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INHIBITION OF CAUDAL FIN ACTINOTRICHIA REGENERATION BY ACETYLSALICYLIC ACID (ASPIRIN) IN TELEOSTS†

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ABSTRACT

Various substances have been used to investigate physiological and physiopathological processes in animals. In this study, we investigated the effects of acetylsalicylic acid (ASA, aspirin) on the regeneration of actinotrichia, skeletal structures of the caudal fin of teleosts. Two groups of fish (*Tilapia rendalli*) were maintained in aquaria with dechlorinated water at 24ºC, with one group being exposed to ASA (0.1 g/l) for 24 h. Thereafter ASA-treated and untreated (control) fishes were anesthetized and their tail fin amputated. After periods ranging from 4-12 days, the fishes were sacrified and the regenerating tissue was processed for light and transmission electron microscopy and picrosirius-hematoxylin staining. Control specimens showed normal regeneration of the actinotrichia, whereas all (except one) of the ASA-treated fishes showed no regeneration. The 20 ASA-treated fishes devoid of actinotrichia had varying degrees of caudal fin regeneration. These results indicate that, as in mammals, aspirin also affects biological processes in fish. Based on reports in the literature, we hypothesize that ASA interfered with the transcription of the fibroblast genes necessary for the synthesis of elastoidin, or altered the typical rapid turnover of this protein, thereby affecting regeneration of the actinotrichia. The use of ASA and other drugs to study the regeneration of actinotrichia could be a valuable approach for investigating cell-matrix interactions. This model could also be useful for evaluating the toxic effects of river pollution and chemical damping.

Key words: Actinotrichia, aspirin, regeneration, teleosts

INTRODUCTION

Teleost fins consist of skeletal structures known as lepidotrichia (fin rays) and actinotrichia, both of which are surrounded by loose connective tissue containing blood vessels and nerves and covered by an epidermal layer [8]. The lepidotrichia are segmented, bifurcate structures made up of collagen fibrils surrounded by a calcified amorphous ground substance whose main component is chondroitin sulphate [32]. The actinotrichia are assemblages of very tiny, thin spicules irradiating from the distal endings of each lepidotrichium to the edge of the fins. A collagen-like protein, elastoidin, is the main component of these structures [23].

When amputated, teleostean fins rapidly regenerate [34] via a process which greatly resembles the regeneration of amphibian appendages. The complex mechanisms of wound healing, differentiation, blastema formation, growth and complete morphological recovery involve various types of tissues [3,17,18,19,36,42,44].

The primordium of the actinotrichium extends into the connective matrix beneath the wounded epidermis around the fifth day after amputation, and is partially or fully surrounded by fibroblasts [7]. The full size and final morphological organization of the bundle of actinotrichia is achieved by the tenth day after injury. During the whole process of regeneration, the actinotrichia maintain their characteristic distal location [28].

†Deceased. This article is dedicated to the memory of Prof. Gregorio Santiago Montes

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Various studies have shown the influence of drugs on collagen metabolism. Synthetic glycocorticoids, such as dexamethasone, inhibit the synthesis of collagen [1,12,47], and negatively affect the growth and structure of bone tissue [4]. The chelating agent, D-penicillamine, directly blocks the aldehyde radicals of the collagen molecule and chelates the Cu^{2+} from lysyl-oxidase [38]. D-penicillamine may also act indirectly [27] to increase the solubility of collagen in tissue [20,37]. At low concentrations, D-penicillamine decreases the biosynthesis of collagens I and III [11,53]. Beta-aminopropionitrile (BAPN), a lathyrogen nitrile derived from the sweet pea, *Lathyrus odoratus*, inhibits the cross-linking of collagen and induces lathyrism [2,10,13,25]. This pathology is characterized by the impairment of cartilage calcification, delayed endochondral ossification and weakening of the insertion of the tendon and ligament [5]. BAPN forms an irreversible linkage bond with lysyl-oxidase, abolishing the capacity to establish cross-reactions between collagen molecules [33], thereby increasing the solubility of collagen [45,46]. In BAPN-treated chick embryos, there is a 20% increase in the hydration of cartilage and other tissues. The most likely explanation for this is that disruption of the cross-links allowed the proteoglycans in cartilage to express their hydrophilic nature when freed of their collagenous network [26].

Two non-steroidal anti-inflammatory drugs, indomethacin and aspirin, decrease collagen synthesis in cultured chondrocytes by reducing gene transcription [14]. Acetylsalicylic acid (ASA) inhibits the remodeling and growth of bone and skin in young rats [48-52]. Studies *in vitro* have shown that ASA can decrease the biosynthesis of the two major constituents of the cartilage matrix (collagen II and proteoglycans) in rats [30]. In cultured human chondrocytes, ASA decreases the synthesis of proteoglycans but not that of collagen II [6,21]. Bechara *et al*. [9] investigated the effects of dexamethasone, D-penicillamine, beta-aminopropionitrile and aspirin on the regeneration of the lepidotrichial matrix (the fin rays) of two teleostean species, and observed that these drugs caused marked disorganization of all regenerating structures of the lepidotrichial matrix. These effects were attributed to interference in the synthesis and/or metabolism of collagen during fin regeneration.

In this study, we examined the effects of aspirin on the regeneration of actinotrichia, soft unmineralized structures made up of fibrils of elastoidin in the caudal fin of *Tilapia rendalli.*

MATERIAL AND METHODS

Forty-two *Tilapia rendalli* 5-7 cm in length were purchased from commercial suppliers, and were kept in plastic aquaria containing aerated dechlorinated water (24°C), half of which was replaced daily. The fishes were fed twice a day with flakes of standard chow for aquarium fish. After 40 days, the fishes were transferred to two glass aquaria (n = 21 each), one of which contained 0.1 g of acetylsalycilic acid (ASA-aspirin, Sigma) per liter. Twenty-four hours later, the fishes of both aquaria were anesthetized with benzocaine (1:10,000) and their tail fin was amputated 3 mm from the muscular peduncle [7].

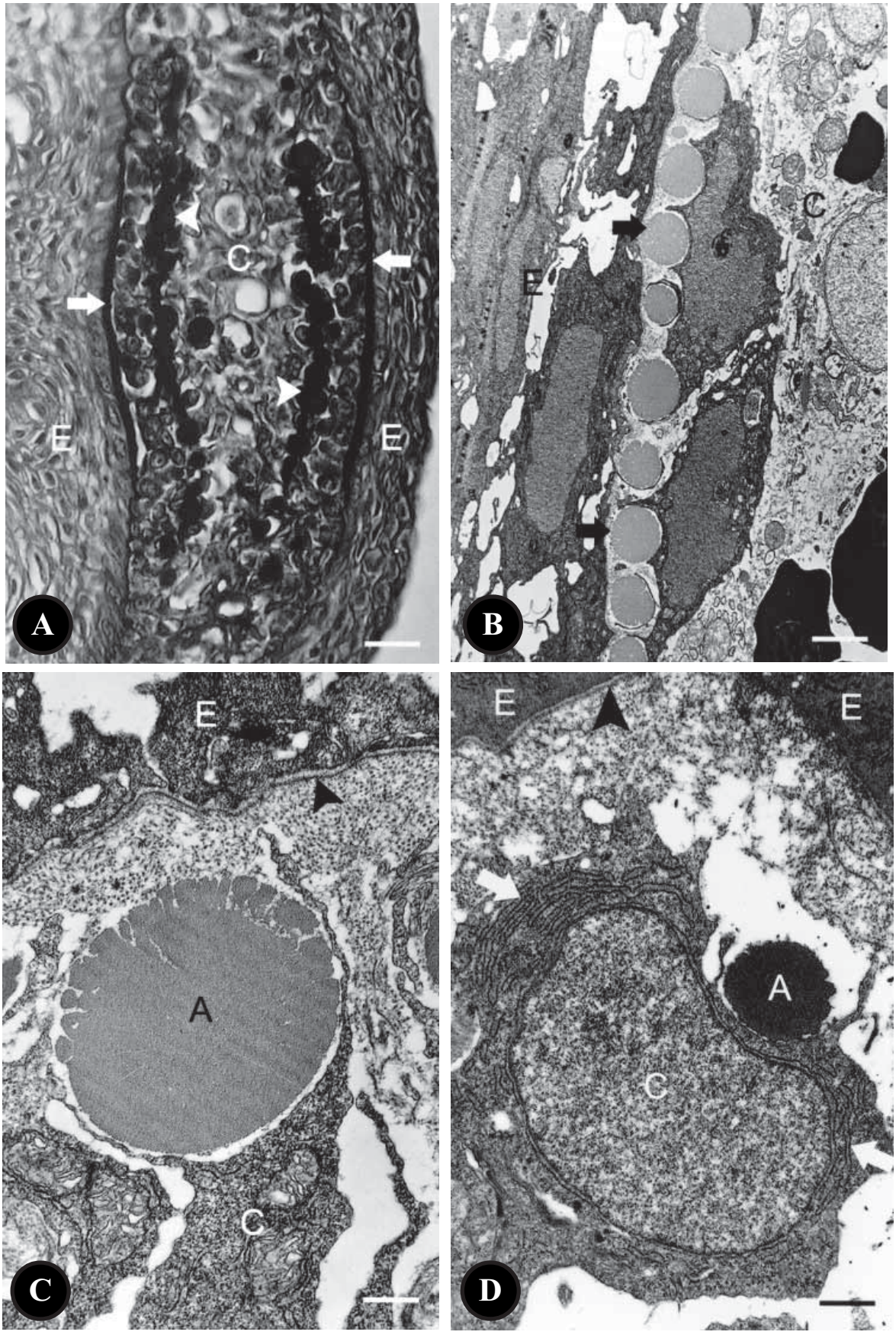
The fishes were returned to their respective aquarium, and each day half of the aquarium water was replaced by fresh dechlorinated water, as described above. Aspirin was added at half the concentration to maintain the initial levels of ASA (0.1 g/l). At 4, 5, 6, 7, 8, 10 and 12 days (n = 3 each) after amputation, the regenerated fins were removed from anesthetized fish. For each period, samples from control and ASA-treated fish were fixed either in Bouin fixative (6 h) for light microscopy, or according to Becerra *et al.* [7] and Montes [31] for transmission electron microscopy. Paraffin sections (6 μm thick) were stained with picrosirius-hematoxylin [22] and examined under conventional light microscopy.

RESULTS

Control (untreated) fish

No anomalies were observed during the regeneration of caudal fin lepidotrichia, connective tissue and epidermis from 4 to 12 days after amputation. By day 5 after amputation, the actinotrichia had started to regenerate at the distal fin stump, within the connective tissue matrix subjacent to the covering epidermis (Fig. 1A and 1B). Connective tissue cells (fibroblasts) were seen in the vicinity of developing

Figure 1. A. Transversal section of the distal region of a tail fin of control *Tilapia rendalli* after eight days of regeneration. Note the regenerating lepidotrichial matrix (**arrows**) and the regenerating actinotrichia (**arrowheads**). Typical connective tissue (**C**) is seen within the ray. (**E**) - epithelium. Picrosirius-hematoxylin. Bar = 15 μm. **B**. Transversal section of the distal region of a tail fin of control *T. rendalli* observed by electron microscopy after eight days of regeneration. Note the row of transversely sectioned regenerating actinotrichia (**arrows**) lying immediately beneath the epidermis (**E**). (**C**) - connective tissue. Bar = 2 μm. **C.** Transversal section of the distal region of a tail fin of control *T. rendalli* observed by electron microscopy after ten days of regeneration. Note the basal lamina (**arrowhead**) of the epidermis (**E**) and the transversely sectioned regenerating actinotrichium (**A**) surrounded by cytoplasmic processes from connective tissue (fibroblast - like) cells (**C**). Bar = 0.5 μm. (**D**) Detail of a fibroblast-like cell (**C**) Note the rough endoplasmic reticulum-rich cytoplasm of these cells (**arrows**). (**A**) - actinotrichium, (**E**) **-** epidermis. Bar = 0.4 μm.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actinotrichia (Fig. 1C). These cells had a well-developed rough endoplasmic reticulum (Fig. 1D), which suggested involvement in the protein synthesis needed for the formation of actinotrichia. Transmission electron microscopy of longitudinal sections of actinotrichia showed that they were traversed by cross striations typical of collagen-like fibers (Fig. 2A). By day 12 after fin amputation, the size and distribution of the actinotrichia had almost completely returned to normal.

Aspirin-treated fish

Of the 21 fish treated with aspirin, only one showed the normal regeneration of actinotrichia, seen in control fishes (Fig. 2B). In this specimen, the epidermis and connective tissue recovered completely. In the remaining 20 fishes, there was no sign that the actinotrichia were being regenerated, or that *de novo* formation had started, as shown by light (Fig. 2C) and transmission electron (Fig. 2D) microscopy. Ten of these 20 aspirin-treated fishes showed no formation of the blastema, nor there was any reconstitution of the connective tissue. In the remaining 10 specimens, the blastema was poorly developed and connective tissue neoformation was delayed compared to control (untreated) fish. As a consequence, the fin appeared atrophic 12 days after fin amputation. Actinotrichia were absent in these 20 abnormally-regenerated fin.

DISCUSSION

Fin regeneration in teleosts is a common event aimed at restoring the loss of part or all of the fin and, when amputated, fins rapidly regrow to replace the ablated portions [3,7,9].

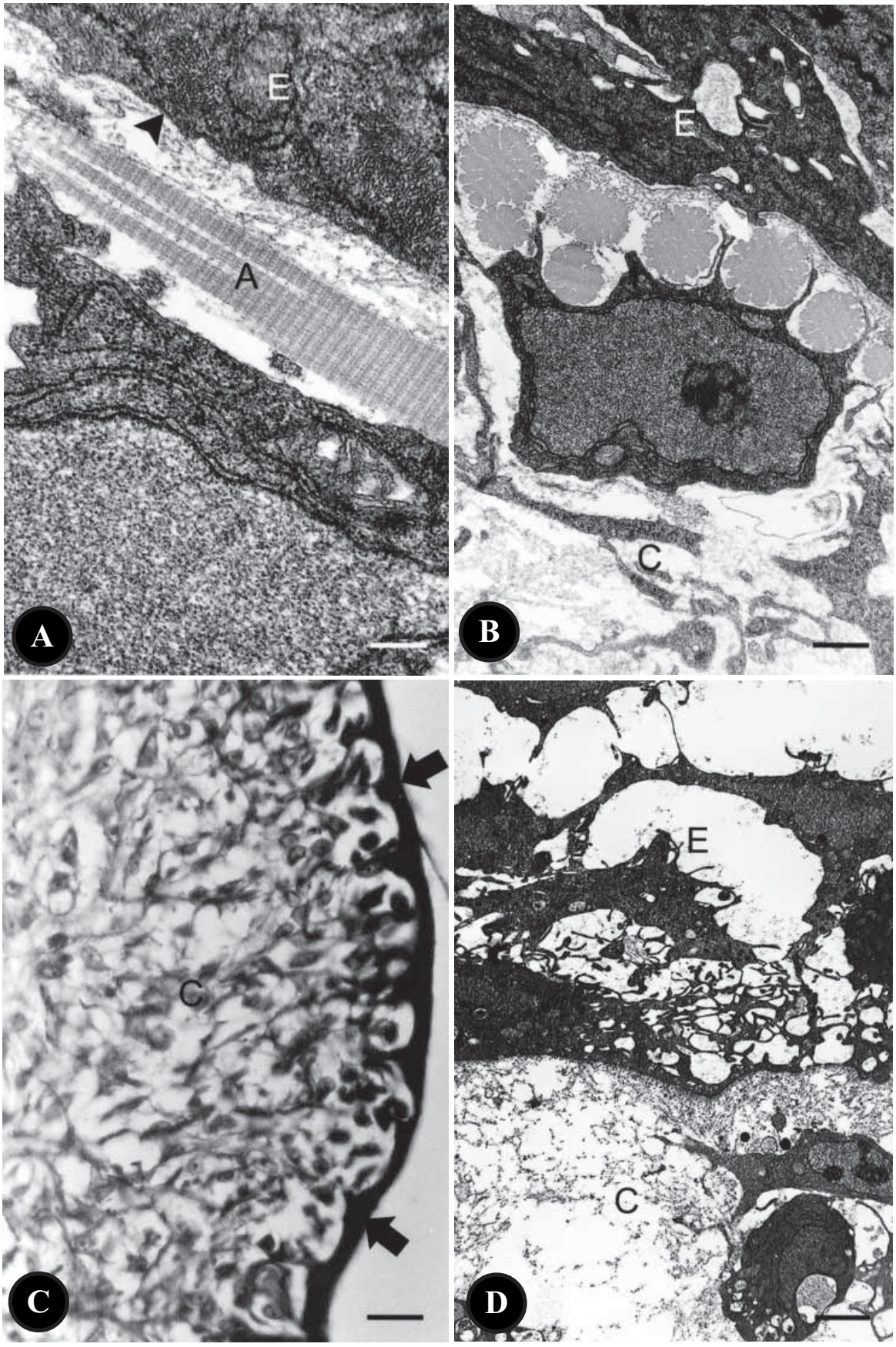
Ontogenetically, the actinotrichia are the first skeletal, non-mineralized structures which appear in developing fins [15,16]. In contrast, when regeneration is involved, the actinotrichia appear latter, around the fifth day after the lepidotrichia start to develop. The actinotrichia arise distally and always maintain close contact with the blastema, an assembly of mesenchymal-like cells inserted between the stump tissues and wounded epidermis [7,28]. Radioautographic, histochemical and ultrastructural studies have shown that actinotrichia are formed by mesenchymal-producing cells with which the actinotrichia are always closely associated [7]. Our results confirmed this intimate topographical relationship.

The actinotrichia are hyperpolymerized macrofibrils, formed from elastoidin [23]. The collagenous nature of elastoidin was confirmed by Becerra *et al.* [7] who observed a strong radioautographic signal associated with newly-formed actinotrichia after the injection of ^{3}H-proline. In addition, an increased birefringence seen with the picrosirius-polarization method indicated the presence of collagen in these structures [22], and showed that elastoidin was resistant to non-specific protease but was hydrolyzed by collagenase. Finally, the transversal banding pattern, typical of collagen fibers was confirmed by electron microscopy for elastoidin-rich actinotrichia [7].

Since actinotrichia undergo a high turnover of elastoidin during tail fin regeneration [28], and since this response is coordinated by a complex series events in which differentiating blastema (mesenchymal-like) cells are probably involved, it is plausible that a number of substances can modulate the mechanisms of ontogenesis and regeneration.

Bechara *et al.* [9] recently showed that a variety of anti-inflammatory drugs known to impair collagen synthesis/metabolism in mammals, such as dexamethasone, D-penicillamine, indomethacin, aspirin and B-aminopropionitrile, also affected collagen biosynthesis in two species of teleosts. The resulting disorganization of the collagen scaffolding of the lepidotrichial matrix lead to impaired fin regeneration. The authors suggested that the stromal histoarchitecture plays a vital role in fin regeneration. *In vivo* and *in vitro* studies have described the negative effect of ASA on collagen metabolism in mammals and mammalian cells [6,21,30,48-52]. ASA depresses

Figure 2. A. Longitudinal section of the distal region of a tail fin of control *Tilapia rendalli* observed by electron microscopy after ten days of regeneration. Note the basal lamina (**arrowhead**) of the epidermis (**E**) and an actinotrichium in longitudinal view (**A**). The regular cross-banding characteristic of collagen is also seen. Bar = 0.3 μm. **B**. Transversal section of the distal region of a tail fin of aspirin-treated *T. rendalli* after eight days of regeneration. This electron micrograph shows the epidermis (**E**), the connective tissue (**C**) and the regenerating actinotrichia (**arrows**) in specimen that had regenerated. Note the row of transversely sectioned regenerating actinotrichia immediately beneath the epidermis. Bar = 0.7 μm. **C.** Transversal section of the distal region of a tail fin of aspirin-treated *T. rendalli* after eight days of regeneration. Note the connective tissue (**C**) and the regenerating lepidotrichial matrix (arrows), and the absence of actinotrichia. Compare this figure with figure 1A. Picrosirius-hematoxylin. Bar = 40 μm. **D**. Transversal section of the distal region of a tail fin of aspirin-treated *T. rendalli* after twelve days of regeneration. This electron micrograph shows the epidermis (**E**) and connective tissue (**C**). Note the absence of actinotrichia. Bar = 0.2 μm.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the synthesis of collagen II in cultured chondrocytes by down regulating gene transcription [14]. The anti-inflammatory drug lysine acetylsalicylate also has a marked dose-dependent anti-proliferative effect on the proliferation and matrix gene expression (procollagen I and III mRNA synthesis) of keloid fibroblasts derived from human donors genetically predisposed to keloid formation [39].

Epidermis-blastema interactions during fin regeneration [29] may regulate genes such as *ptc-1* (membrane-receptor *patched1*) by the *shh* (*sonic hedgehog*) pathway [24], *msxC* (a member of the zebrafish *msx* homeobox gene family) via FGFR1 activity [40], or *msxD/msxA* in the epidermis [35]. Quint *et al*. [41] observed that amputation of the caudal fin of zebrafish stimulated regeneration of the dermal skeleton and reexpression of *shh*-signaling pathway genes. These authors studied the inhibition of *shh* signaling using cyclopamine, a steroidal alkaloid that interrupts *shh* signaling by acting on smoothened, a component of the receptor complex present on the surface of the target cell. The resulting inhibition of cell proliferation in the blastema ultimately leads to the arrest of fin growth. The exposure of regenerating fins to cyclopamine initially reduced and then inhibited fin outgrowth, and resulted in the formation of fewer or no actinotrichia along with a distal accumulation of pigment cells. These effects were accompanied by a reduction in cell proliferation within the blastema and a diminution in blastema size [41]. The phenotype of the fin regenerated after cyclopamine treatment [41] is similar to that observed after treatment with SU5402, a specific chemical inhibitor of FGFR1 phosphorylation signaling [40]. In both cases, there is a significant decrease in cell proliferation accompanied by an arrest of fin growth.

In the present study, we found that except for one case, all the other 20 fish specimens treated with ASA (0.1 g/l) failed to regenerate actinotrichia, i. e. the synthesis of elastoidin was antagonized by aspirin. Marí-Beffa *et al.* [28] showed that there is a rapid turnover of elastoidin in regenerating actinotrichia of amputated fins, and suggested that in intact fins there is probably a continuous synthesis of elastoidin distally, with a degradation of these molecules proximally. In ten ASA-treated fishes, there was no regeneration of the blastema or connective (stromal) tissue, while in the remaining 10, the blastema was unconspicuous and stromal tissue development was delayed. Thus, acetysalicylic acid probably interfered with gene transcription, with the differences in the responses to treatment reflecting individual variability. Alternatively, the absence of actinotrichia could have resulted from a loss of distalization caused by the ability of the drug to interfere with the balance between the distal synthesis and proximal degradation of elastoidin. Further experiments are needed to discriminate between an inhibitory effect on elastoidin synthesis and the activation of its degradation.

As with the *sonic hedgehog* (*shh*) gene which is involved in the formation of actinotrichia and is inhibited by the alkaloid cyclopamine, aspirin probably also interfered with the *shh* signaling pathway to adversely affect the regeneration of these structures [41]. Another experiments with a larger population of fish may substantiate the findings now obtained. On the other hand, the finding that the actinotrichia were restored only when the blastema and connective tissue were present [43], raises the possibility that the blastema is also a key element in the regeneration of elastoidin in ASA-treated fish. Our results, together with those of Bechara *et al.* [9] demonstrate that a variety of drugs can interfere with the synthesis and deposition of collagen. They also reinforce the idea that the regeneration of teleostean fins provides an excellent model for studying the effects of drugs *in vivo.* This approach could be useful for assessing the toxic effects of river pollution and chemical dumping.

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