Cyclosporine A Promotes Regeneration in Larval Stages of the Egyptian Toad, *Bufo regularis*

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Abstract: The regeneration of the amputated hind limbs of stage (54) of tadpole larvae of *Bufo regularis* was studied after amputating their hind limbs at the mid shank level. The immunomodulator Cyclosporine A (CsA) was selected to determine its effect on regeneration. Tadpole larvae were immersed in a solution of CsA (0.02, 0.2 and 0.5 mg/mL) for five hours daily for three consecutive days starting the treatment twelve hours after amputation. Cyclosporine A significantly promoted regeneration and it was dose-dependent. Histologically, Cyclosporine A was associated with acceleration in the processes of dedifferentiation, differentiation and histogenesis in comparison with their control counterparts. It also delayed the dermal differentiation underneath the epithelium. CsA treatment was also associated with early dedifferentiation of mesenchyme cells forming a well-formed blastema, and early differentiation of skeletal elements of the regenerates in comparison with their control counterparts. The result of the present work was that regeneration of hind limbs was significanlty promoted and accelerated by Cyclosporine A. In addition, Cyclosporine A effect was dose-dependent. We also consider that these data will give us deeper insights on the mechanisms taking place in higher vertebrates and in human.

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1. Introduction

Regeneration is the ability of the fully developed organism to replace lost part by growth or remodeling of somatic tissue. When lost parts are built (from a population of undifferentiated cells) into correctly patterned structures, it is called epimorphosis. This occurs in the tails or limbs of amphibia, in the head and tails of different types of worms and in the arms of starfishes (Baguña, 2001; Tuma *et al*., 2008).

Mammals can regenerate more than 1/3 of the liver, but not in the same shape as the original, the liver is able to accomplish the goals of re-establishing its mass while it maintains its functional capacity during regeneration (Taub, 1996; Fouzas *et al*., 2008).

Limb regeneration in amphibians provides system for the study of the formation of a complex pattern of skeletal and soft tissue elements. Information for this pattern is believed to lie in the cells close to the amputation plane and to be present in both the dermis and underlying muscles (Tank and Holder, 1981; Abbott *et al*., 2008).

The immune system affects morphogenetic or differentiation events of regeneration primarily and possible have secondary effects on proliferation and growth of the regenerate (Sicard, 1981a). Many investigators noted differential effects of immune status on tumor growth. Based on certain parallels between regeneration and tumor, it was suggested that the immune system might exert influence over the events of regulation of limb regeneration in amphibians. The mild immunostimulation should enhance limb regeneration in a fashion not unlike that by which mild immunostimulation enhances tumor growth (Sicard, 1981b).

Cyclosporine A (CsA) is the most frequent immunosuppressant drug widely used in post-allogeneic organ transplant to reduce the activity of the patient's immune system and so decreases the risk of organ rejection. It is also used in treatment of autoimmune disease. It is known to decline the immune system in mammals (Alvira *et al*., 2002; Andres and Cascales, 2002; Merlini *et al*., 2007). It has been studied in transplants of skin, heart, kidney, liver, lung, pancreas, bone marrow, and small intestine.

Several studies suggested that immunostimulation can promote tumor growth (Prehn, 1970, 1972; Fidler, 1973) and might enhance the rate of regeneration (Andrew, 1976). Thus the immune system affects morphogenic or differentiative events of regeneration primarily and might possibly have secondary effects on proliferation and the growth of regenerating limbs in adult newts. Sicard (1983) also noted slight elevation in granulocytes/monocytes counts through limb regeneration in newts. Moreover, Sicard *et al*. (1985) showed that the immune status was altered during regeneration. Dhawan *et al*. (2010) also found that

the role of CsA was in augmenting the regenerative potential of allograft by eliminating immune reactions.

Previous studies showed manupulations and/or drugs that delayed or inhibited forelimb regeneration in adult newts possibly by altering the animal's immune status. For example, splenectomy (Fini and Sicard, 1980), X-irradiation (Sicard and lombard, 1990), Cobra venum factor and anti-lymphatic serum (Sicard, 1981 a&b), and skin allograft challenge itself delayed regeneration when presented at the time or before amputation (Sicard *et al*. 1986). Since these manipulations could affect the immune status in mammals and did produce effects on regeneration, it was unclear whether the effect on regeneration was achieved by alteration of the immune status in amphibians on not.

Stage 54 of the tadpole larvae of *Bufo regularis* was selected to investigate the effect of immunomodulator Cyclosporine A (CsA) on hind limb regenerative ability.

The aim of the present study was to investigate the effect of immunomodulator CsA on the hind limb regenerative ability of the Egyptian tadpole larvae, *Bufo regularis*. The present work certainly clarified the effect of CsA and its correlation with the progress of limb regeneration in anuran tadpoles. We also consider that these data will give us deeper insights on the mechanisms taking place in higher vertebrates and in man.

2. Material and Methods

Animals: Groups of tadpole larvae of *Bufo regularis* were reared in number of glass aquaria with fairly large quantities of water with Elodea plant which act as natural ventilator. The tadpoles were provided with boiled spinach and small liver tissue for feeding. Stage 54 was selected according to the normal table of Sedra and Michael (1961).

Experimental Design: Before the operation each individual was transferred to an anesthetic medium of chloretone solution (0.5 mg/mL). The operations as well as the experiments were initiated by amputation through the midshank level of the left hind limb, while the right hind limb was left intact. Amputation was performed using a pair of iridectomy scissors under a binocular microscope. Each operated case was then transferred to a petri-dish containing half-concentration of the anesthetic medium (0.25 mg/mL), where it was kept for 5 – 10 minutes for partial recovery, and then it was transferred to dechlorinated tap water for complete recovery.

Tadpoles (10/dish) were immersed in 100 mL of CsA solution (0.02, 0.2 and 0.5 mg/mL) for 5 hours daily for three consecutive days. The first treatment was administered twelve hours after amputation. Control cases were bathed in stock of tap water.

Histological Procedure: The final operated cases were reared for a period ranging from 2 to 6 weeks. To clarify the early post-operative histological changes, several individuals were fixed at regular interval on time series 1, 3, 5 and 7 days for each experiment. The limb stumps of the time series were sectioned at 7 microns and stained with Haematoxylin and Eosin for histological investigation. The skeletal configuration of regenerates ending was studied by using Victoria blue stain.

Statistical Analysis:

Statistical analysis was done using Z-test, used for comparisons between two parameters. Differences will be considered significant at (p<0.05).

3. Results

1- Normal cases (control):

a. Histogenesis of the time series:

By the first day, the wound area was covered with two layers of cuboidal cells forming the epidermis having apical knob at the apex of epidermis, which consisted of five to six layers of cells. No dermis was found. Few amount of cellular debris were also found.

By the third day, the epidermis still consisted of two layers of cuboidal cells. Large number of embryonic cells accumulated proximal to stump, forming a poorly formed blastema (Fig. 1).

By the fifth day, the epidermis thickening didn't change. Basement membrane was found. The dermis was well developed underneath the entire epidermis. The tibiofibula was restored and differentiation of skeletal elements was obvious at the distal part of tibiofibula.

By the seventh day (Fig. 2), differentiation process was obvious. The tibiofibula restored its missing part, several skeletal elements of the autopdial region were found forming the astragalus and calacaneum and phalanges. Large amount of myoblasts were differentiating forming muscle.

b- General morpholgical characteristics of final cases (see Table 1 and Fig. 5)

Eighty nine cases were operated. Of these, five cases developed limb outgrowths ending with five toes with a prehallux each, in which one of these cases had the first and the second syndactylous toes. Thirty five cases completed regeneration ending with four toes. Seventeen cases of them were deformed. Four cases showed syndactyly either between fourth and fifth toes or second and third toes. Another four cases showed bent terminal phalanges either in all toes or fourth or fifth toe. Five cases showed smaller sized foot alone or foot and shank together. Another

four cases showed either thicker or thinner tarsus in comparison with the normal size. One of the thick tarsus cases formed an empty distance between the fourth and the fifth toes.

Nine of the control cases regenerated the shank and the foot region ended with three toes. Two of them ended with normal shank, foot and toes. The rest of cases were malformed. Two cases had smaller sized foot, in which one of them showed an empty distance between the third and the fourth toes. Five cases showed bent foot axially, dorsally, or ventrally. Five of them regenerated two toes, all of them regenerated part of foot, one of them was bent towards the right and the other had the two toes syndactylous towards the left side. Fifteen of them had regenerated one toe (Fig. 3), five of them regenerated reduced foot with obvious toe. Another case regenerated shank with knob end. Nine of fifteen cases regenerated shank region carrying either toe like protrusion, skin fold or spike like protrusion. Six cases had regenerated shank region only. Three cases had regenerated part of shank region. Nine cases failed to regenerate.

Victoria blue transparencies induced the skeletal structure of four cases. The first case showed reduced tarsus ending with two toes one of them had only metatarsus; the other had metatarsal parts and four phalanges. In the second case (Fig. 4) the tibiofibula was completely restored, but the autopodial skeletal elements were highly reduced carrying one toe that has four phalanges. The third case showed restored tibiofibula, but at the amputation level the tibia became separated from tibiofibula. The foot was restored carrying a small protrusion, metatarsus and their phalanges were present supporting one toe.

Table 1: Summary of CsA treated and control final cases of stage 54 after transection of the hind limb at the mid shank level:

CsA	Numbers of regenerates with toes (5-1)					Number and categories without toes			
Conc. of CsA	5	4	3	2	1	A	B	C	D
0.02 mg/mL	54.5%* (12)	31.8%* (7)	-	9.1% * (2)	-	4.6% (1)	-	-	-
0.2 mg/mL	33.3%* (4)	33.3%* (4)	16.8%* (2)	16.3% * (2)	-	-	-	-	-
0.5 mg/mL	81.8%* (9)	18.2%* (2)	-	-	-	-	-	-	-
Control	5.6% (5)	39.3% (35)	10.1% (9)	5.6% (5)	16.9% (15)	6.8% (6)	2.2% (2)	3.4% (3)	10.1% (9)

A: the shank region and base of foot paddle region had been restored, B: the shank region only had been restored, C: part of the shank region had been restored, D: negative cases. * $p<0.05$ vs controls

2- Treatment with Cycosporine A:

i. 0.02 mg/mL

a. Histogenesis of the time series:

By the first day the wound area was covered with two to four layers of cuboidal cells. Neither basement membrane nor the dermis was found. A large amount of cellular debris was observed.

By the fifth day (Figs. 6a and b), epidermis consisted of two layers of cuboidal cells. Basement membrane and dermis were sepreading distally at the sides of the regenerate. A fair amount of embryonic cells accumulated distal to the tibiofibula forming blastema. Signs of differentiation started where the beginning of condylar cap and periosteum were formed. Furthermore, skeletal accumulation was taking place forming the digital primordia.

By the seventh day, dermis and basement membrane were still found on the sides of the regenerate. The differentiating cells increased in size and in number forming a more developed distal primordia than the fifth day.

b. General morphological characteristics of final cases (see Table 1 and Fig. 5):

Twenty two cases were operated. Twelve cases developed limb outgrowth ending with five toes, two cases of them showed bent terminal phalenges either in second or third toe and showed syndactyly between the first and second toes. Another two cases showed either preaxial bent foot or small sized foot. Seven cases regenerated limb outgrowths with four toes, two cases of them showed either small size limb or axial bent and abnormal toes, other two cases showed bent terminal phalanges either in second (Fig. 7) or third toe, another two cases showed preaxial bent toe either first or fourth toe. Two cases regenerated outgrowth ending with two toes one case showed bent toes. One case regenerated shank region and base of foot region.

Demonstration with Victoria blue transparencies indicated the skeletal structure of four cases. The first case showed restoration of the entire missing skeletal parts of the limb. The second case showed a complete

restoration of four digited limb (Fig. 8). The fourth case was one of the two-toed cases, but after Victoria blue stain, it was found that there was a small third toe under the skin.

ii. 0.2 mg/mL

a. Histogenesis of the time series:

By the first day, epidermis consisted of two layers of cuboidal cells and few spindle-shaped cells were found migrating from stump to regenerating part.

By the fifth day (Figs. 9 a&b), epidermis consisted of two layers of cuboidal cells. Signs of foot regions started to appear by the formation of digital primordia. Few unicellular glands were found in the epidermis. Dermis and basement membrane were found at the sides of the regenerating part and they were missing in its apical part. Differentiated cells were accumulating forming a well formed blastema.

By the seventh day (Figs. 10 a&b), the condylar cap was in the process of differentiation and toes primordia were obvious. In addition, the blastema increased in size and the unicellular glands increased in number.

b. General morphological characteristics of final cases (see table 1 and Fig. 5):

Twelve cases were operated. Four cases developed limb outgrowths ending with five normal toes with prehallux. Four cases regenerated normal limb ending with foot carrying four toes. Two of them showed bent foot. Two cases regenerated normal limb ending with three toes. Two cases restored shank region and part of foot ending with two toes.

Demonstration with Victoria blue transparencies indicated the skeletal structure of two cases. The first case regenerated five toes having normal skeletal elements. The second case was one of the two-toed cases but after Victoria blue stain it was obvious that there was a small third toe.

iii. 0.5 mg/mL

General morphological characteristics of final cases (see table 1 and Fig. 5):

Most of the treated cases weren't able to complete their lives during the experiment time interval. Eleven cases only were able to live until the end of the experiment. Of these nine cases developed limb outgrowth ending with five toes, seven of them had normal five toes (Fig. 11) with prehallux, two had bent toes or had empty distances between toes. Two cases restored the entire regenerate ending with four normal toes.

Demonstration with Victoria blue transparencies indicated the skeletal structure of one case (Fig. 12) which restored the tibiofibula, the whole foot and the digital skeletal elements.

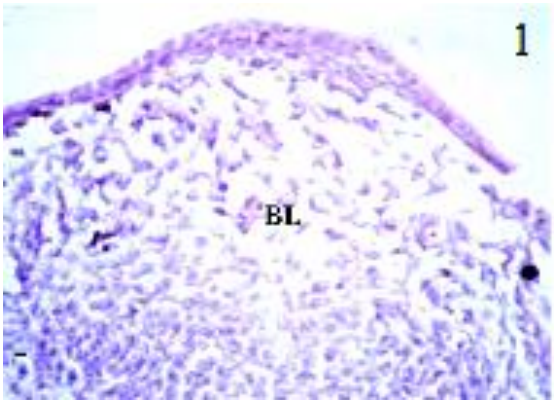


Fig 1: A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 3 days after amputation. BL: blastema (Control, X 400).

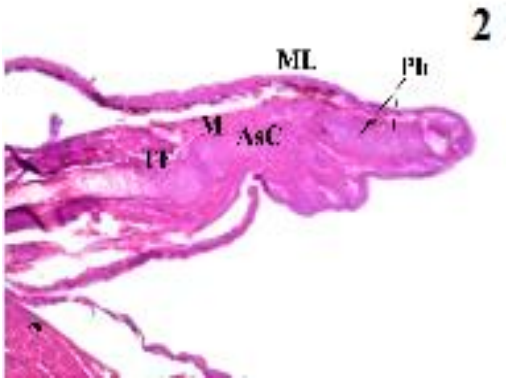
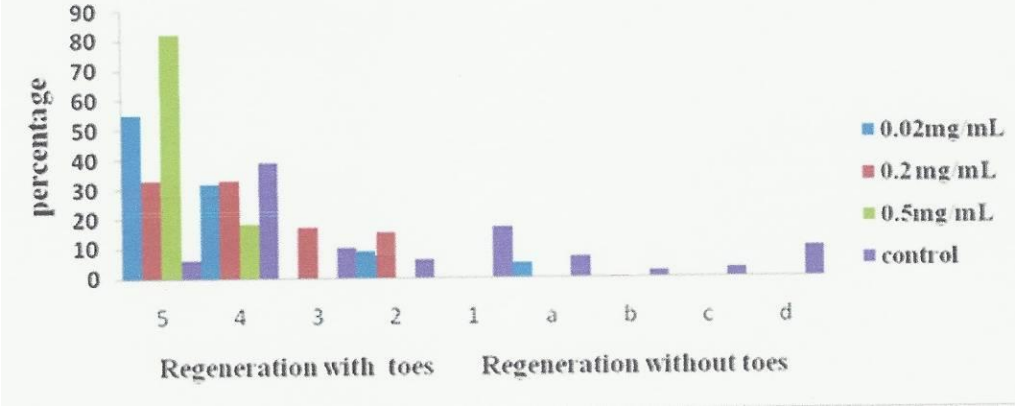


Fig 2: A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 7 days after amputation. AsC: Astragulus and Calcaneum, M: mesenchyme cells, ML: melanophores, Ph: phalanges, TF: Tibiofibula (Control, X100)

Fig 3: A photomicrograph of a regenerate of left hind limb ending with one toe, fixed 21 days after amputation. (Control, X 120)

Fig 4: A photomicrograph of a regenerate of left hind limb, fixed 23 days after amputation. Skeleton was stained with Victoria blue (Control, X 120).

Fig. 5: Histogram showing the effect of CsA on hind limb regeneration of larval stage 54 of *Bufo regularis*



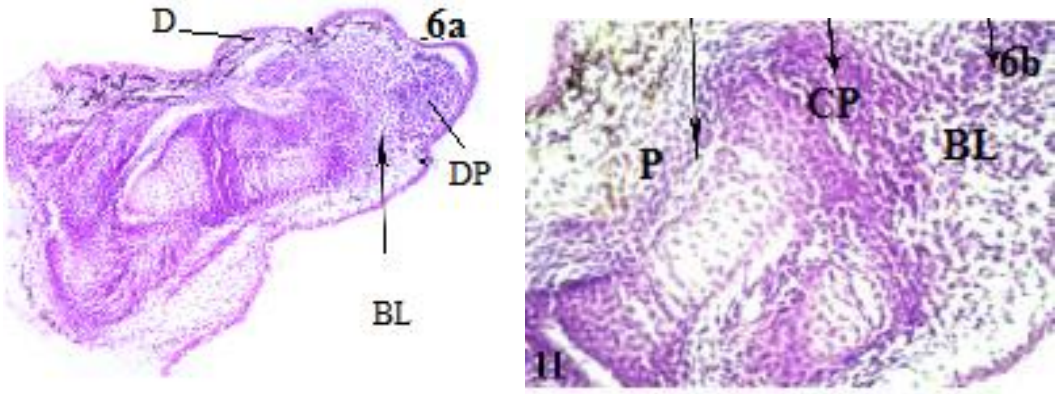


Fig. (6a): A photomicrograph of a regenerate of a longitudinal section through the left hind limb, fixed 5 days after amputation. BL: blastema, D: dermis, DP: digital primordia (0.02 mg/mL, X 100)
Fig. (6b): Enlarged portion of Fig.6a. BL: blastema, CP: condylar cap, P: periosteum (X400)

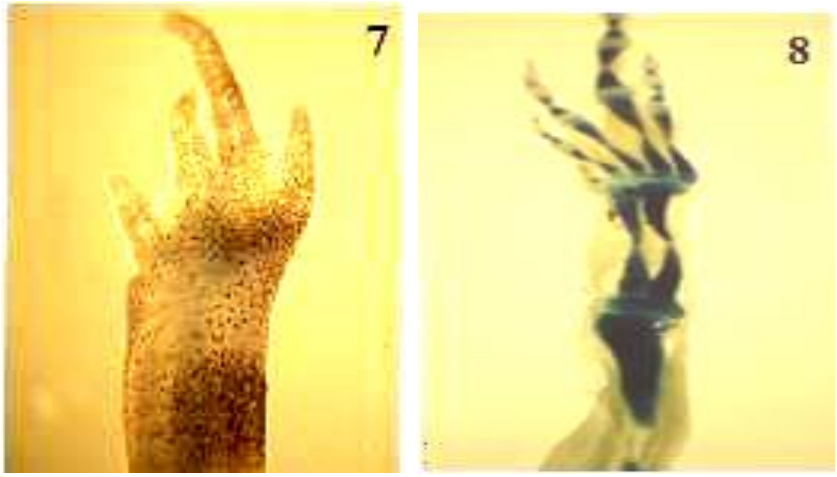


Fig. 7: A photomicrograph of a regenerate of left hind limb ending with four toes, fixed 23 days after amputation.
Fig. 8: Skeleton was stained with Victoria blue (0.02 mg/ml, X 120).

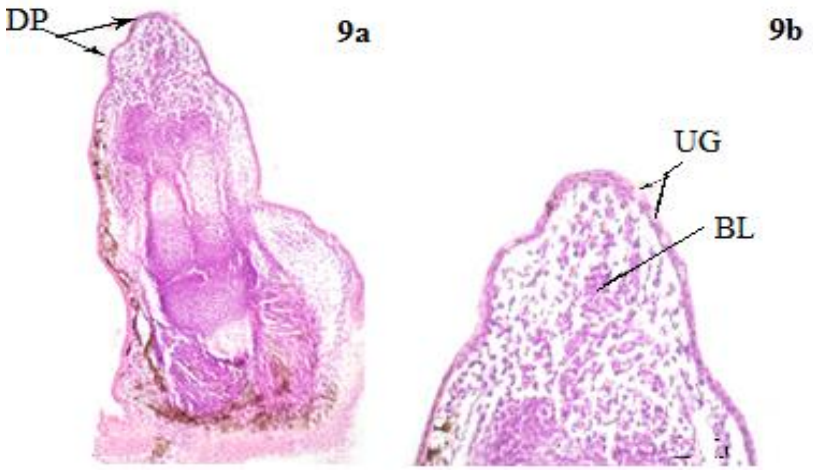


Fig. 9a: A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 5 days after amputation. DP: digital primordia (0.2 mg/mL, X 100)
Fig. 9b: Enlarged part of Fig. 9a. BL: blastema, UG: unicellular glands (X 400)

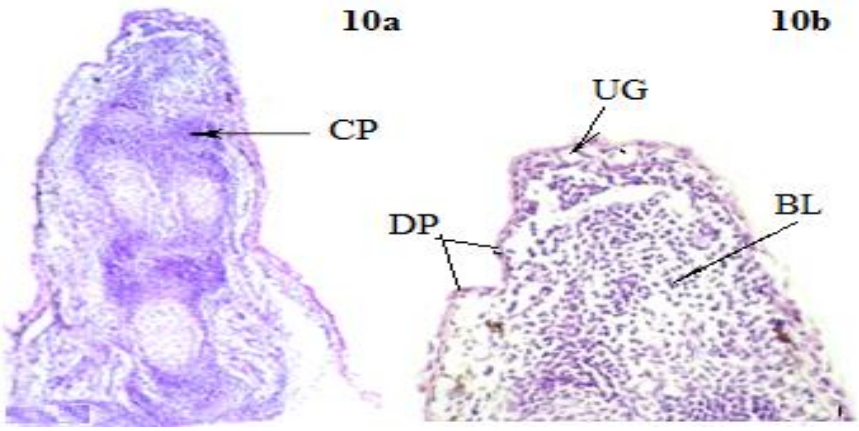


Fig. 10a: A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 7 days after amputation. CP: condylar cap (0.2 mg/mL, X100)
Fig. 10b: Enlarged part of Fig 10a. BL: blastema, DP: digital primordia, UG: unicellular glands (X 400)

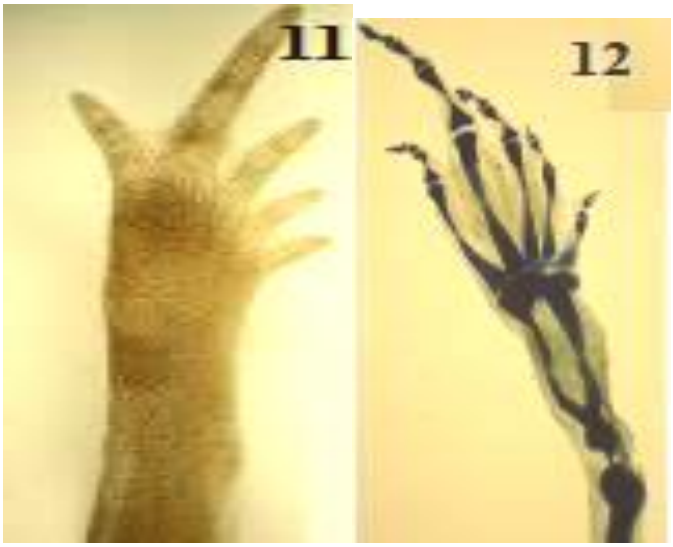


Fig. 11: A photomicrograph of a regenerate of left hind limb ending with 5 toes, fixed 23 days after amputation (0.5 mg/mL, X120).
Fig. 12: Skeleton was stained with Victoria blue (0.5 mg/mL, X120).

4. Discussion

The immune response is one of the important factors that influence limb regeneration in amphibians. The present study revealed interesting results exhibited by both morphogenesis and histogenesis observations of the larval stage 54 of the Egyptian toad *Bufo regularis*. The observations proved that CsA do certainely have its effective share in the enhancement of the regenerative ability of the amputated limbs compared with the normal limb regeneration.

Previous studies revealed that the immunosuppressant drug Cyclophosphamide (CY) inhibited regeneration in different amphibians. Aziz (1978) found that CY retarted regenertion and caused deviation from its normal course in tritons and axolotls. Schotté and Sicard (1982) observed that CY suppressed the initial formation of blastemas in fore limb regeneration of the adult newts *Notophthalmus viridescens*. Fahmy (1993) and Fahmy and Sicard (2002) found that certain doses of CY inhibited regeneration of hind limbs of *Bufo regularis* tadpoles. The present study has attempted to extend these observations by exploring the potential effects of another immunomodulator on regeneration using another experimental model.

CsA is a potent immunomodulator that has no adverse effect on wound healing (Golderberg and Hardy 1983). In previous studies, CsA was observed to have no effect on nerve regeneration. Moreover, Wang *et al.* (1997) found that CsA didn't alter the rate of sciatic nerve regeneration in rat. CsA significantly delayed regeneration of adult newt forelimbs (Fahmy and Sicard, 2002). In contrast, CsA didn't delay regeneration in injured kidney in rats (Ysebaert *et al*., 1997).

In vitro studies investigating whether CsA may have an inhibitory effect on smooth muscle cell proliferation in rabbits or not (Ferns *et al*., 1990). In another study, the effect of CsA was stimulatory or inhibitory, dependent on the dose of CsA (Tavares *et al*., 1998). In a third experiment, CsA was without any effect on arteries smooth muscle cells in rats (Jonasson *et al*., 1988). Finally in a fourth study, CsA was reported to inhibit smooth muscle cell proliferation indirectly via endothelial cell-derived factors on smooth muscle cells in rats (Leszczynski *et al*. 1993).

In the present study, CsA effect was unexpected; it significantly promoted regeneration ($p<0.05$). In addition, the effect of CsA on limb regeneration at stage 54 was dose-dependent; as the concentration of CsA increased the regeneration outcomes were enhanced.

Administration of CsA to tadpoles in the highest concentration (0.5 mg/mL) (table 1), 100% of cases regenerated the whole limb (shank and foot region) ending with either five (81%) or four toes (19%), while only 45% of the control cases ended with five or four toes. The rest of the control cases had all different regenerates starting from three toes to regenerates without toes. 10% of the control cases totally failed to regenerate. At the medium concentration (0.2 mg/mL), 66.6% of the regenerates ended with either five or four toes, 33.6% of the cases ended with either three or two toes. Finally, at the lowest concentration (0.02 mg/mL), 86.3% of cases ended with either five or four toes, 9% of cases ended

with two toes and 4.6% of cases restored the shank and foot region without toes.

Collectively, the present study showed that the immune system apparently had an effect on epimorphic regeneration, and that CsA had positive infuence on the enhancement of hind limb regeneration at that stage. Therefore, the present study disagreed with the ealier mentioned researches, which observed that immuonosuppressant drugs inhibited or delayed regeneration, e.g. Sicard, 1981 a and b; Schotté and Sicard, 1982; Fahmy, 1993 and Fahmy and Sicard, 2002. Furthermore, the present results confirmed the findings of Tanaka *et al*. (2002), who reported that CsA had been found to induce cancer by a cell-autonomus mechanism. Irintchev *et al*. (2002) showed that high numbers of fiber profiles were found in CsA treated-regenerated muscle. CsA treatment had a hyperplastic effect on regenerating muscles, and drug inducing phenotype alteration was much prominent in regenerated muscle. Schincaglia *et al*. (1992) showed a direct stimulatory action of CsA on collagen synthesis, but not on DNA synthesis in human gingival fibroblasts. Masuhara *et al*. (1993) found that CsA stimulated rat liver cell proliferation *in vitro* without inducing liver cell necrosis and that the carcinoma found was due to feeding rats with CsA diets.

Considering the known effect of CsA on immunoregulatory cells, it is possible that CsA may modify the functions of these cells and interfere with production of growth factors (Yabu *et al*., 1991). It is worth note to mention the relation between the concentration variables and the result observed. As the concentration of CsA increased, the enhancement and promotion of hind limb regeneration increased. The dose-dependent effect of CsA on regeneration (Table 1, Fig. 5) suggested that the immune system might influence the regeneration processes, and that it could have exerted a growth-promoting influence on the dedifferentiating cells.

Histological observation supported the morphological gross of the regenerating tadpole's hind limbs. It showed accleration in dedifferentiation, differentiation and histogenesis process after CsA treatment in comparison with control outcomes. By the early days after amputation, it was obviously noticed that the basement membrane and the dermis formation were delayed till the seventh day post amputation in all concentrations used. On the other hand, it was noticed in the control cases that the basement membrane was found from the first day post amputation, and the dermis was well-formed underneath the entire epidermis from the fifth day post amputation. It is well-known that the early differentiation of dermal layer causes regeneration failure (Rose, 1970; Goss and Holt, 1992). The dermis-free wound epithelium which migrated across the amputation surface from the cut edge of the epidermis promoted the continued proliferation of the cells in the area of injury. Delaying the dedifferentiation caused accumulation of blastema (Tassava and Mescher, 1975; Mescher, 1976; Globus *et al*., 1980). So the early formation of dermis could prematurely inhibited regeneration. Thus, it could be suggested that CsA contributed to the delay of dermal differentiation. In tadpoles, the epithelial covering the wound is not underlain by dermis, allowing direct communication between epidermis and mesenchyme, leading to the formation of a regeneration blastema (Rose, 1970; Goss and Holt, 1992).

Another histological observation was detected; the CsA treatment was associated with early dedifferentiation process. Embryonic spindle-shaped mesenchyme cells were noticed to migrate distally from the stump tissues to the amputation level at the first day post amputation. These mesenchyme cells accumulated distally forming a well formed blastema at day three post amputation. This observation was noticed in all concentrations of CsA used except at the lower concentrations (0.02mg/mL). On the contrary, no signs of dedifferentiation process were noticed in the control cases until the third day post amputation, where accumulation of mesenchyme cells occurred forming a poorly-formed blastema. Once blastema was formed, regeneration seemed to largely recapatulate the events of limb ontogenesis (Stocum, 2004). It was also noticed that CsA treatment was associated with early chondrogenesis. Tadpoles treated with CsA started their skeletal differentiation from the third day post amputation and continued their tibiofibula and foot skeletal histogenesis from the fifth day, where the tibiofibula restored its missing parts. In addition, the condylar cap, periostium and the digital skeletal primordia were formed. On the other hand, differentiation process of control cases started on the fifth day post amputation, where the restoration of tibiofubula took place. On the seventh day, histogenesis of the foot and toes regions took place.

Thus it was clear that CsA accelerated all processes of regeneration of tadpole's hind limb at stage 54. Fahmy (1993) found that the effect of the least concentration used of Cyclophosphamide on tadpoles only enhanced regeneration. The general immunosuppressant CsA was generally attributed to its inhibitory action on T-cell activation by blocking the induction of mRNA for several lymphokines, such as Interlukine-2 (IL-2) (Borel, 1983; Kahan, 1989) and IL-6 (Namavari *et al*., 2012). IL-2 was reported to inhibit liver regeneration in hepatomized rats (Wadamori *et al*., 1996). Therefore, the present work suggested that CsA had blocked the production

of IL-2 and thus arrested its inhibitory effect in regeneration of *Bufo regularis* tadpole's hind limbs.

In general, the present work achieved its major objectives. The effect of immunomodulator CsA treatment to the tadpole larvae of *Bufo regularis* was surprising and unexpected. CsA significantly promoted and accelerated regeneration of hind limbs after amputation at the mid shank level of tadpole larvae of *Bufo regularis*.

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