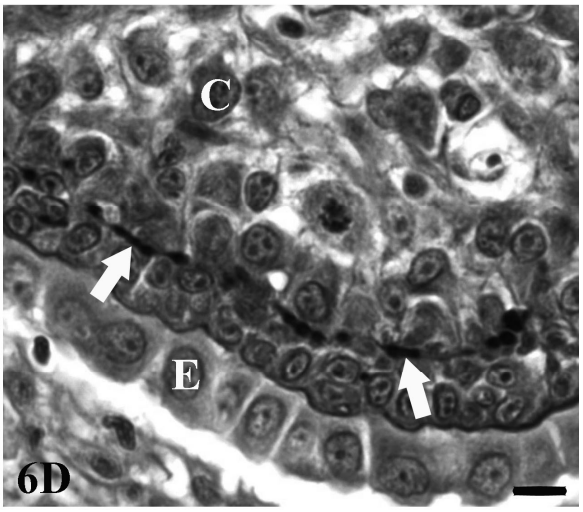
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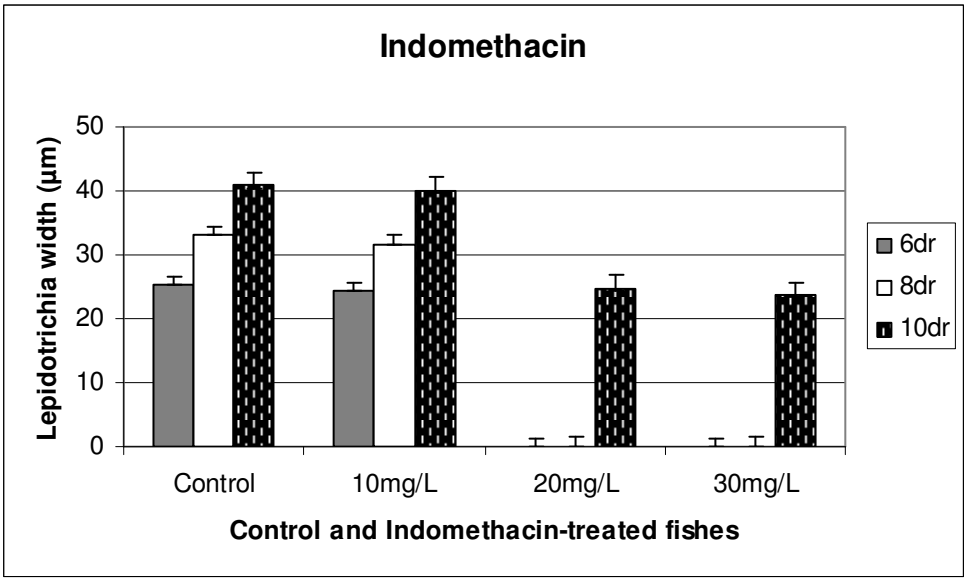

Figure 7. Graphic of the morphometric analysis done compared the mean of the widths of lepidotrichia regenerating of the control and indomethacin treated fishes, on the intervals of 6, 8 and 10 days after amputation of tail fin. The morphometric analysis indicate extremely significant alteration ($p<0.0001$).

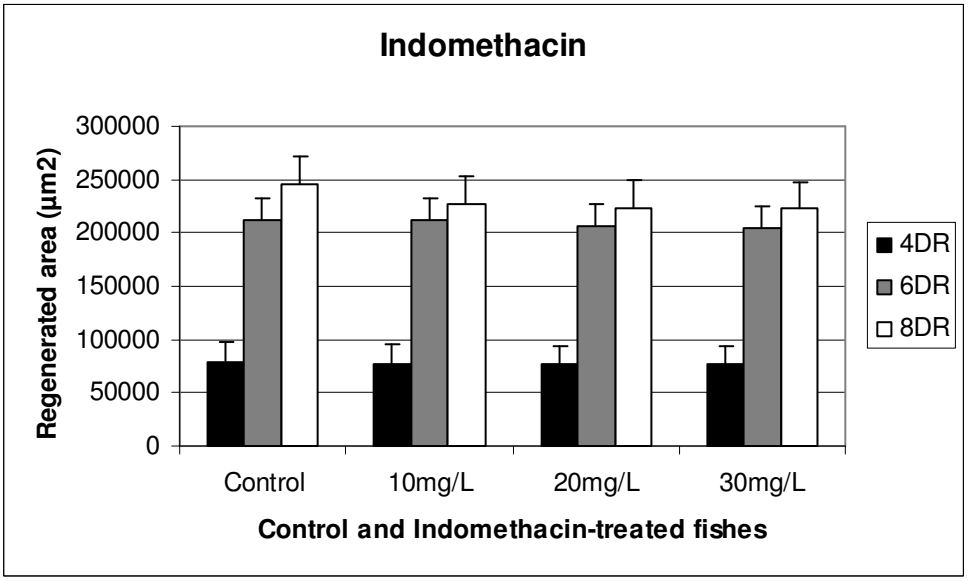

Figure 8. Graphic of the morphometric analysis done compared the mean of the areas of regenerating fins of the control and indomethacin treated fishes, on the intervals of 4, 6 and 8 days after amputation of tail fin.

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REGULAR PAPER

HISTOCHEMICAL AND ULTRASTRUCTURAL ANALYSIS OF THE ACTION OF NAPROXEN ON TAIL FIN REGENERATION IN CARP (*Cyprinus carpio*)

Petra Karla Böckelmann and Ivanira José Bechara

Department of Histology and Embryology, Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil.

ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of rheumatoid arthritis and osteoarthritis, and are also indicated for periarticular and musculoskeletal diseases. However, the use of NSAIDs is limited by their toxicity. NSAIDs have a variable effect on the regeneration of cells and extracellular matrix that depends on the dose used. In this work, we examined the effect of naproxen, a NSAID, on tail fin regeneration in carp (*Cyprinus carpio*), a teleost fish that is a good model for studying the growth of connective tissue *in vivo*. We used histochemical, ultrastructural and morphometric analyses to assess the synthesis, deposition and organization of the lepidotrichial extracellular matrix components and the total area of regenerating fins, including lepidotrichia, epidermis and connective tissue. Naproxen (15.6 mg/L in the tank water) did not affect the formation of the epidermal cap and blastema, the differentiation of blastemal cells in scleroblasts or the synthesis, deposition, organization and mineralization of lepidotrichial matrix components. In addition, there was no significant difference in the area of regenerated tissue between control and naproxen-treated fishes. These results indicate that at the concentration tested, naproxen had no effect on tail fin regeneration.

Key words: Fin, lepidotrichia, naproxen, regeneration, teleost

INTRODUCTION

The tail fin of teleosts consists of two structural units known as lepidotrichia and actinotrichia. The lepidotrichia originate from the base of the fin, extend distally and branch dichotomically towards the margin of the fin. Each lepidotrichium consists of a pair of elongated, curved structures (demirays) that are arranged bilaterally and are subdivided longitudinally into lepidotrichial hemisegments separated by articulations [2]. The lepidotrichial hemisegments are filled with extracellular matrix containing collagen type II fibrils surrounded by a calcified substance that contains chondroitin sulfate [32]. The actinotrichia form clusters of small, fusiform, rigid spicules extending from the distal portion of each lepidotrichium towards the margin of the fin. The actinotrichia are formed by hyperpolymerized macrofibrils of elastoidin that support the border of the tail fin [7,30]. The lepidotrichia and actinotrichia are surrounded by connective tissue containing blood vessels, nerves, pigment cells and fibroblasts and by a multistratified epidermis [7].

The partial amputation of teleost fins results in a process known as epimorphic regeneration. This process involves the recruitment of mesenchymal cells to form a blastema that is followed by the differentiation of these cells into scleroblasts, the synthesis and deposition of extracellular matrix, and morphological restoration [1,21,24,40,41].

Morphological restoration during regeneration depends on internal (biotic) and external (abiotic) factors [13]. Internal factors such as the activity of the thyroid gland and the hypophysis regulate the rate of regeneration [25], whereas external factors such as temperature, light intensity [13,27], certain environmental contaminants [17] and drugs such as β-aminopropionitrile, penicillamine, dexamethasone, indomethacin and acetylsalicylic acid may interfere with the regenerative capacity of teleost fins [8,9].

Naproxen, a nonsteroidal anti-inflammatory drug (NSAID) derived from propionic acid, inhibits

Correspondence to: Dr. Ivanira José Bechara, Laboratório de Histofisiologia e Histopatologia Experimental em Animais Ectotérmicos, Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, SP, Brazil. Tel: (55) (19) 3521-6245, Fax: (55) (19) 3289-3124. E-mail: ibechara@unicamp.br.

the enzyme cyclooxygenase (COX) involved in the formation of prostaglandins at inflammatory sites, thereby reducing swelling, pain and fever [46]. Naproxen is commonly prescribed for the treatment of rheumatoid arthritis, osteoarthritis, gout and soft tissue rheumatism and is also indicated for periarticular and musculoskeletal diseases [26,31,37]. However, the inhibition of COX in various organs results in side effects that include gastrointestinal damage [19], renal failure [14] and the inhibition of platelet aggregation [18].

In addition to its anti-inflammatory action, naproxen may also affect the composition of the extracellular matrix, depending on the dose used [3,4,10,23,33,37-39, 42-44]. Naproxen represses bone formation when administered during bone growth and tissue differentiation in a titanium chamber implanted in rabbit tibia [23], and also inhibits the synthesis of glycosaminoglycans by human tendon *in vitro* [38]. In contrast, naproxen does not affect collagen synthesis in adult human articular cartilage chondrocytes *in vitro* [42].

In this study, we investigated the effect of naproxen on the regeneration of teleost fins, including the synthesis, deposition and organization of the lepidotrichial extracellular matrix and the total area of regenerated lepidotrichia, epidermis and connective tissue.

MATERIAL AND METHODS

Eighty carp (*Cyprinus carpio*) alevins (~1.5 g, ~4 cm long) obtained from a fish farm were used in this study. The fish were maintained in quarantine in plastic aquariums with constantly aerated clean dechlorinated water at 26°C and fed daily with appropriate fish feed.

At the beginning of the experiment, the fish were randomly allocated to two glass aquaria (n=40/aquarium), each containing 10 L of water. The alevins were anesthetized with benzocaine (1:10000; SYNTH, São Paulo, SP, Brazil) and their tail fins were amputated transversely (dorsoventrally) 3 mm from the tail muscle peduncle using a sharp razor, according to Becerra *et al.* [6]. The fish in one aquarium were treated with naproxen (SYNTEX, Rio de Janeiro, RJ, Brazil) by dissolving the drug in the aquarium water to a final concentration of 15.6 mg/L while the fish in the other aquarium (not treated with naproxen) served as the control. The fish were monitored until the cut fin had regenerated. Half of the water in each aquarium was replaced daily with fresh clean dechlorinated water and, with each change of water, half the dose of naproxen (7.8 mg/L) was added to the aquarium containing the treated fish to maintain the initial drug concentration.

At various intervals (1, 2, 4, 5, 6, 8, 10 and 12 days) after fin amputation, five fish from each group (control and naproxen-treated) were anesthetized, sacrificed and the regenerating fins excised. Fragments of regenerating fins from each fish were processed for light and transmission electron microscopy. For light microscopy, the samples were fixed in Bouin solution for 6 h, embedded in paraffin, and sections 6 µm thick were cut, as described by Böckelmann *et al.* [11]. Longitudinal sections were stained with picrosirius-hematoxylin [28] and observed and photographed using conventional and polarized light in conjunction with Image Pro-Plus image analysis software (version 4.1.12; Media Cybernetics, Bethesda, MD, USA). Twenty-five longitudinal sections from each fish were examined. Morphometric analysis was used to assess the area of regenerated tissue 4, 6 and 8 days after amputation (these intervals were chosen as representative of the entire process of tail fin regeneration). For transmission electron microscopy, the tissues were processed as described elsewhere [11].

The results were expressed as the mean ± S.E.M and statistical comparisons were done using one-way analysis of variance (ANOVA) followed by Tukey's mean comparison test. A value of $p<0.05$ indicated significance. All statistical analyses were done using INSTAT v 2.01 (GraphPad, San Diego, CA, USA).

RESULTS

Blood loss after fin amputation was minimal and repair started immediately after the lesion. One day after amputation, epidermal cells completely covered the cut edge at the distal end of the tail fin (amputated region of the fin) to form the epidermal cap (Fig. 1A,B). On the second day of regeneration, a blastema (a homogenous mass of cells) began to form immediately below the regenerating epidermis (Fig. 1C,D).

Some of the blastema cells formed a row of cells arranged side by side immediately below the epidermis in close association with the basement membrane on both sides of the fin. These cells, known as scleroblasts, initiated the synthesis and deposition of lepidotrichial extracellular matrix in the region facing the basement membrane.

There was growth throughout the fin four days after regeneration (Fig. 1E,F). By this time, the scleroblasts had migrated to the other side of the hemisegment of the regenerating lepidotrichia and were interposed between the epidermis and the hemisegment, where they formed a single cell layer that secreted extracellular matrix towards the side facing the hemisegment (Fig. 2A,B).

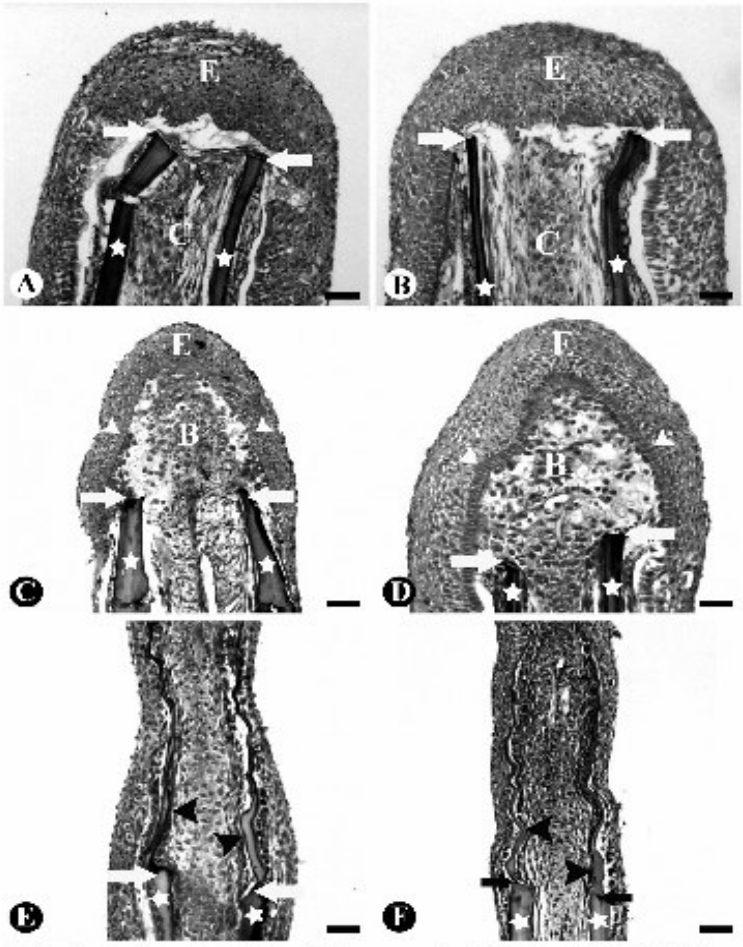


Figure 1. A. Longitudinal section through the distal end of a regenerating tail fin (control fish) 24 h after amputation (one day of regeneration). Note that the regenerating epidermis (**E**) has already fully covered the amputated region of the tail fin (**arrows**) to form the epidermal cap. Note also the loose connective tissue (**C**) between the old lepidotrichial hemisegments (**stars**), i.e., lepidotrichia that did not undergo regeneration. Picrosirius-hematoxylin. Bar = 60 µm. **B.** Longitudinal section through the distal end of the regenerating tail fin of a naproxen-treated fish (after one day of regeneration). Note the epidermal cap (**E**) and the loose connective tissue (**C**) between the old lepidotrichial hemisegments (**stars**) and the amputated region of the tail fin (**arrows**). Compare with panel **A**. Picrosirius-hematoxylin. Bar = 59 µm. **C.** Longitudinal section of the tail fin of a control fish after two days of regeneration. Note the epidermal cap (**E**), the blastema (**B**) and the lepidotrichial hemisegment (**stars**). The arrows indicate the site of amputation. Note also the basal epidermal layer composed of cuboidal cells adjacent to blastemal tissue (**arrowheads**). Picrosirius-hematoxylin. Bar = 58 µm. **D.** Longitudinal section of the tail fin of a naproxen-treated fish after two days of regeneration. Note the same structures as in panel **C**. Picrosirius-hematoxylin. Bar = 63 µm. **E.** Longitudinal section of the tail fin of a control fish after four days of regeneration. Note the old lepidotrichia (**stars**), the amputated region of the tail fin (**arrows**) and the regenerating lepidotrichial hemisegment (**arrowheads**). Picrosirius-hematoxylin. Bar = 58 µm. **F.** Longitudinal section of the tail fin of a naproxen-treated fish after four days of regeneration. Note the same structures as in panel **E**. Picrosirius-hematoxylin. Bar = 60 µm.

After 5, 6, 8, 10 and 12 days of regeneration, the lepidotrichia became elongated as a result of proximal-distal growth and an overall increase in length. There was also an increase in thickness through the deposition of extracellular matrix. This deposition accounted for the thickening of the hemisegment through appositional growth (the central lepidotrichial layers are older than the outer layers).

The deposition of extracellular matrix on the side facing the hemisegment resulted in two lighter (less electron-dense) regions, one facing the basement membrane and the other facing the hemisegment, and a central dark (more electron-dense) region (Fig. 2C,D). The two light regions corresponded to newly synthesized extracellular matrix in which the collagen fibrils were easily visualized because there was no deposition of calcium salts. In contrast, the dark central region, described by Kemp *et al.* [29] as a nucleation center (with calcium salt deposits), showed a fundamental mineralized substance that obscured the collagen fibrils, indicating that this region was in a more advance level of regeneration than the two lighter regions surrounding it (Fig. 2E,F).

In picrosirius-stained sections examined under conventional light old lepidotrichia (those not undergoing regeneration) and regenerating lepidotrichia were reddish in color, whereas polarized light revealed bright red or yellow regions (against a dark background) that corresponded to the extracellular deposition of collagen (Fig. 3A,B).

Comparison of the foregoing histological and ultrastructural features between control and naproxen-treated fish showed that treatment with this drug did not affect fin regeneration at any of the intervals examined. Similarly, morphometric analysis done 4, 6 and 8 days after amputation showed that there was no significant difference in the total area of regenerated tissue between the two groups (Fig. 4).

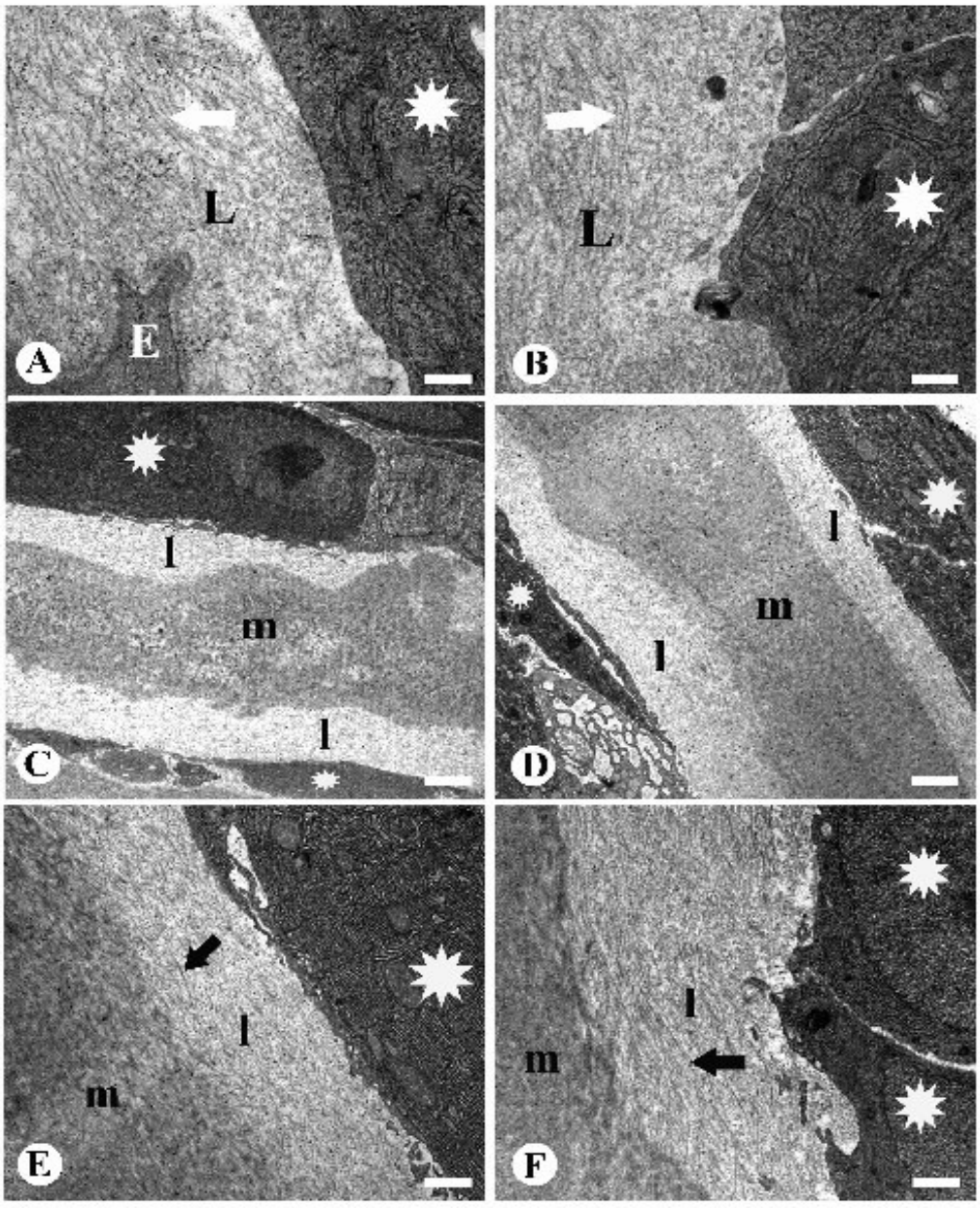
DISCUSSION

Nonsteroidal anti-inflammatory drugs are commonly prescribed for painful and inflammatory conditions, including traumatic injuries, arthritis and dysmenorrhea [15,16,22,45]. Naproxen is a non-specific NSAID that inhibits the two isoforms of cyclooxygenase (COX), COX-1 and COX-2 [46,47]. COX-1 is generally considered a constitutive enzyme that is expressed in practically all tissues and is responsible for producing prostaglandins involved in basic physiological functions, whereas COX-2 is an inducible enzyme that produces prostaglandins involved in tissue injury and inflammation [23,48].

According to literature reports, naproxen may or may not exercise an effect antiproliferation. Mendias *et al.* [31] showed that naproxen decreased differentiation and fusion in cultured skeletal muscle satellite cells from rats and interfered with muscle regeneration. Similarly, Aybar *et al.* [5] observed naproxen inhibited DNA synthesis, cell multiplication and viability in fibroblasts *in vitro*. In contrast, naproxen has no significant effect on the synthesis of glycosaminoglycans or proteins in canine articular cartilage *in vitro* [12] and does not affect the mechanical properties and healing process of transected rat medial collateral ligaments [26].

Böckelmann *et al.* [11] reported that naproxen (10.4 mg/L) did not affect the synthesis, deposition, organization and mineralization of extracellular matrix components in the lepidotrichial matrix of carp tail fin, whereas a concentration of 20.8 mg/L was lethal to the fish. As shown here, a naproxen concentration of 15.6 mg/L also did not interfere with tail fin regeneration, as assessed by the formation of

Figure 2. Electron micrographs of longitudinal sections of: **A.** Regenerating tail fin of a control fish four days after amputation. Note the epidermis (**E**), the regenerating lepidotrichial hemisegment (**L**) with the collagen fibrils arranged in various directions (**arrow**) and the cytoplasm of the scleroblast (asterisk) rich in granular endoplasmic reticulum. Bar = 0.6 µm. **B.** Tail fin of a naproxen-treated fish after four days of regeneration. Note the regenerating lepidotrichial hemisegment (**L**) with the collagen fibrils arranged in various directions (**arrow**) and the cytoplasm of the scleroblast (asterisk) rich in granular endoplasmic reticulum. Compare with panel **A**. Bar = 0.6 µm. **C.** Tail fin of a control fish eight days after amputation. Note that the scleroblasts now coat both sides of the regenerating lepidotrichial hemisegment (asterisks). Note the two lighter (less electron-dense) regions in the lepidotrichial matrix (**l**) and a dark central region that is more electron-dense (**m**). Bar = 1.8 µm. **D.** Tail fin of a treated fish after eight days of regeneration. Note the same structures as in panel C. Bar = 1.8 µm. **E.** Tail fin of a control fish after 10 days of regeneration. Note the scleroblast (asterisk) responsible for the synthesis and deposition of the lepidotrichial matrix. Note also the presence of a less electron-dense region (**l**) with the collagen fibrils arranged in various directions (**arrow**) and a more electron-dense region (**m**) known as the nucleation center. Bar = 0.8 µm. **F.** Tail fin of a treated fish after 10 days of regeneration. Note the same structures as in panel E. Bar = 0.8 µm.



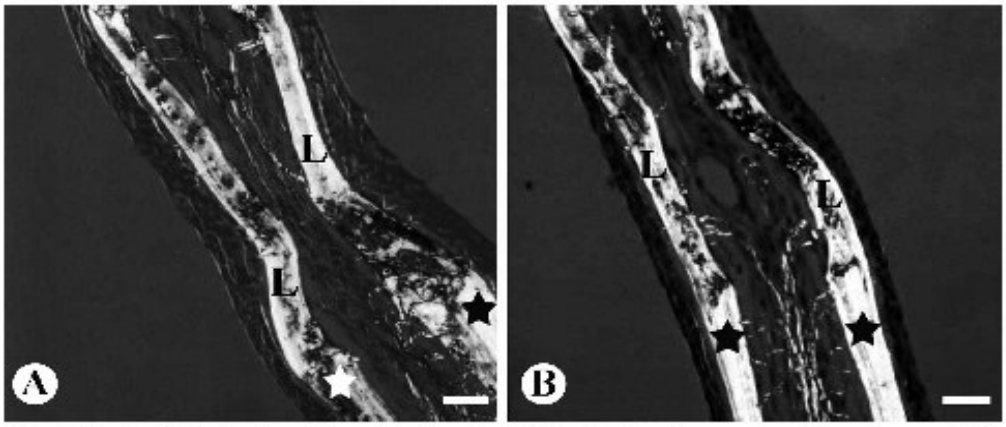


Figure 3. A. Longitudinal section of the tail fin of a control fish after 12 days of regeneration. The section was stained with picrosirius-hematoxylin and observed under polarized light. Note the old lepidotrichium (stars) and the regenerating lepidotrichium (L). The collagen molecules in the lepidotrichial matrix are brightly colored against a dark background. Bar = 28 µm. **B.** Longitudinal section of the tail fin of a treated fish after 12 days of regeneration. The section was stained with picrosirius-hematoxylin and observed under polarized light. Note the same structures as in panel **A**. Bar = 24 µm.

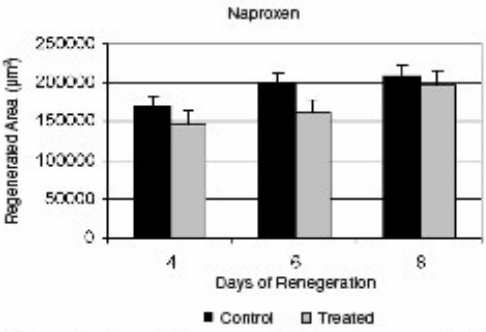


Figure 4. Area of regenerated tissue in control and naproxen-treated fish 4, 6 and 8 days after amputation of the tail fin. The columns are the mean±S.E.M. of 30 fish.

an epidermal cap and blastema, differentiation of the blastemal cells in scleroblasts and the synthesis, deposition, organization and mineralization of lepidotrichial matrix components. Morphometric analysis confirmed that there were no quantitative differences in the total area of regenerated tissue between control and naproxen-treated fish. In contrast to our results, Bechara *et al.* [8,9] reported that two other non-specific NSAIDs, indomethacin and aspirin, impaired the deposition and organization of collagen fibrils that resulted in the formation of abnormal or poorly developed lepidotrichia.

Tissue regeneration in teleost fins involves a cascade of genes that participate in wound healing, epidermal cap and blastema formation, scleroblast differentiation, tissue outgrowth and morphological restoration. One of the earliest molecular markers for fin regeneration is β-catenin, the expression of which is induced in epidermal cells in the first few hours after amputation and is maintained throughout regeneration [35]. The increase in β-catenin is presumed to function in maintaining cell-cell interactions that facilitate migration [34].

The mature wound epidermis is suspected to be a source of outgrowth factors that stimulate formation of the regeneration blastema and maintain its function. One factor that may stimulate blastema formation is Wnt5, a gene product detectable in the wound epidermis and at later stages in the basal epidermal layer [35]. Various other genes, in addition to that for Wnt5, are expressed during formation of the blastema. Poss *et al.* [36] showed that SU5402, an inhibitor of Fgfr1, potently inhibited blastema formation and blocked the induction of *msxb* and *msxc*, as well as mesenchymal cell proliferation.

Another outgrowth factor expressed in wound epidermis is the *shh* gene that is responsible for scleroblast differentiation, alignment and proliferation [34]. In addition, during fin regeneration, *hoxa11b* and *hoxa13b* genes are

induced exclusively in all undifferentiated blastema cells during establishment and growth of the blastema. During the growth of each regenerating lepidotrichium, these two *hoxa* genes are expressed in scleroblasts, and the *hoxa13b* gene may participate in ray patterning during fin regeneration [20].

Based on the foregoing considerations, it is possible that the deleterious action of aspirin and indomethacin on tail fin regeneration described above may result from the inhibition of several of the genes required for regeneration. In contrast, we can suggest that naproxen, in the dose applied, could not influence the expression of genes required during stages of regeneration.

The difference of action of those drugs upon the tail fin regenerative process could be due to divergent actuations of those drugs upon the expression of the genes required for the regeneration. Studies on the mechanism of action of those non-specific nonsteroidal anti-implammatory drugs in the expression of the genes could be performed in the future and may answer the reason of differences of effects between those drugs.

ACKNOWLEDGMENTS

The authors thank Baltazar Pereira de Paula and Cleusa de Oliveira Franco for technical assistance, Patrick Vianna Garcia for help with the morphometric analysis and the Recanto dos Peixes Fishing Club for providing the fish. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundo de Apoio ao Ensino e à Pesquisa (FAEP/UNICAMP).

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Received: December 7, 2006
Accepted: February 13, 2007

ARTIGO SUBMETIDO À REVISTA "BRAZILIAN JOURNAL OF BIOLOGY"

"The regeneration of the tail fin actinotrichia of Teleost fish, *Cyprinus carpio* (carp), under the action of naproxen"

Petra Karla Böckelmann *; Ivanira José Bechara *

Department of Histology and Embryology, Institute of Biology, UNICAMP, Campinas (SP), 13083-970, Brazil.

Running Title: Actinotrichia under the action of naproxen

Mailing address: Prof. Dr. Ivanira José Bechara, Laboratório de Histofisiologia e Histopatologia Experimental em Animais Ectotérmicos, Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), CP 6109, CEP 13083-970, Campinas, SP, Brasil. Tel: (55) (19) 3521-6245, Fax: (55) (19) 3289-3124, E-mail: ibechara@unicamp.br.

KEY WORDS: naproxen; carp; regeneration; actinotrichia; elastoidin

ABSTRACT

A conglomerate of small, rigid, fusiform spicules known as **actinotrichia** sustains the border of the tail fin of teleost. After amputation, these structures show an extremely rapid regenerative capacity. In this study we observed the effect of a nonsteroidal anti-inflammatory drug, the naproxen, used in the treatment of degenerative articular diseases, during the process of actinotrichia regeneration. For this purpose, regenerating tissue from animals in contact with the drug was submitted to histochemical and ultra-structural analyses and compared to tissue from animals under normal conditions, i.e., not in contact with the drug in question. Actinotrichia regeneration was similar in animals in contact or not with the drug, indicating that naproxen, at the dose used in the present study, did not interfere with actinotrichia synthesis during the regenerative process of the tail fin. This could be because naproxen did not influence the expression of the genes required for the regeneration process, such as the *Sonic hedgehog (Shh)* gene, that is involved in actinotrichia formation.

INTRODUCTION

The tail fin of teleost fish is composed of multiple skeletal rays, the **lepidotrichia**, covered by the skin (Becerra et al., 1983; Geraudie and Singer, 1990; Montes et al., 1982; Santamaría and Becerra, 1991). These rays originate from the base of the fin and extend distally, branching dichotomically towards the margin. Extending from the distal portion of each lepidotrichia towards the margin of the tail fin there is a cluster of small, fusiform, rigid and slender spicules called **actinotrichia**, which support the border of the tail fin (Becerra et al., 1983).

Lepidotrichia are structures composed of collagen type II fibrils surrounded by a mineralized fundamental substance whose major component is chondroitin sulfate (Montes et al., 1982). Actinotrichia are formed of hyperpolymerized macrofibrils of elastoidin, a protein with characteristics similar to those of collagen (Krukenberg, 1885)**.** Both the lepidotrichia and actinotrichia are surrounded by connective tissue containing blood vessels, nerves, pigment cells and fibroblasts and by a multistratified epidermis (Becerra et al., 1983).

After amputation, the regeneration that the fin underwent is a situation in which the mechanisms of control of the form and structural pattern of the fin start functioning. The phases

of the process are: formation of a multistratified epidermal cap, disorganization and distal migration of mesenchymal cells near to the amputation plane, proliferation of these mesenchymal cells forming the blastema, continuous proliferation of the distal blastema to facilitate growth, and differentiation of the proximal blastemal cells to replace the lost structures (Akimenko et al., 2003; Géraudie and Singer, 1990; Goss and Stagg, 1957; Johnson and Weston, 1995; Poss et al., 2000; Santamaría and Becerra, 1991; Santos-Ruiz et al., 2002).

Fin regeneration in teleost fish is extremely sensitive to external physical and chemical factors, such as variations in temperature (Johnson and Weston, 1995), light intensity (Buser and Blanc, 1949), action of some environmental contaminants such as TCDD (2,3,7,8-Tetrachlorodibenzo-*p*-dioxin) (Fingerman, 1980; Zodrow and Tanguay, 2003) and the action of some drugs, for example beta-aminopropionitrile, penicillamine, dexamethasone and acetylsalicylic acid, which inhibit collagen synthesis and consequently regeneration (Bechara et al., 2003; 2000).

Naproxen is a nonsteroidal anti-inflammatory drug derived from propionic acid, which has three main types of action: antipyretic, analgesic and anti-inflammatory. It is used in the treatment of osteoarthritis, rheumatoid arthritis, gout and rheumatism of soft tissue and is also indicated for periarticular and musculoskeletal diseases (Hanson et al., 2005; Mendias et al., 2004; Ratcliffe et al., 1993).

However, there are controversial reports in the literature about the action of naproxen on connective tissue, principally with respect to collagen synthesis (Arumugham and Bose, 1981 and 1982; Bjelle and Eronen, 1991; Muscará et al., 2000; Ratcliffe et al., 1993; Smith et al., 1995; Solheim et al., 1986; Srinivas et al., 1994). Goodman et al. (2002) analysed the effect of naproxen on bone growth and tissue differentiation in a titanium chamber implanted in a rabbit tibia, and showed that the drug, a potent non-specific inhibitor of cyclooxygenase 1 and 2, repressed bone formation.

Riley et al. (2001) observed that naproxen inhibited the proliferation of human tendon cells and inhibited the *in vitro* synthesis of glycosaminoglycans, indicating that the drug has a deleterious effect on the tendon matrix metabolism and on repair processes.

In addition, Sadowski and Steinmeyer (2001) verified that naproxen interfered with the expression of mRNA of metalloproteinase-1 so as to reduce its activity, thus interfering with the production of the type II collagen by chondrocytes of bovine articular cartilage.

Since actinotrichia are formed by a protein with collagen-like characteristics and have the ability to regenerate quite rapidly, and since controversial reports exist in the literature regarding the action of naproxen on the extracellular matrix, more precisely collagen synthesis, the objective of the present study was to observe the possible effects of this drug on actinotrichia regeneration.

MATERIALS AND METHODS

Animals

Eighty carp alevins, *Cyprinus carpio*, obtained from a fish farm, weighing on average 1.5 g and measuring on average 4 cm in length, were used in this experiment. The fish were maintained in quarantine in plastic aquariums with clean dechlorinated water at 26ºC with constant aeration, and fed daily with appropriate fish feed.

Experiment

Two glass aquariums, each containing 10 litres of water, were prepared at the beginning of the experiment. The water was clean and dechlorinated, the temperature maintained at 26ºC, the pH at 8.0 and aeration was constant. Naproxen (SYNTEX, Brazil) was dissolved in the water of one of the aquariums at a rate of 15.6mg/L. The fish in the other aquarium, containing only water, served as the control. Eighty alevins were anaesthetized with benzocaine (SYNTH, Brazil) (1:10000) and their tail fins were amputated transversally (in the dorso-ventral direction) at a distance of 3 mm from the tail muscle peduncle using a sharp razor (according to Becerra et al., 1996). After amputation, the fish were divided into two groups of 40 animals each. One group was placed in the aquarium containing dissolved naproxen and the other was placed in the control aquarium. The fish were left in this aquarium until the occurrence of regeneration.

The animals were then anaesthetized, sacrificed and the regenerating fins excised and fixed at intervals of 1, 2, 4, 5, 6, 8, 10 and 12 days after amputation, using 5 specimens for each time interval.

Histology

Histochemical Process – The regenerating fins were fixed in Bouin's solution for 6 hours and embedded in paraffin. Transversal 6 μm thick fin sections were stained with Picrosirius-Hematoxylin and observed and photographed under microscopes (Nikon Eclipse E800, Japan) using conventional light and polarized light.

Ultrastructural Process – Small fragments of regenerating fins were fixed in Karnovsky for 4 hours at 4°C, washed with a 0.1 M phosphate buffer solution (pH 7.4) containing 7.5% saccharose and subsequently postfixed with 1% osmium solution in 0.2 M phosphate buffer for 1 hour at 4°C. The samples were then washed with glycosated saline, dehydrated with increasing acetone concentrations, pre-embedded in a mixture of acetone and epon (1:1) for 3 hours and embedded in pure epon for 24 hours. The tissue fragments included, transversally to the rays, were polymerized in an incubator at 60ºC for 48 hours. Semi-fine sections (1 μm) were obtained using a LEICA ultramicrotome (Germany) and stained in hot Toluidine Blue. Further ultra-fine sections (60-70 nm) were subsequently obtained, contrasted with uranyl acetate and lead citrate, and examined and micrographed in a transmission electron microscope (LEO 906, Germany).

RESULTS

Regeneration in the control animals

Repair of the remainder tissue started immediately after the amputation of the tail fin in the fish in the control aquarium. Regeneration of the epidermis, connective tissue, lepidotrichia and actinotrichia proceeded as reported in the literature (Becerra et al., 1996; Böckelmann and Bechara, 2007).

One day after amputation, the epidermal cells completely covered the cut edge. On the second day a blastema was beginning to form just below the regenerating epidermis.

Actinotrichia were initially observed 4 days after excision of the fin, or in other words, after 4 days of regeneration (4D.R.). Their regeneration started in the distal region of the fin, on the inside of the connective tissue matrix adjacent to the epidermis. They were very thin and arranged in bilateral lines, as expected (Fig. 1).

Connective tissue cells, similar to fibroblasts, were observed near the regenerating actinotrichia, indicating that probably these are the cells involved in the synthesis of elastoidin. Cytoplasmic prolongations of these cells, partially surrounding the actinotrichia, could also be observed (Fig. 3). When observed under microscope with conventional light, these cells presented basophilic cytoplasm, and when observed under transmission electron microscope, they presented a large amount of rough endoplasmic reticulum (Figs. 3 and 5), suggesting their involvement in the synthesis of proteins necessary for the formation of actinotrichia.

The actinotrichia became increasingly thicker after 5, 6, 8, 10 and 12 days of regeneration, due to elastoidin synthesis, until reaching their normal size (Figs. 7 and 9).

Longitudinal sections of the actinotrichia, when observed under the transmission electron microscope, showed that these structures, like the collagen, also presented a transversal striation pattern (Fig. 5), and when observed under polarized light using the Picrosirius-Polarization method, which is a histochemical method for the detection of collagen in tissue sections, the normal birefringence was greatly increased, thus demonstrating their collagen nature (Fig. 11).

Regeneration in the animals treated with naproxen

Regeneration in the naproxen treated fish proceeded in a manner similar to the control fish. One day after amputation, the epidermis had completely covered the edge and on the second day a blastema was beginning to form.

Four days after amputation, regeneration of the actinotrichia started in the distal region of the fin, on the inside of the connective tissue matrix adjacent to the epidermis (Fig. 2), increasing in thickness over the subsequent days (5, 6, 8, 10 and 12 D.R.) (Figs. 8 and 10).

Cells probably involved in the synthesis of the elastoidin of the regenerating actinotrichia were also observed in these treated animals (Fig. 4), and, as in the case of the control animals, these cells presented a large amount of rough endoplasmic reticulum in their cytoplasm (Figs. 4, 6 and 10).

The actinotrichia of the treated animals when observed under polarized light using the Picrosirius-Polarization method exhibited an increased birefringence, once again showing their collagen nature (Fig. 12). In addition, transmission electron microscopy of the regenerating actinotrichia of the treated animals showed the transversal striation characteristic of collagen (Fig. 6).

DISCUSSION

For many years amphibians were the chosen organisms for the study of regeneration in vertebrates since they are capable of regenerating limbs, tail, eyes, optic nerve, spinal cord and part of the heart (Brockes et al., 2001; Brockes and Kumar, 2002). However, the teleost fish also regenerate various structures (fins, optic nerve, heart, spinal cord and scales) (Becker et al., 1997; Bereiter-Hahn and Zylberberg, 1993; Bernhardt et al., 1996; Broussonet, 1786; Morgan, 1900; Poss et al., 2002), and in addition offer numerous advantages as a biological model, such as: present a large number of descendents from a single spawning making it possible to obtain a large number of related examples at low cost and thus amplify sampling; the diet can be carefully

controlled as soon as they begin to accept fish food; various ways of administration chemicals can be used, such as addition to the feed, injection or addition to the water in the tank; and the high regeneration velocity of the fins of these animals. All these factors make these structures a good biological model for the *in vivo* study of the growth of connective tissue, and also for the study of substances which might possibly interfere in the regenerative process (Bailey et al., 1984; Dawe, 1982).

The tail fin of teleost is composed of two structural units known as lepidotrichia and actinotrichia. The actinotriquia form a group of small, rigid, fusiform spicules, found at the apex of each lepidotrichium (Marí-Beffa et al., 1989).

The actinotrichia are formed from hyperpolymerized macrofibrils of elastoidin, a protein with characteristics similar to those of collagen (Krukenberg, 1885). The collagen nature of elastoidin was determined from the fact that, when viewed under polarized light using the Picrosirius-Polarization method, the normal birefringence of these structures was greatly increased (Junqueira et al., 1979), in addition to the fact that the transmission electron microscope showed that the actinotrichia presented a transversal striation pattern characteristic of collagen (Becerra et al., 1996), as also observed in the present study.

When they suffer injury or are amputated, the fins of teleost fish show a capacity to quickly regenerate the lost structures. This regeneration is known as epimorphic regeneration, a process involving the presence of a proliferative mass of pluripotent, progenitorial cells denominated the blastema, and the reconstitution of the tissue complex surrounding multiple cell types (Akimenko et al., 2003; Morgan, 1901).

Both the lepidotrichia and actinotrichia are formed during the regeneration process. However, whereas the lepidotrichia are formed by the addition of new material, the actinotrichia are synthesized distally at the amputation site, and are maintained by a continuous turnover movement, that is, regeneration of these structures can be described by way of the synthesis and degradation of the elastoidin, as a regulatory mechanism of the number and distribution of the actinotrichia (Marí-Beffa et al., 1989).

Bechara et al. (2000), showed that a variety of drugs, such as: dexamethasone, D-penicillamine, beta-aminopropionitrile and aspirin, altered the collagen metabolism in regenerating lepidotrichia, resulting in different types of disorganization in the lepidotrichial matrix. These same researchers inferred that penicillamine could have affected the union of the

elastoidin molecules in the actinotrichia of the distal blastema in such a way that their degradation was also weakened. More recently, Bechara et al. (2003) demonstrated in a histochemical and ultra-structural study, that aspirin inhibited elastoidin synthesis in the actinotrichia of the tail fin in the species *Tilapia rendalli*.

According to reports in the literature (Arumugham and Bose, 1981 and 1982; Bjelle and Eronen, 1991; Goodman et al., 2002; Muscará et al., 2000; Ratcliffe et al., 1993; Riley et al., 2001; Sadowski and Steinmeyer, 2001; Smith et al., 1995; Solheim et al., 1986; Srinivas et al., 1994), naproxen may affect, or not, the composition of the extracellular matrix in mammals depending on the dose applied.

Bockelmann and Bechara (2004), studied the effect of naproxen on the lepidotrichial matrix of the carp tail fin and, at a dose of 10.4 mg/L, observed no alterations in the synthesis, deposition and organization of collagen fibrils, or in the synthesis of the glycosaminoglycans or mineralization of the fundamental substance. These same authors had previously verified that a dose of 20.8 mg/L was lethal for all fish.

Thus the objective of this study was to evaluate a dose intermediate between the lethal dose (20.8 mg/L) and that failing to alter the regeneration of the carp tail fin (10.4 mg/L). The chosen dose was 15.6 mg/L and the aim was to evaluate the effects of naproxen on the synthesis of the actinotrichia during the tail fin regeneration process, based on a histochemical and ultra-structural evaluation of possible alterations occurring in the treated animals as compared to the control animals. A study utilizing the same dose (15.6 mg/L) was carried out in order to evaluate the effect of the naproxen on the synthesis of the lepidotrichial collagen during the regenerative process (Böckelmann and Bechara, 2007). After histochemical and ultra-structural studies and morphometric analysis it was verified that the drug had no affect the lepidotrichial collagen synthesis.

The first indication of actinotrichia regeneration in both the control and naproxen treated animals was observed on the fourth day after excision (4 days of regeneration), and on the following days (5, 6, 8, 10 and 12 days), the structure was seen to increase in thickness, becoming more and more evident and occupying a distal position during the whole process. In the present study, the fish treated with 15.6 mg/L of naproxen presented no alteration in the regeneration of the tail fin actinotrichia when compared to the control fish.

Although reports can be found in the literature of the inhibitory effects of naproxen in collagen and glycosaminoglycan synthesis, it appears that this drug does not affect the synthesis of elastoidin during regeneration.

It is now known that fin regeneration in teleost is intimately related to the expression of some genes, and that the inhibition of these genes could alter the configuration of the newly formed fin. Akimenko et al. (1995) studied the expression of four homeobox genes from the *Msx* family: *MsxA, MsxB, MsxC* and *MsxD* during fin regeneration, and observed that the expression of these genes was strongly reinduced during the regenerative process. The levels of expression of these genes increased greatly during the formation of the blastema, and subsequently fall progressively and disappeared when the fin reached normal size. The same authors also observed that the blastema cells expressed *MsxB* and *MsxC* and the epidermal cells covering the fin expressed *MsxA* and *MsxD*. However, no expression of these four genes was detected in the region of fins that had already suffered differentiation.

Laforest et al. (1998) observed that retinoic acid could directly regulate the expression of the *Shh (Sonic Hedgehog)* gene during fin regeneration, since treatment with retinoic acid caused inhibition of the expression of the *Shh* gene, highly upsetting development of the fin rays. At the end of the treatment, ray synthesis was reinitiated, coinciding with reactivation of the *Shh* expression. Quint et al. (2002) found similar results when investigated the effects of cyclopamine, an alkaloid that blocks the *Hedgehog (HH)* transduction signal, under the expression of the *Shh* signalling genes, and observed a reduction in fin regeneration followed by inhibition, as well as the formation of little or no actinotrichia and a distal accumulation of pigmented cells. These effects were accompanied by a reduction in cell proliferation within the blastema and a reduction in its size.

It was also observed that the signalling pathway of *Fgf (Fibroblast Growth Factors)* was essential for blastema formation and for the regenerative growth of the tail fin of Zebrafish. It has been suggested that *Fgf* signalling induces the blastema by stimulating the gene expression of *MsxB* and *MsxC* (Poss et al., 2000).

These data suggest that a delay, or even inhibition, of the fin regeneration process is closely connected to alterations in the expressions of these genes.

According to the results of the present research, in the dose applied naproxen did not affect regeneration of the actinotrichia. This could be because naproxen did not influence the

expressions of the genes required for the regeneration process, such as the *Sonic hedgehog (Shh)* gene, that is involved in actinotrichia formation.

Bechara et al. (2003) observed that aspirin, a nonsteroidal anti-inflammatory drug like naproxen, inhibited actinotrichia formation, and they suggested that this inhibition could have been because the aspirin probably interfered with the *Shh* signalling pathway. Although aspirin and naproxen are both members of the same group of nonsteroidal anti-inflammatory drug, both inhibiting cyclooxygenases 1 and 2, the difference in action of the two drugs on actinotrichia regeneration may have been due to variations in how these pharmacological agents act locally or are metabolized, leading to variable activities within the same organ.

ACKNOWLEDGEMENTS

The authors are grateful to CAPES/DS and to the UNICAMP Teaching and Research Support Foundation (FAEP/UNICAMP) for their financial support of this research. The authors also wish to thank the "Recanto dos Peixes" Fishing Club for providing the fish, and Sr. Baltazar Pereira de Paula for technical assistance with the fish.

FIGURE CAPTIONS:

Fig. 1. Transversal section of the distal region of a regenerating tail fin of a control animal, 4 days after amputation (4 D.R.). Observe the epidermis (E), the connective tissue (C) and the regenerating actinotrichia (arrows). Picrosirius-Hematoxylin. Bar: 17μm.

Fig. 2. Transversal section of the distal region of the tail fin of a naproxen-treated animal after 4 days of regeneration. Observe the same structures that **Fig. 1.** Picrosirius-Hematoxylin. Bar: 24μm.

Fig. 3. Electron micrograph of a transversal section of the tail fin of a control animal after 6 days of regeneration. Observe the regenerating actinotrichia (A) surrounded by cytoplasmic processes of connective tissue cells, similar to fibroblasts (arrows). Observe the basal lamina of the epidermis (arrowhead). Bar: 4μm.

Fig. 4. Electron micrograph of a transversal section of the tail fin of a treated animal after 6 days of regeneration. Observe the same structures that **Fig. 3.** Bar: 1.4μm.

Fig. 5. Electron micrograph of a transversal section of the tail fin of a control animal after 6 days of regeneration. Observe the longitudinal section of the actinotrichium and the regular transversal striation characteristic of collagen proteins (A). Note the amount of rough endoplasmic reticulum

in the cell cytoplasm involved in the synthesis of the actinotrichium (arrow) and the basal lamina of the epidermis (arrowhead). Bar: 0.3µm.

Fig. 6. Electron micrograph of a transversal section of the tail fin of a treated animal after 6 days of regeneration. Observe the same structures as in **Fig. 5.** Bar: 0.5µm.

Fig. 7. Transversal section of a regenerating tail fin of a control animal, 10 days after amputation (10 D.R.). Observe the epidermis (E) and the regenerating actinotrichia (arrows). Picrosirius-Hematoxylin. Bar: 10µm.

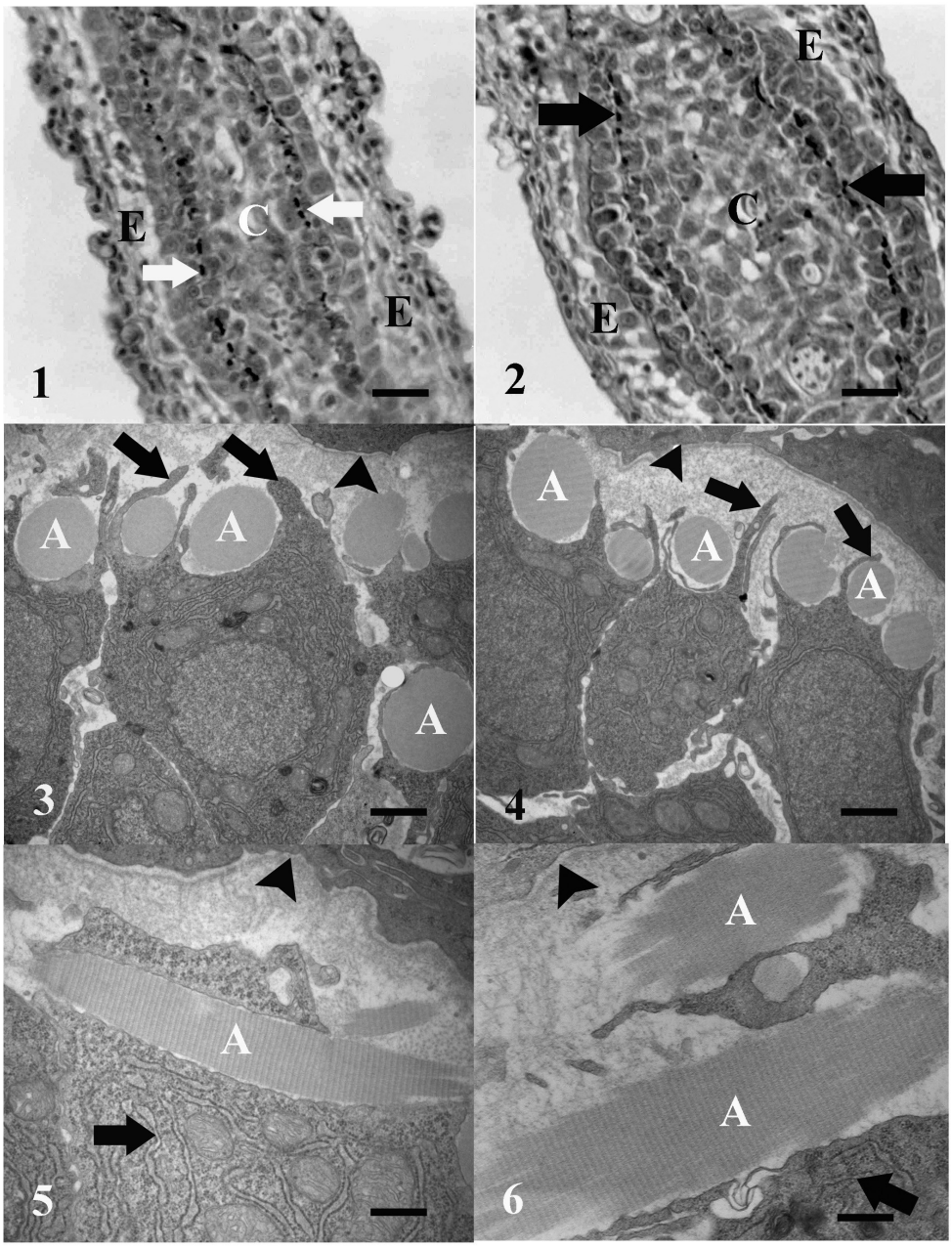
Fig. 8. Transversal section of a regenerating tail fin of a treated animal, 10 days after amputation (10 D.R.). Observe the same structures that **Fig. 7.** Picrosirius-Hematoxylin. Bar: 13µm.

Fig. 9. Electron micrograph of a transversal section of the tail fin of a control animal, 10 days after amputation (10 D.R.). Observe the actinotrichia in an advanced regeneration stage (A) and the cells involved in their synthesis (C). Bar: 0.7µm.

Fig. 10. Electron micrograph of a transversal section of the tail fin of a treated animal, 10 days after amputation (10 D.R.). Observe the same structures that **Fig. 9.** Note the considerable amount of rough endoplasmic reticulum within the cell cytoplasm (arrow). Bar: 1µm.

Fig. 11. Transversal section of the tail fin of a control animal after 12 days of regeneration, stained with Picrosirius-Hematoxylin and observed under polarized light. The arrows indicate the regenerating actinotrichia, bright against a dark background. Observe the presence of transversal (arrowhead) and oblique (arrows) actinotrichia. Bar: 19µm.

Fig. 12. Transversal section of the tail fin of a treated animal after 12 days of regeneration, stained with Picrosirius-Hematoxylin and observed under polarized light. Observe the same structures that **Fig. 11.** Bar: 14µm.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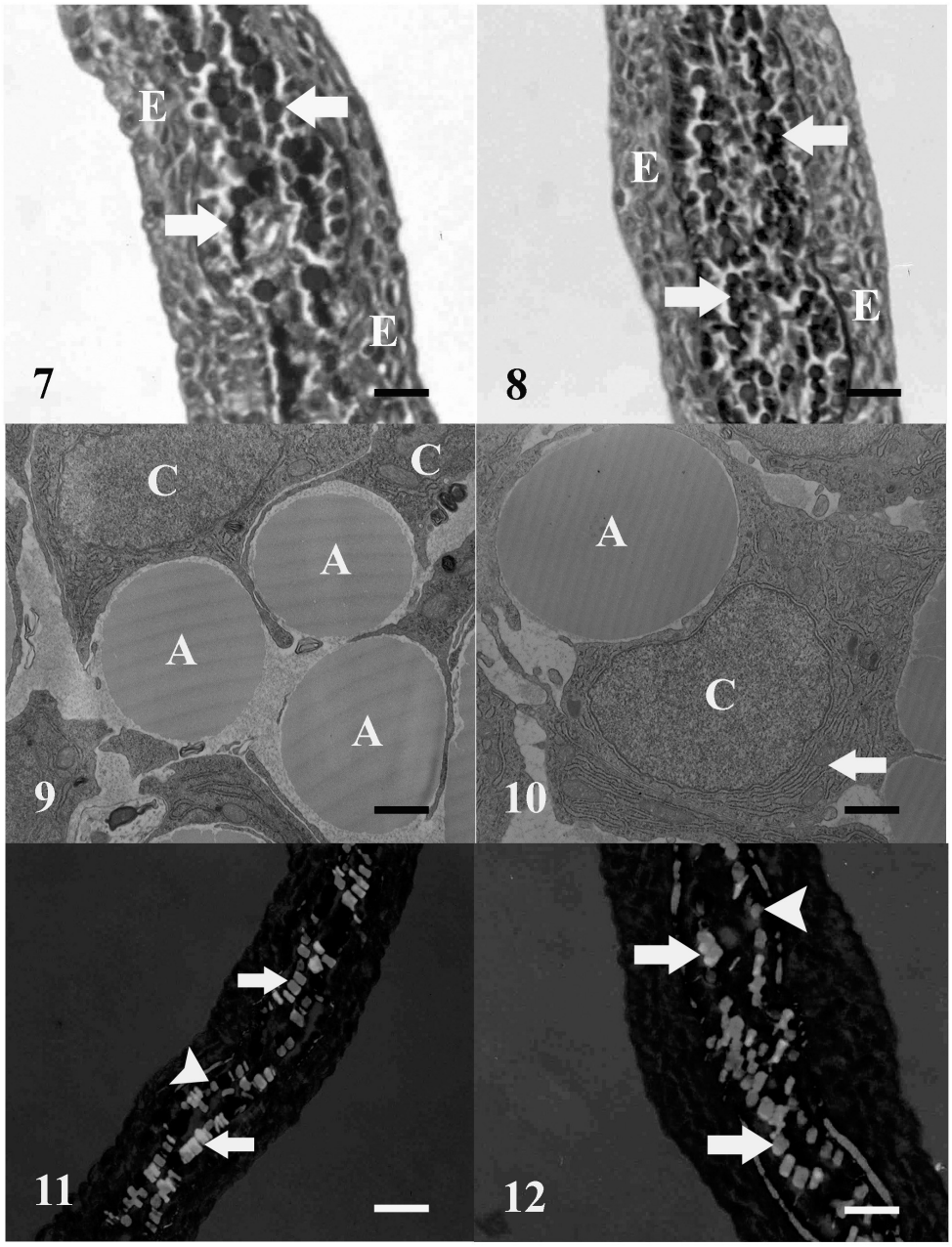
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CONCLUSÃO GERAL

A partir das análises histoquímica, ultra-estrutural e morfométrica, este trabalho permite as seguintes conclusões:

- Os peixes tratados com o naproxeno, na dose de 15,6 mg/L, e os peixes tratados com a indometacina, na dose de 10 mg/L, apresentaram o processo regenerativo de forma semelhante aos peixes do Grupo controle, ou seja, essas doses não afetaram a formação da capa epidermal, do tecido conjuntivo, a formação do blastema, a diferenciação das células blastemais em escleroblastos e fibroblastos, a regeneração das actinotriquias, bem como a síntese, deposição, organização e mineralização dos componentes da matriz lepidotriquial. De forma semelhante, os peixes tratados com as doses de 20 mg/L e 30 mg/L apresentaram a completa formação da capa epidermal, a formação do blastema e a regeneração completa do tecido conjuntivo. No entanto, esses peixes apresentaram um atraso no processo de regeneração das unidades esqueléticas (lepidotriquias e actinotriquias) das nadadeiras caudais. Essas conclusões foram observadas a partir das análises histoquímica e ultra-estrutural.

- A análise morfométrica feita tanto nos peixes do Grupo controle como nos peixes dos Grupos tratados com o naproxeno e com as três doses da indometacina, não indicaram nenhuma alteração significativa quanto à **área total** da nadadeira em regeneração. No caso dos peixes tratados com as doses de 20 e 30 mg/L, isto ocorreu porque a região que deveria ser ocupada pelas unidades esqueléticas em regeneração foi preenchida pelo tecido conjuntivo em regeneração, não alterando a área total da nadadeira. No entanto, a morfometria feita analisando a **espessura da lepidotriquia em regeneração** dos peixes do grupo controle e dos peixes tratados com as doses de 10, 20 e 30mg/L da indometacina mostrou de forma quantitativa o atraso no processo regenerativo da lepidotriquia nos peixes tratados com as doses de 20 e 30mg/L.

CONSIDERAÇÕES GERAIS

- Estudos mais detalhados sobre os mecanismos de ação das drogas anti-inflamatórias não esteróides e a ação dessas drogas sob a expressão ou a inibição da expressão de alguns genes envolvidos no processo de regeneração da nadadeira caudal de teleósteo talvez possam responder a razão das diferenças de efeitos entre o naproxeno e a indometacina.

- Além disso, drogas anti-inflamatórias não esteróides (AINEs) convencionais são inibidoras das duas isoformas da enzima ciclooxigenase (COX-1 e COX-2) e é sugerido que os efeitos colaterais gastrointestinais dessas drogas estão associados com a inibição de COX-1, enquanto os efeitos analgésico, anti-inflamatório e antipirético são derivados da COX-2 (Hinz & Brune, 2002). Desta forma, estudos no futuro seriam viáveis no objetivo de analisar o efeito de AINEs específicas, inibidoras apenas de COX-2, na regeneração da nadadeira caudal de teleósteo e, também, para observar possíveis diferenças entre a ação de AINEs convencionais e AINEs específicas sob o processo regenerativo.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de doutorado intitulada "Análise histoquímica, ultraestrutural e morfométrica do efeito de drogas antiinflamatórias não esteróides (Naproxeno e Indometacina) sob a regeneração da nadadeira caudal de teleósteo, *Cyprinus carpio* (carpa)":

( ) não se enquadra no Artigo 1º, § 3º da Informação CCPG 002/06, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo nº_____), intitulado _____

(X) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº 1135-1 (A)). aprov. 29/11/2006

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo nº_____).

Aluno(a): Petra Karla Böckelmann

Orientador(a): Profa. Dra. Ivanira José Bechara

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Nome:
Função: Profa. Dra. ANAMARIA A. GUARALDO
Presidente
Comissão de Ética na Experimentação Animal
CEEA/IB - UNICAMP