The Journal of Basic & Applied Zoology (2012) **65**, 240–248

The Egyptian German Society for Zoology

The Journal of Basic & Applied Zoology

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Morphogenetic effects of vitamin A on the regenerating tail fin of the teleost fish, *Oreochromis niloticus*

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Received 12 March 2012; accepted 26 April 2012
Available online 24 September 2012

KEYWORDS

Regeneration;
Fin;
Teleost;
Vitamin A

Abstract The present study was performed to investigate the effects of vitamin A (VA) on tail fin regeneration of the teleost fish, *Oreochromis niloticus*. Following amputation, the tail fin undergoes a regenerative process which leads to an apparently faithful replacement, both in shape and size of the missing part. However, analysis of the fin skeleton of whole mount preparations of normal (unamputated), control, and treated tail fins revealed that, fin regenerates were not perfect copies of the missing part, and that the distance and the number of ray segments between the amputation plane and the first dichotomy were higher in treated tail fins than in control and unamputated fins. This suggests that VA can affect patterning formation of the regenerating tail fin. Vitamin A has morphogenetic effects on the regenerating fin by increasing the number of ray segments as well as it exhibits a marked reduction in the amount of tissue between rays that leads to fusion of adjacent rays.

Introduction

Human organs are subjected to a variety of injuries, but have a limited ability to heal and regenerate the damaged or lost tissue. Natural scientists have actively pursued the problem of regeneration since the 17th century, largely by utilizing lower vertebrate species possessing exceptional regenerative capacities (Dinsmore, 1991). Newts are the primary experimental model used to study vertebrate regeneration, as they can regrow a striking number of adult structures including limbs, tail, spinal cord, jaws, tongue, lens and optic nerve (Brockes, 1997; Ferretti and Géraudie, 1998).

Zebrafish has proved to be a valuable laboratory model of teleost fish for understanding many aspects of vertebrate embryogenesis. Small- and large-scale mutagenesis screens have yielded hundreds of interesting mutants, from which dozens of genes essential for ontogeny have been identified (Driever et al., 1996; Gaiano et al., 1996; Haffter et al., 1996; Zhang et al., 1998). Somewhat overlooked is the fact that, teleost fish as in zebrafish, can regenerate an impressive number of structures as adults, such as spinal cord, optic nerve, heart muscle, scales, and each of five types of fins (Johnson and Weston, 1995; Poss et al., 2000; Raya et al., 2003; Becker et al., 2004).

Fin regeneration can be broken down into four stages. First, epidermal cells migrate to cover the wound and form a

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Peer review under responsibility of The Egyptian German Society for Zoology.

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2090-9896
http://dx.doi.org/10.1016/j.jobaz.2012.04.001

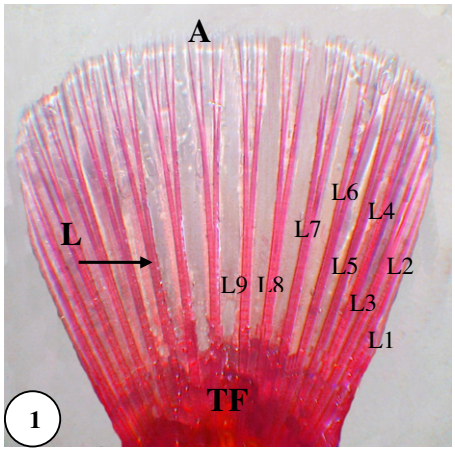

Figure 1 Photomicrograph of whole-mount Alizarin preparation of the normal (unamputated) tail fin (TF) of *O. niloticus* showing actinotrichia (A) and lepidotrichia (from L_1 to L_9). X: 10.

multilayered cap. Second, mesenchymal tissue beneath the new epidermis disorganizes, or dedifferentiates, and mesenchymal cells migrate distally toward the amputation plane. Third, these cells proliferate and accumulate to form the regeneration blastema, a tissue mass from which the new fin structures are ultimately derived. Fourth, regeneration is completed by a phase of outgrowth, composed of exquisitely integrated proliferation, patterning and differentiation events (Poss et al., 2000; Poleo et al., 2001; Abdel-Karim et al., 2003).

It is well documented that, the positional memory of the limb blastema can be respecified by treatment with the retinoid VA and its derivatives (Niazi and Saxena, 1978; Maden, 1982; Brockes, 1989; Stocum, 1991; Bryant and Gardiner, 1992). One of these compounds, retinoic acid (RA), has stimulated particular interest because of its multiple effects on many developing systems (Tickle et al., 1982; Tamarin et al., 1984; Sulik et al., 1988; Holder and Hill, 1991; Ruizi-Altaba et al., 1991; Sundin and Eichele, 1992; Morriss-kay, 1993), and of the identification of numerous genes encoding for RA nuclear receptors (Giguere et al., 1987; Petkovich et al., 1987; Benbrook et al., 1988; Zelent et al., 1989; Mangelsdorf et al., 1990, 1992; Rowe et al., 1991; Ragsdale et al., 1992) and cytoplasmic RA binding proteins (Shubeita et al., 1987; Stoner and Gudas, 1989), that suggest an endogenous role for this compound during morphogenesis. VA induces the complete regeneration of lung alveoli that have been destroyed by various noxious treatments. In these cases, VA is required for the development of the organ (Maden and Hind, 2003, 2004). Also, retinyl esters (Res), the major storage form of VA (retinol), provide substrates for the production of bioactive retinoids, including RA, which are known to promote lung development and maturation (Ross and Ambalavanan, 2007).

Retinoic acid is the biological active derivative of VA. It is an important signal for patterning the hind brain, the branchial arches and the limb bud. RA is thought to act on the posterior hindbrain and the limb bud; at somitogenesis stages in chick and mouse embryos (Grandel et al., 2002). Also, it is required to determine proximodistal identity in the developing mouse fore limb (Yashiro et al., 2004). Retinoic acid is required for a variety of processes during vertebrate embryonic development. Its effect is transmitted by RA receptors (RARs) at the level of regulating the expression of target genes. Three enzymes, Aldh 1a 1–3, catalyze the final oxidative step by which VA (retinol) is converted to RA, but only Aldh 1a 2 has been shown to be responsible for RA synthesis during the early stage of embryogenesis (Gibert et al., 2006; Bokelmann et al., 2010; Tu and Johnson, 2011).

Materials and methods

The present investigation was performed on the freshwater teleost *Oreochromis niloticus*. Specimens of the fish were collected from El-Abbasa fish farm, near Zagazig city. The body length ranged from 4.6 to 10.3 cm and the weight from 3.8 to 19.3 g. The fishes were placed into aerated aquaria under conditions of room temperature at about 27 ± 3 °C, the photoperiod was 12 h of light per day. Prior to fin amputation, fishes were anaesthetized with MS222 (ethylaminobenzoate methane sulfonate) dissolved in tap water (1 mg/l). Tail fins were amputated with microscissors at a proximal level; removing 70% of the fin. Two hundred specimens of the fish have been studied for their capacity for morphological restoration of the fin following amputation. Fishes were divided into two groups. The first (Control) group was exposed to a water medium containing 1 ml/l (ethyl alcohol), the same concentration of the solvent. The second (treated) group, was exposed to VA palmitate dissolved in 1 ml/l ethyl alcohol. The rearing medium containing VA palmitate at the definitive concentration has been previously prepared as follows: an ampule of VA palmitate containing 1 ml of vitamin A (equivalent to 300,000 IU), was

Table 1 The number of segments of different lepidotrichia of the tail fin of normal, control and vitamin A-treated fishes after 15 days of amputation.

Lepidotrichia (L)	Number of segments (means ± SD)				
	Normal (unamputated)	Control	Vitamin A-treated groups		
			2 IU/ml of VA	4 IU/ml of VA	8 IU/ml of VA
L_1	15.6 ± 0.6	15.2 ± 1.25	15.0 ± 1.32	15.2 ± 1.32	15.4 ± 1.37
L_2	18.0 ± 0.8	19.0 ± 1.41	22.2 ± 1.26	20.4 ± 1.23	21.4 ± 1.28
L_3	17.0 ± 0.5	15.6 ± 1.34	18.6 ± 0.6	17.4 ± 0.8	21.6 ± 0.96
L_4	15.4 ± 0.9	14.2 ± 1.27	18.4 ± 0.9	16.6 ± 1.26	17.8 ± 1.27
L_5	15.2 ± 1.07	11.4 ± 0.6	18.8 ± 1.45	15.4 ± 0.85	16.2 ± 0.93
L_6	17.6 ± 1.09	12.8 ± 0.9	14.2 ± 1.31	15.2 ± 1.09	19.0 ± 1.24
L_7	19.8 ± 1.1	12.2 ± 1.5	13.0 ± 1.22	18.6 ± 0.99	17.0 ± 1.23
L_8	18.0 ± 1.32	13.6 ± 1.05	15.4 ± 1.03	15.4 ± 1.3	20.2 ± 1.12
L_9	18.4 ± 1.46	14.0 ± 1.45	14.8 ± 1.5	17.4 ± 0.75	21.4 ± 1.01

Table 2 The number of segments of different lepidotrichia of the tail fin of normal, control and vitamin A-treated fishes after 30 days of amputation.

Lepidotrichia (L)	Number of segment (means ± SD)				
	Normal (unamputated)	Control	Vitamin A-treated groups		
			2 IU/ml of VA	4 IU/ml of VA	8 IU/ml of VA
L_1	15.6 ± 0.6	15.4 ± 1.5	15.2 ± 0.9	15.2 ± 1.5	15.8 ± 1.49
L_2	18.0 ± 0.8	21.0 ± 1.3	21.6 ± 1.3	23.4 ± 1.02	24.4 ± 1.45
L_3	17.0 ± 0.5	14.6 ± 1.2	18.4 ± 1.07	16.6 ± 1.03	25.6 ± 1.25
L_4	15.4 ± 0.9	15.2 ± 0.9	16.0 ± 1.09	15.8 ± 1.34	23.4 ± 0.55
L_5	15.2 ± 1.07	14.4 ± 1.4	17.4 ± 1.11	14.6 ± 0.6	18.2 ± 1.3
L_6	17.6 ± 1.09	15.8 ± 0.5	15.6 ± 0.8	16.6 ± 1.26	21.2 ± 1.2
L_7	19.8 ± 1.1	16.2 ± 1.07	16.2 ± 1.36	17.2 ± 0.8	17.4 ± 1.06
L_8	18.0 ± 1.32	17.6 ± 0.9	18.0 ± 1.47	17.4 ± 1.45	18.0 ± 1.03
L_9	18.4 ± 1.46	16.0 ± 1.11	18.6 ± 0.5	18.0 ± 1.2	18.2 ± 0.85

Table 3 The number of segments of different lepidotrichia of the tail fin of normal, control and vitamin A-treated fishes after 45 days of amputation.

Lepidotrichia (L)	Number of segment (means ± SD)				
	Normal (unamputated)	Control	Vitamin A-treated groups		
			2 IU/ml of VA	4 IU/ml of VA	8 IU/ml of VA
L_1	15.6 ± 0.6	15.2 ± 1.25	15.0 ± 1.32	15.2 ± 1.32	15.4 ± 1.37
L_2	18.0 ± 0.8	19.0 ± 1.41	22.2 ± 1.26	20.4 ± 1.23	21.4 ± 1.28
L_3	17.0 ± 0.5	15.6 ± 1.34	18.6 ± 0.6	17.4 ± 0.8	21.6 ± 0.96
L_4	15.4 ± 0.9	14.2 ± 1.27	18.4 ± 0.9	16.6 ± 1.26	17.8 ± 1.27
L_5	15.2 ± 1.07	11.4 ± 0.6	18.8 ± 1.45	15.4 ± 0.85	16.2 ± 0.93
L_6	17.6 ± 1.09	12.8 ± 0.9	14.2 ± 1.31	15.2 ± 1.09	19.0 ± 1.24
L_7	19.8 ± 1.1	12.2 ± 1.5	13.0 ± 1.22	18.6 ± 0.99	17.0 ± 1.23
L_8	18.0 ± 1.32	13.6 ± 1.05	15.4 ± 1.03	15.4 ± 1.3	20.2 ± 1.12
L_9	18.4 ± 1.46	14.0 ± 1.45	14.8 ± 1.5	17.4 ± 0.75	21.4 ± 1.01

dissolved in 1 ml of absolute ethyl alcohol in a test tube. After shaking well to ensure complete dissolving, the content was transferred to a dark bottle and completed to 500 ml with tap water. With great care, the bottle was enveloped in a sheet of aluminum foil paper and kept in a refrigerator to be used at the time of operation.

Three different concentrations of the VA were prepared for the present study using tap water; 2, 4 and 8 IU/ml. Immediately after amputation of the tail fins, the operated fishes were transferred to water medium for recovery (about 5 min), then gently to the desired concentrations of VA where they were kept for three days, and renewed the concentrations in the second and third days in order to maintain proper concentrations of the vitamin. For each experimental type, after handling with the vitamin, the fishes were allowed to continue their development in normal tap water.

Mortality

Three high concentrations of VA, 70, 30 and 10 IU/ml were used, respectively to determine the mortality rate of fishes immediately after amputation of the tail fins.

Morphological analysis

Whole-mount preparations of the normal (control) and treated tail fin specimens were stored in 95% alcohol for studying of gross morphological changes. In order to analyze the fin skeleton, whole-mount preparations of regenerated fins were stained with Alizarin Red-S (Taning, 1944).

Results

Morphological structure of the normal (unamputated) tail fin

The tail fin is a medial structure consisting of two lobes, which are nearly perfect mirror images of each other along the dorsoventral axis. The skeletal structure of the adult tail fin is clearly visible in whole-mount preparation. The skeleton of each lobe is made up of nine major lepidotrichia. These lepidotrichia of both dorsal and ventral lobes, except the outermost ones, are branched. A variation in the number of dichotomies in equivalent rays from different tail fins is observed in all of the other rays furthermore, a different number of dichotomies is occasionally apparent within the same tail fin between rays in the dorsal lobe and their counterparts in the ventral one (Fig. 1).

The arrangement of the lepidotrichia (L) and their numbers (number of segments before the first dichotomy) of normal (unamputated) tail fin is represented in Table 1.

Effect of vitamin A on the morphology of the regenerating fins

It was observed that the number of segments proximal to the first dichotomy was rather constant, and that of tail fin

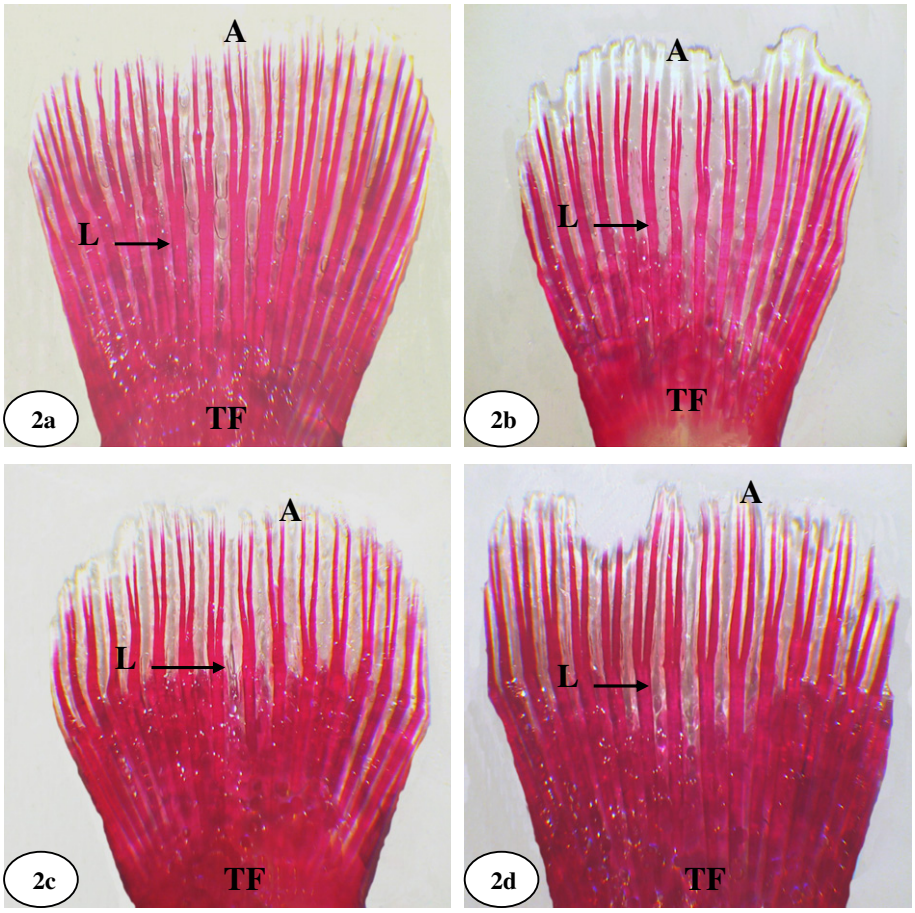

Figure 2 Photomicrographs of whole-mount Alizarin preparation of the regenerating tail fins (TF) of *O. niloticus* after 15 days of amputation showing actinotrichia (A) and lepidotrichia (before the first dichotomy) from L_1 to L_9. (a) The tail fin of the control. X: 10. (b) The tail fin of a fish treated with 2 IU/ml of VA. X: 10. (c) The tail fin of a fish treated with 4 IU/ml of VA. X: 10. (d) The tail fin of a fish treated with 8 IU/ml of VA. X: 10.

specimens, treated with VA was significantly increased following amputation. Morphometrical analysis was performed in tail fins and it was observed that VA can induce distalization of the first dichotomy by increasing the number of segments proximal to it. There was a clear difference between the number of segments counted in the tail fins of control fishes and those treated ones with VA after 15, 30 and 45 days of amputation (Tables 1–3).

Mortality and abnormality resulted by vitamin A treatment

Vitamin A can modify patterning of the regenerating fins of fishes which were treated with different concentrations of VA immediately after amputation for three days. Under all the conditions of treatment, there was no observed difference between fishes, which are left to regenerate in water or in water containing the VA solvent (alcohol). On the contrary, when treated with 70 IU/ml of VA immediately after amputation all fishes died immediately. However, 80% of fishes died within 1 h from the amputation when treated with 30 IU/ml of VA and 50% of fishes died within 12 h from the amputation when treated with 10 IU/ml of VA. On the other hand, the treatment with lower concentrations (2, 4 and 8 IU/ml) of VA did not affect survival, but can promote the regeneration process of the amputated tail fins.

It was observed that, VA apparently does not induce any significant change of pattern on the anteroposterior axis, but it can affect both the proximodistal and dorsoventral axes of the regenerating fins. Vitamin A appears to induce morphogenetic effects that do not result in any external malformation, seems specific to the proximodistal axis and is targeted to the bone, where the formation of extra segments was induced. However, none of the rays grows longer than its respective control, that VA cannot alter the presumably tight regulation controlling the concerted growth of individual rays. The major difference between control and treated fins lies in the position of the first dichotomy, which is always more distally located in the treated than in control specimens (Figs. 2–4).

Also, VA induced effects along the dorsoventral axis by reducing the amount of tissue between rays (Fig 5a and d). Unlike growth of interray tissues, growth of the skeleton was not inhibited, and the skeletal abnormalities observed were seen rather to be a consequence of decreased amount of tissue between rays, that leads to fusion of adjacent rays (Fig. 6a and d).

Discussion

The present work is concerned with the study of the effect of VA on the tail fin regeneration in the teleost, *O. niloticus*. The amputation was carried out through the tail fins at a prox-

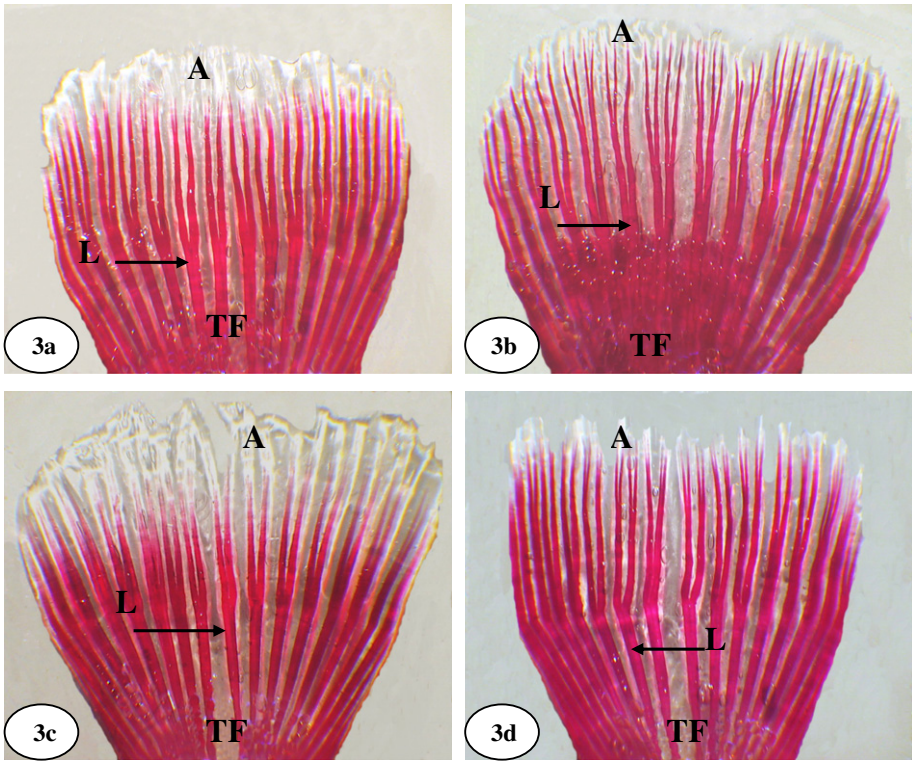

Figure 3 Photomicrographs of whole-mount Alizarin preparation of the regenerating tail fin (TF) of *O. niloticus* after 30 days of amputation showing lepidotrichia (L_1 to L_9) before the first dichotomy and actinotrichia (A). (a) The tail fin of the control. X: 8. (b) The tail fin of a fish treated with 2 IU/ml of VA. X: 8. (c) The tail fin of a fish treated with 4 IU /ml of VA. X: 8. (d) The tail fin of a fish treated with 8 IU/ml of VA. X: 8.

imal level (approximately 70% of the fin was removed). Regeneration process was described morphologically during various regeneration stages.

It is known that, the fish fin is the system of non-proliferating or slowly dividing tissue, but has a capacity for rapid cell proliferation in an emergency when partial amputation of the tissue is carried out (Hama, 1979).

The present investigation confirmed the previous studies concerning the morphological structure of the fin. The current work has demonstrated that the tail fin consists of a number of bony fin rays (lepidotrichia). Most of these rays are branched into dichotomies ended finally with actinotrichia. The same description was observed in the zebrafish caudal fin which is composed of multiple bony fin rays or lepidotrichia, most of which are bifurcated at the ends (Montes et al., 1982; Becerra et al., 1983; Santamaría and Becerra, 1991; Géraudie and Singer, 1992).

The present investigation revealed that VA can modify patterning of the regenerating fins of fishes, which were treated with different concentrations of VA immediately after amputation for about three days. The present results are in agreement with the observations of Brockes (1990), who reported that the alterations in regeneration pattern caused by treatment with RA have been well characterized in the newt, along the proximodistal axis of the regenerate, and results in serial pattern duplication. Also, Holder and Hill (1991) showed that RA affects pattern formation during development and regeneration of the ray in zebrafish embryos. Grandel et al. (2002) reported that much earlier requirements for RA signaling during presegmentation stage for proper development of these structures in zebrafish, is necessary for anteroposterior patterning in the pectoral fin. Maden and Hind (2003) observed that RA acts on the nucleus to induce gene transcription. In amphibians and mammals, it induces the regeneration of several tissues and organs. Retinoic acid induces the "super-regeneration" of organs that can already regenerate such as the urodele amphibian limb by respecifying positional information in the limb. Retinoic acid is used for both development and regeneration. This suggestion, therefore might serve as a strategy for identifying potential tissue or organ targets that have the capacity to be stimulated to regenerate. Yashiro et al. (2004) suggested that a gradient of RA signaling is required to determine proximodistal identity in the developing mouse forelimb. Similar results were obtained by Gibert et al. (2006), they have found that RA is required for a variety of processes during vertebrate embryonic development.

The present work revealed that the morphogenetic effect of VA, that does not result in any external malformation, seems specific to the proximodistal axis and is targeted to the bone, where formation of extra segments is induced. The major differences between control and treated fishes lie firstly, in the distance between the planes of amputation and the first dichotomy was significantly longer in different concentrations of VA treated (2, 4 and 8 IU/ml) than in control fishes. Then, VA treatment can induce distalization of the first dichotomy by increasing the number of segments proximal to it and second, in the absence or reduce number of segments of the second dichotomy. Furthermore, the possibility that normal number of segments of the second dichotomy may be re-established over a long period of time during the regeneration process. This assumption was suggested also by Crawford and Stocum

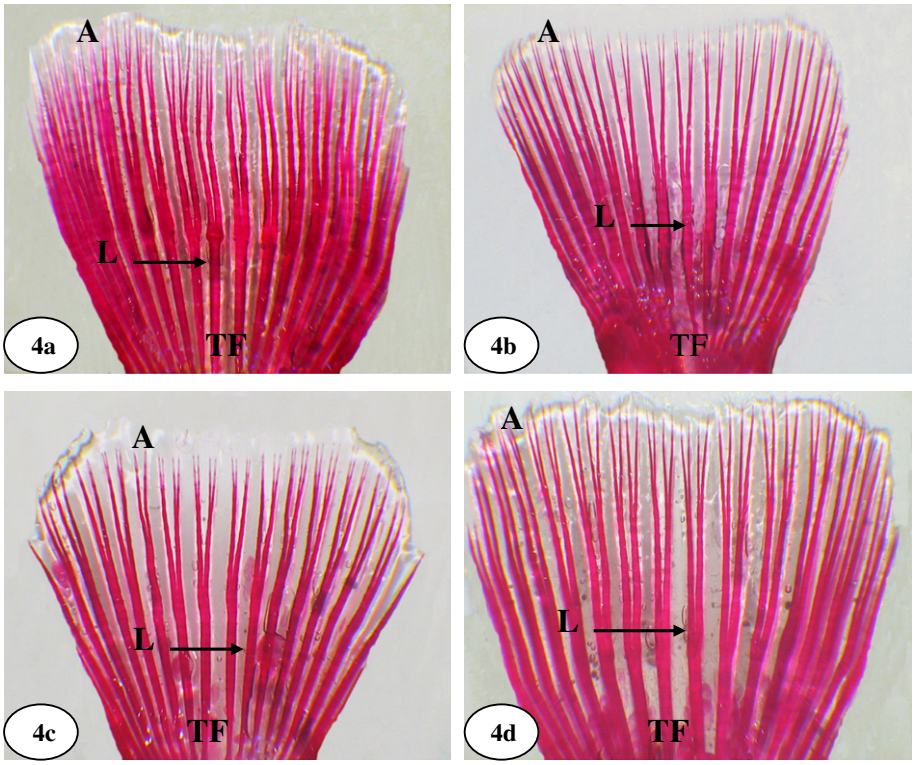

Figure 4 Photomicrographs of whole-mount Alizarin preparation of the regenerating tail fin (TF) of *O. niloticus* after 45 days of amputation showing the number of lepidotrichia (L_1 to L_9)and actinotrichia (A). (a) The tail fin of the control. X: 8. (b) The tail fin of a fish treated with 2 IU /ml of VA. X: 8. (c) The tail fin of a fish treated with 4 IU /ml of VA. X: 8. (d) The tail fin of a fish treated with 8 IU/ml of VA. X: 8.

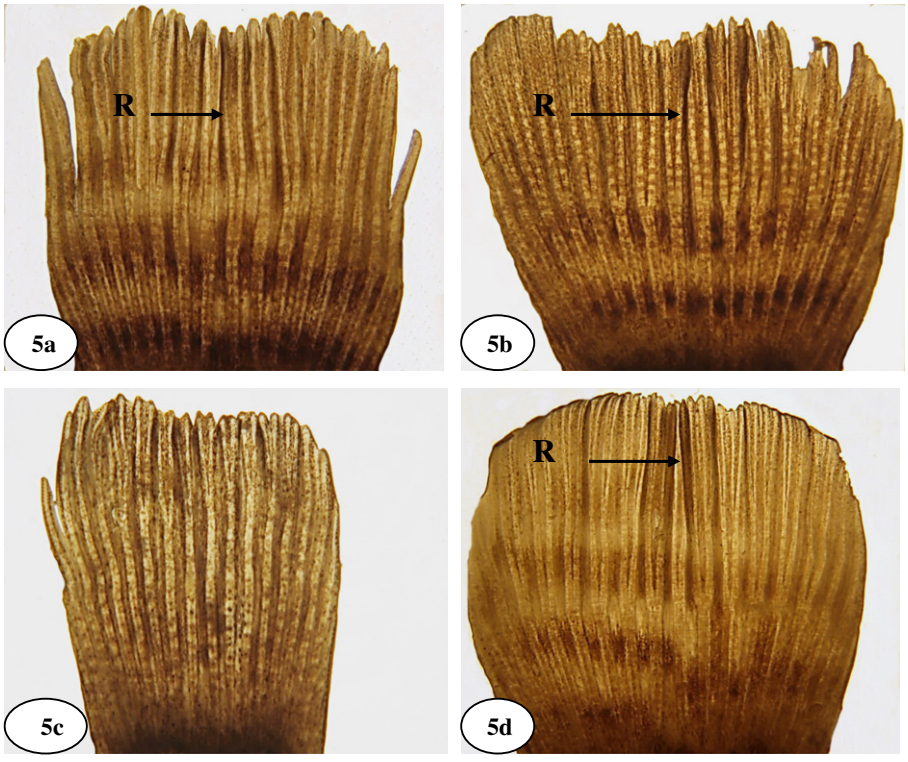

Figure 5 Photomicrographs of whole-mount preparation of regenerating tail fin (TF) of *O. niloticus* showing reduction (R). (a) The tail fin of a fish treated with 2 IU/ml of VA, after 30 days of amputation. X: 8. (b) The tail fin of a fish treated with 2 IU/ml of VA, after 30 days of amputation. X: 8. (c) The tail fin of a fish treated with 4 IU/ml of VA, after 30 days of amputation. X: 8. (d) The tail fin of a fish treated with 8 IU/ml of VA, after 45 days of amputation. X: 8.

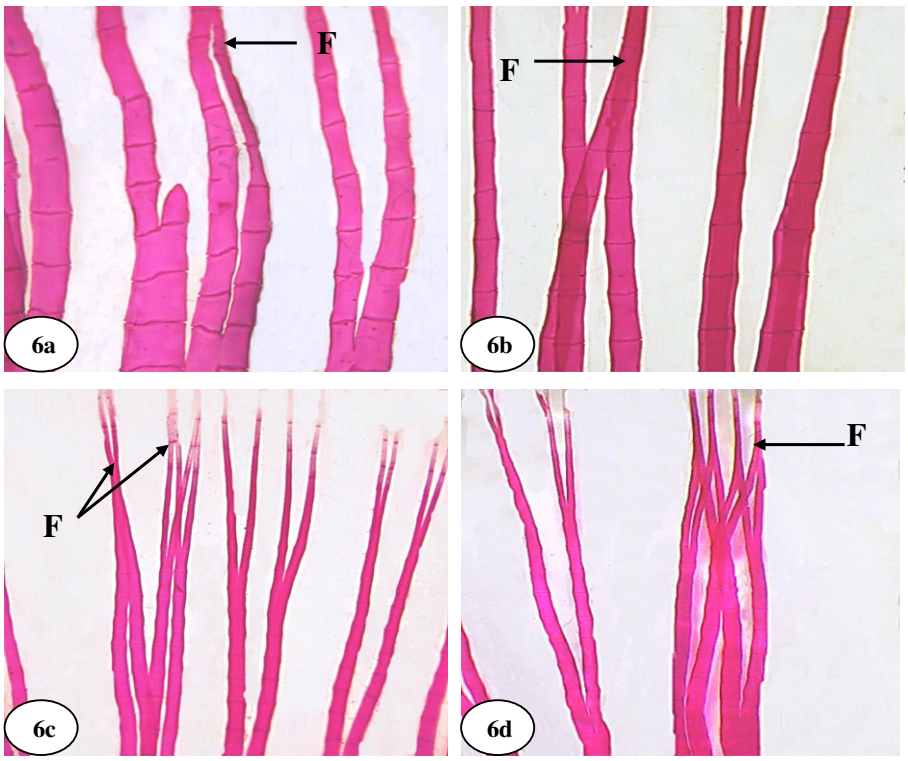

Figure 6 Photomicrographs of whole-mount Alizarin preparation of regenerating tail fin (TF) of *O. niloticus* showing fusion of fin rays (F). (a) The tail fin of a fish treated with 2 IU/ml of VA, after 30 days of amputation. X: 30. (b) The tail fin of a fish treated with 2 IU/ml of VA, after 45 days after amputation. X: 30. (c) The tail fin of a fish treated with 4 IU/ml of VA, after 45 days of amputation. X: 20. (d) The tail fin of a fish treated with 8 IU/ml of VA, after 45 days of amputation. X: 20.

(1988), they have reported that, the increased number of segments present before the first dichotomy in control and RA treated regenerates represent a proximalization of the positional memory of the blastema as in the newt limb. Moreover, it is possible that the origin of the extra segments differs in normal and RA treated regenerates. Similar results were obtained by Stocum (1991). The author reported that in the regenerating limb, RA induces formation of supernumerary structures that are more proximal than those removed by amputation, and this has been interpreted as respecification of the blastema positional memory. Again, Géraudie et al. (1994, 1995) reported that treatment of a regenerating pectoral fin in zebrafish with RA, however, does not induce formation of an extra long fin, but at certain doses and times of treatment it induces formation of more segments between the amputation level and the first fork (morphogenetic effect) than that observed in control regenerates.

The results of the present work indicated that VA induced effects along the dorsoventral axis by reducing the amount of tissue between rays, that leads to fusion of adjacent rays. Also, Santamaría et al. (1993) observed the results of experimental manipulations such as partial, hemiray, or individual ray amputations and chemical administration (RA), which cause fin rays fusions in teleost fish (zebrafish). Similar results were obtained by Géraudie et al. (1994, 1995). They demonstrated that RA could affect the regeneration of pectoral fin in zebrafish and has teratogenic effect on the regenerating fin (narrowing of the fin and fusion of ray segments). White et al. (1994) reported that treatment of zebrafish with RA during fin regeneration, where a single ray bifurcates into two individual 'daughter' rays. Retinoic acid treatment causes a dichotomy reduction where the two 'daughter' rays fuse once again to form a single ray. The single ray subsequently bifurcates in a comparatively normal manner. Also, this assumption was suggested by Ferretti and Géraudie (1995, 1998). The latter authors stated that RA is most effective in inducing teratogenesis after the phase of accumulation of blastema cells has been completed. The teratogenic effects observed may be the result of significant RA induced apoptosis in the wound epidermis, which would decrease its width and consequently affects patterning of the underlying blastemal mesenchyme in the zebrafish fin. It was also described by Laforest et al. (1998), that amputations of caudal fins immediately after the first branching point of the lepidotrichia, and global administration of all-trans-retinoic acid, two procedures known to cause fusion of adjacent rays, result in a transient decrease in the expression of shh (sonic Hedgehog), ptc1 (patched1) and bmp2 (bmp2 genes). The effects of retinoic acid on shh expression occur within minutes after the onset of treatment suggesting direct regulation of shh by RA. These observations suggest a role for shh, ptc1 and bmp2 in patterning of the dermoskeleton of developing and regenerating teleost fins of zebrafish.

The present results have revealed that different concentrations of VA affect fins regeneration. When fishes were treated with lower concentration of VA (2, 4, and 8 IU/ml) immediately after amputation, did not significantly affect survival but can affect the regeneration process of the fins. However, in fishes treated with high concentrations of VA (70, 30 and 10 IU/ml) the mortality rate varies according to the concentration of VA. In fishes treated with 70 IU/ml of VA all fishes

died immediately. In fishes treated with 30 IU/ml of VA, 80% of fishes died within one hour from amputation while only 50% died within 12 h from amputation when treated with 10 IU/ml of VA. The present results are also are in agreement with the observations of Géraudie et al. (1994), who reported that all fishes which were treated with 10^{-4} MRA, immediately after amputation, died within 1 h, while 50% died within 24 h from the amputation when treated with 10^{-5} MRA. Treatment with lower concentrations of RA did not significantly affect survival, but 5×10^{-6} and 10^{-6} MRA could affect regeneration. A little or no effect on regeneration was observed with 10^{-7} and 10^{-8} MRA. The treatment with 10^{-6} MRA always slowed down the regenerative process. Also, Géraudie and Ferretti (1997) found that the injection of 100 mg/g body weight dose of RA into zebrafish was lethal within 24 h after injection. But, in the case of the injection of 10 and 5 mg/g of RA, 25% and 75% of the treated animals, respectively, were still alive 5 days after injection.

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