Effects of immunomodulator "Cyclosporine" on regenerative capacity of amputated hindlimbs in larval stages of the Egyptian toad, *Bufo regularis*.

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Abstract: The regeneration of the amputed hindlimbs of stage (54) of tadpole larvae of *Bufo regularis* was studied after amputing their hind limbs at the mid shank level. The immunomodulator Cyclosporine A (CsA) was selected to determine its effect on regeneration. Two kinds of experiments were desigened. The first of experimental design is by immersing the tadpole larvae 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. The second one is by intraperitoneal injection of CsA solution (0.02 and 0.2 mg/mL) administering 1 μ of CsA solution every other day starting from one day pre-amputation to day eleven post amputation. Regarding the effect of CsA on regeneration on stage 54, CsA significantly promoted regeneration in both kinds of experiments (immersion and injection), and this effect was dose-dependent. The regerative ability increases with increase of CsA concentations. Significant number of cases regenerated complete hind limbs with five or four toes, 86.3%, 66.7% and 100% of CsA treated cases (0.02, 0.2 and 0.5 mg/mL respectively) versus 45% of control cases when treated by immersion. In the other experiment, which is by injection, 90.5% and 93.3% of CsA treated cases (0.02 and 0.2 mg/mL respectively) restored hind limbs with five or four toes versus 45% of control cases. Histologically, CsA was associated with acceleration in the processes of dedifferentiation, differentiation and histogenesis in comparison with their control counterparts. Moreover, CsA treatment delayed the dermal differentiation underneath the epithelium. An observation which certainly favors regeneration as the absence of dermis allows direct communication between the above epithelium and the underlying cells for better blastema formation. CsA treatment in both experiments 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. Adminstration of CsA to the tadepole larvae (54 stage) by injection resulted in better regenerative outcomes than adminstration by immersion. CsA significantly promoted and accelerated regeneration of their hind limbs after ambutation at the mid shank level. In addition, CsA 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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Abbreviations of figures 2-15: AsC: astragalus and calcanum, BL: blastema cells, BM: basement membrane, CP: condylar cap., D: dermis, DP: digit primordia, M: muscle fibers, ML: melanophores, P: periosteum, PH: phalanges, TF: tibiofibula and UG:unicellular glands

Keywords: Immunomodulator, Cyclosporine**,** amputated hindlimbs**,** larval stages and *Bufo regularis*.

1. Introduction

Regeneration is the ability of the fully developed organism to replace lost part by growth or remodelling 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; Tuna *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 and 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 (Abbott *et al*., 2008; Tank and Holder (1978).

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, 1981 b).

Cyclosporine A is the most frequent immunosuppressor used in transplant surgery and in the 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).

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

The aim of the present study is to investigate the effect of immunomodulator CsA on the hindlimb regenerative ability of the Egyptian tadpole larvae, *Bufo regularis*. The present work will certainly clarify 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:

Clusters of eggs will be left in water aquaria in the laboratory for more than one month for acclimatization and to reach the stage of tadpole larvae required for the experiment. Groups of tadpole larvae of *Bufo regularis* will be reared in number of glass aquaria with faily large quatities of water with Elodea plant which act as natural ventillator. The tadpoles will be provided with boiled spinach and and small liver tissue for feeding. Stage 54 was selected according to the normal table of Sedra and Michael (1961).

Cyclosporin A:

Cyclosporin A is an immunosuppressant drug widely used in post-allogeneic organ transplant to reduce the activity of the patient's immune system and, so, the risk of organ rejection. It has been studied in transplants of skin, heart, kidney, liver, lung, pancreas, bone marrow, and small intestine. Initially isolated from a Norwegian soil sample, ciclosporin is a cyclic nonribosomal peptide of 11 amino acids produced by the fungus Beauveria nivea, and contains a single D-amino acid, which are rarely encountered in nature (Borel, 2002). Cylosporine A (Fig. 1) were obtained from Sigma-Aldrich Co. United Kingdom Systematic (IUPAC) name: of CsA:- E)-14,17,26,32-tetrabutyl-5-ethyl-8-(1-hydroxy-2-methylhex-4-enyl) -1,3,9,12,15,18,20,23,27-nonamethyl-11,29-dipropyl-1,3,6,9,12,15,18,21,24,27,30undecaazacyclodotriacontan-2,4,7,10,13,16,19,22,25,28,31-undecaone,
Formula: C62H111N11O12

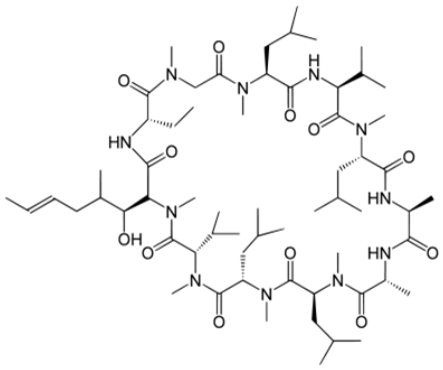


Fig. (1) Chemical formula of Cyclosporine A

Experimantal Design:

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

Two types of experiments will be performed: (1) By Immersion, tadpoles (10/dish) will be 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 will be administered twelve hours after amputation. Control cases will be bathed in stock tap water. (2) By Injection, CsA (0.02 and 0.2 mg/mL) will be administered intraperitoneally (1.0 μL/ injection/ animal) every other day, starting from day one pre-amputation untill day eleven post-amputation. Control cases will receive no injection.

Histological Procedure:

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

Statistical Analysis:

Statistical analysis will be done in the present work 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 consists 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.2, BL).

By the fifth day, epidermis was the same like the third day. Basement membrane was found. The dermis was well developed underneath the entire epidermis. The tibiofibula is restored and differentiation of skeletal elements was obvious at the distal part of tibiofibula.

By the seventh day, differentiation process was obvious. The tibiofibula restored its missing part (Fig. 3,TF), several skeletal elements of the autopdial region were found forming the astragalus and calacaneum and phalanges (Fig. 3, AsC, Ph). Large amount of myoblasts were differentiating forming muscle (Fig. 3, M).

b. General morpholgical characteristics of final cases (see Table 1 and Fig. 15)

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 the right. 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 thired toes. Another four cases showed ben 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 either axial, dorsal, or venteral. 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 syndactous and towards left.

Fifteen of them had regenerated one toe (Fig. 4), five of them regenerated reduced foot with obvious toe. Another cases regenerated shank withknob end. Nine of fifteen cases regenerated shank region carring either toe like prorusion or skin fold or spike like. Six cases had regenerated shank region only. Three cases had regenerated part of shank region. Nine case 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. The second case (Fig. 5), the tibiofibula was completely restored, but the autopodial skeletal elements were highly reduced carring one toe that has four phalanges. The third case showed restored tibiofibula, but at the amputation level the tibia became separated from tibiofibula was restored carring a small protrusion, metatarsus and there phalanges were present supporting one toe.

2- Treatment with Cycosporine-A by Injection:

i- 0.02 mg/mL

a- Histogenesis of the time series:

By the first day, the wound area consisted of two layers of cuboidal cells forming epidermis. Spindle shaped cells were found, migrating to the amputation level, indicating beginning of blastima formation.

By the third day, the epidermis consisted of two layers. A mass of embryonic cells accumulated forming a well formed blastema. Melanophores are present on the sides of the regenetating part. Differentiation of condylar cap of tibiofibula was obvious and periosteum was differentiating as well.

By the fifth day, basement membrane was continuous underneath the epidermis (Fig. 6,BM). No dermis was found.differentiation process has actiely started forming digit primordia in the autopodial region (Fig. 6, DP).

By the seventh day, the shank and foot region had restored their skeletal elements. Dermis was found intermingled with the melanophores at the sides of the regenerating part and missing in the apial part. Cartilaginous parts were obvious, forming digital primordia.

b- General morphological characteristics of final cases (see Table 1 and Fig. 16):

Twenty one cases were operated; fourteen cases developed limb outgrowths ending with five towes each (Fig. 7-a).; one case showed syndactyly in the first and second toes, four cases showed small sized

limb, one case showed reduction in the first and second toes. Five cases regenerated normal limb with four toes, one case showed reduced foot. Two cases regenerated limb ending with one spike-like toe.

Demonstration with Victoria blue transparencies in two cases showed normal restored all skeletal elements of the limb (Fig. 7-b).

i- 0. 2 mg/mL

a- Histogenesis of the time series:

By the first day, the wound area was covered with two layers of cuboidal cells forming epidermis. Basement membrane was found at the sides of the wound area and absent at the apical part. No dermis was found. Few cellular debris were found at the wound area. Spindel-shaped mesenchyme cells were found migrating from the skeletal element and the surrounding tissue to the wound area indicating beginning of differentiation process.

By the third day, the wound area was covered with two layers of cuboidal cells. Accumulation of mesenchyme cells were obvious forming blastema.

By the fifth day (Fig. 8), the basement membrane (BM) was obvious on the sides of regenerate part and absent on the apical part of the epidermis. Differentiation process was intiated. Melanophores (ML) started to migrate in the regenerating part. A large well-formed bended blastema was found (BL), anticipating the knee joint and the base of foot paddel region.

By the seven day, shank region restored its elements. Differentiating cells were found distal to the tibiofibula forming the base of the foot skeletal element.

b- General morphological characteristics of final cases (see Table 1 and Fig. 16):

Thirteen cases were operated. One of these cases developed limb outgrowth ending with five toes. Eleven cases regenerate normal limb ending with four toes (Fig. 9-a), two cases showed either preaxial bent or smaller size in fifth toe. And one regenerated three small toes. Victoria blue transparencies indicated the skeletal structure of one of the four toed cases. This case (Fig. 9-b) showed a well formed three digits with their skeletal elements (metatarsus and phalanges) but the fourth digit was reduced.

2- Treatment with Cycosporine-A by Immersion:

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 were found. A large amount of cellular debris were observed.

By the fifth day, epidermis consisted of two layers of cuboidal cells. Basement membrane and dermis were sepreading distally (D) at the sides of the regenerate (Fig. 10). A fair amount of embryonic cells accumulated distal to the tibiofibula forming blastema (Figs. 10 and 11, BL). Signs of differentiation started where the beginning of condylar cap and periosteum were formed (Fig. 11, CP, P). further more, skeletal accumulationwere taking place forming the digital primordia (Fig. 10, DP).

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. 17):

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 firstand 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 wo cases showed bent terminal phalanges either in second(Fig. 12-a) 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 skeltal 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. 12-b), one of the metaoe appearedtarsus, and the third toe had bent last phalanx. The fourth case was one of the two-toed cases, but after Victoria blue stain, it was obvious that there was a small third toe appeared.

ii- 0.2 mg/mL

a- Histogenesis of the time series:

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

By the fifth day epidermis consisted of two layers of cuboidal cells. Signs of foot regions started to appear by the formation of digit primordia (Fig. 13-a, DP). Few unicellular glands (Fig. 13-b, UG) were found in the epidermis. Dermis and basement membrane wew 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.differentiated cells were accumulating forming a well formed blastema (Fig. 13-b,BL).

By the seventh day, the condylar cap was in the process of differentiation (Fig. 14-a, CP) and toes primordia were obvious (Fig. 14-a, DP). In addition, the blastema increased in size (Fig. 14-b, BL). The

unicellular glands increased in number (Fig. 14-b,UG).

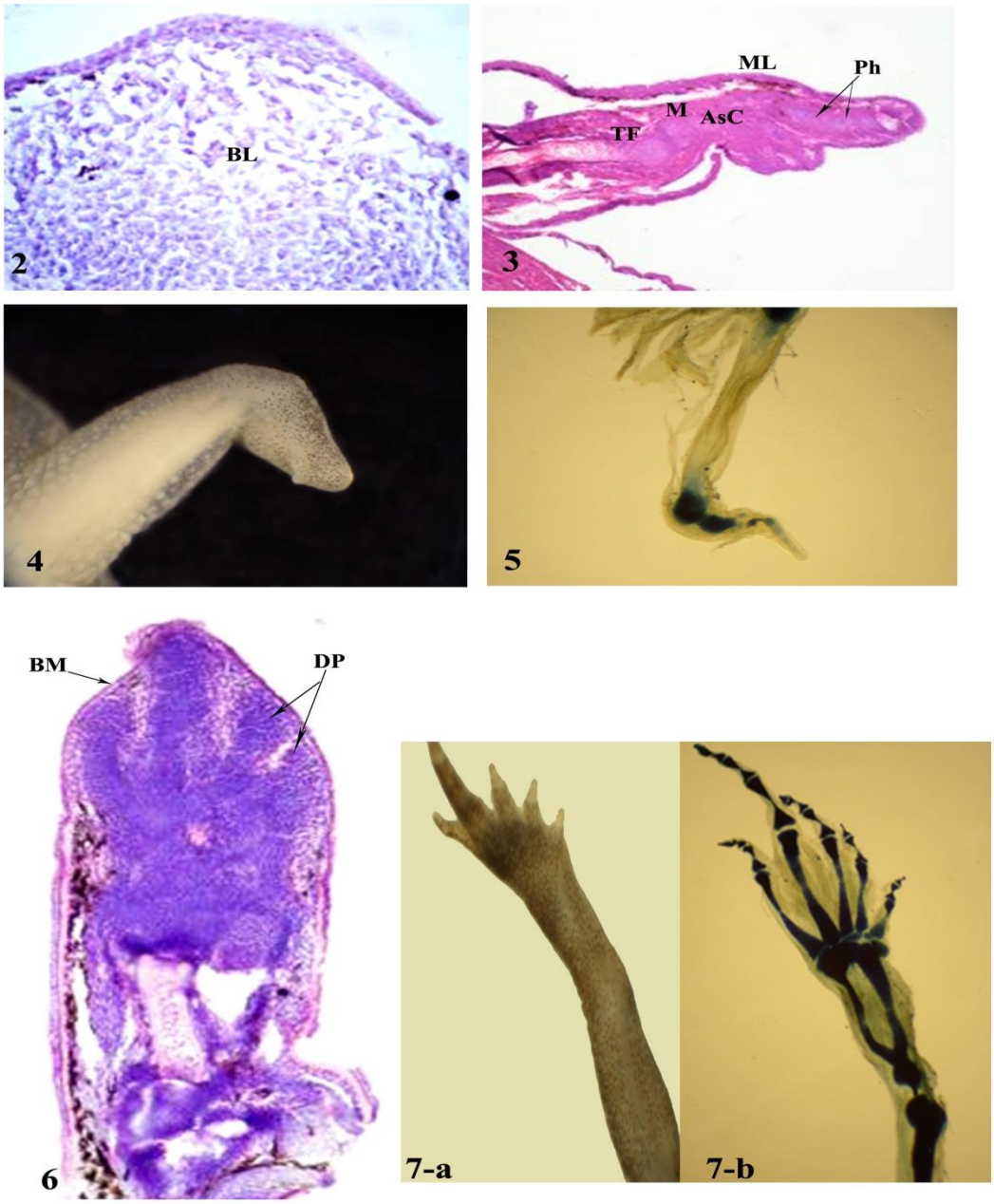


Fig 2: A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 3 days after amputatio n (Control- X400)
Fig (3): A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 7 days after amputation(Control -X100)
Fig (4): A photomicrograph of a regenerate of left hind limb ending with one toe, fixed 21 days after amputation. (Control- X120)
Fig (5): A photomicrograph of a regenerate of left hind limb, fixed 23 days after amputation. Skeleton was stained with Victoria blue(Control - X120).
Fig (6): A photomicrograph of a regenerate of of a longitudinal section through a regenerate of left hind limb, fixed 5 days after amputation. (By Injection: 0.02 mg/mL X100)
Fig (7-a): A photomicrograph of a regenerate of left hind limb ending with five toes. Fixed 23 days after amputation.(By Injection: 0.02 mg/mL X112)
Fig (7-b): A photomicrograph of a regenerate of left hind limb, fixed 23 days after amputation. Skeleton was stained with Victoria blue(By Injection: 0.02 mg/mL X120)

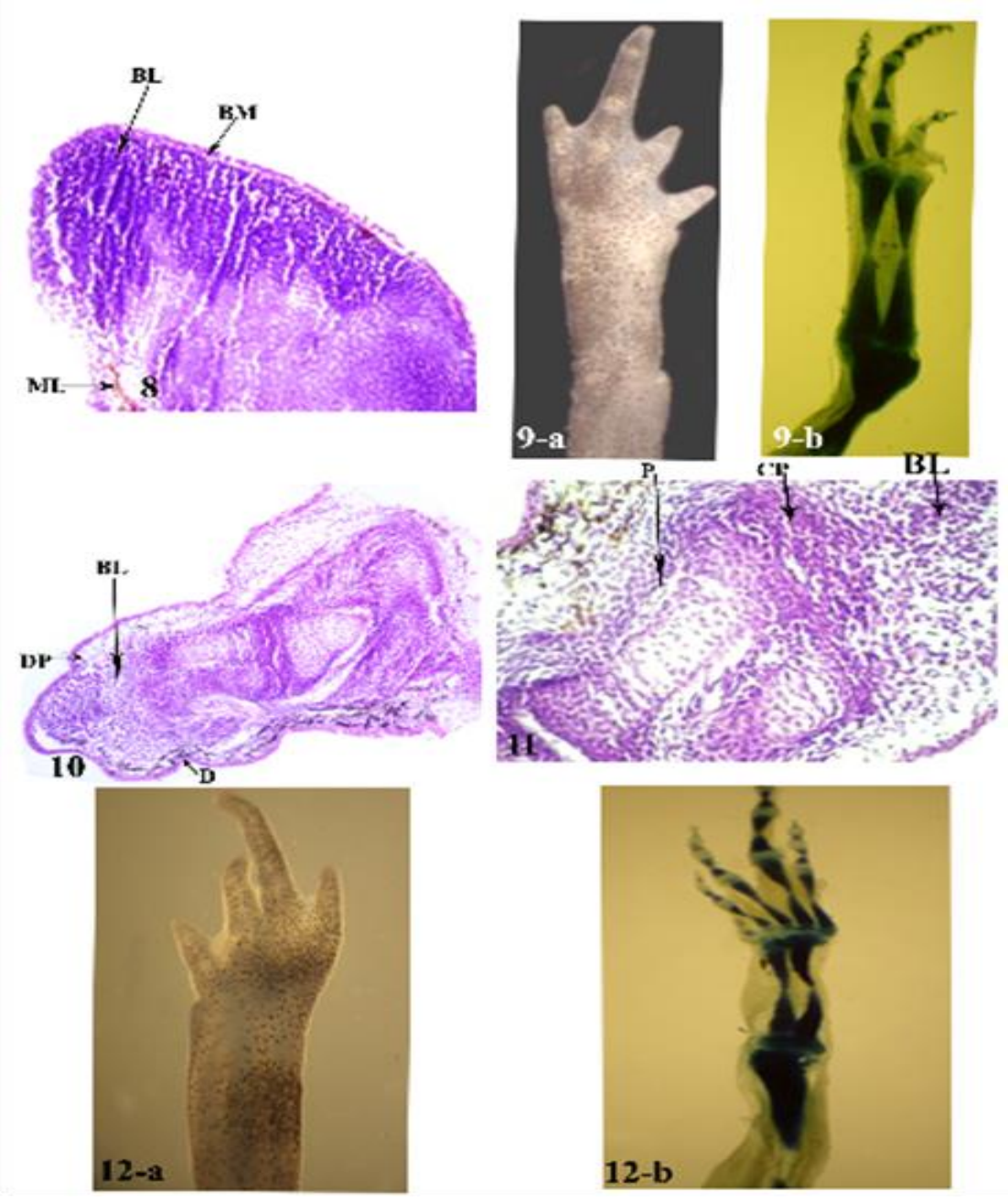


Fig (8): A photomicrograph of a regenerate of a longitudinal section through a regenerate of left hind limb, fixed 5 days after amputation. (By Injection: 0.2 mg/mL X400)
Fig (9-a): A photomicrograph of a regenerate of left hind limb ending with four toes, fixed 23 days after amputation. (By Injection: 0.2 mg/mL X120).
Fig (9-b): A photomicrograph of a regenerate of left hind limb, fixed 23 days after amputation. Skeleton was stained with Victoria blue(By Injection: 0.2 mg/mL X120).
Fig(10): A photomicrograph of a regenerate of of a longitudinal section through a regenerate of left hind limb, fixed 5 days after amputation. (By immersion 0.02 mg/ml X100)
Fig(11): Enlarged portion of Fig(9) through a regenerate of left hind limb, fixed 5 days after amputation. (by immersion 0.02 mg/ml X400)
Fig (12-a&b): A photomicrograph of a regenerate of left hind limb ending with four toes, fixed 23 days after amputation. (11-b)Skeleton was stained with Victoria blue(By immersion 0.02 mg/ml X120).

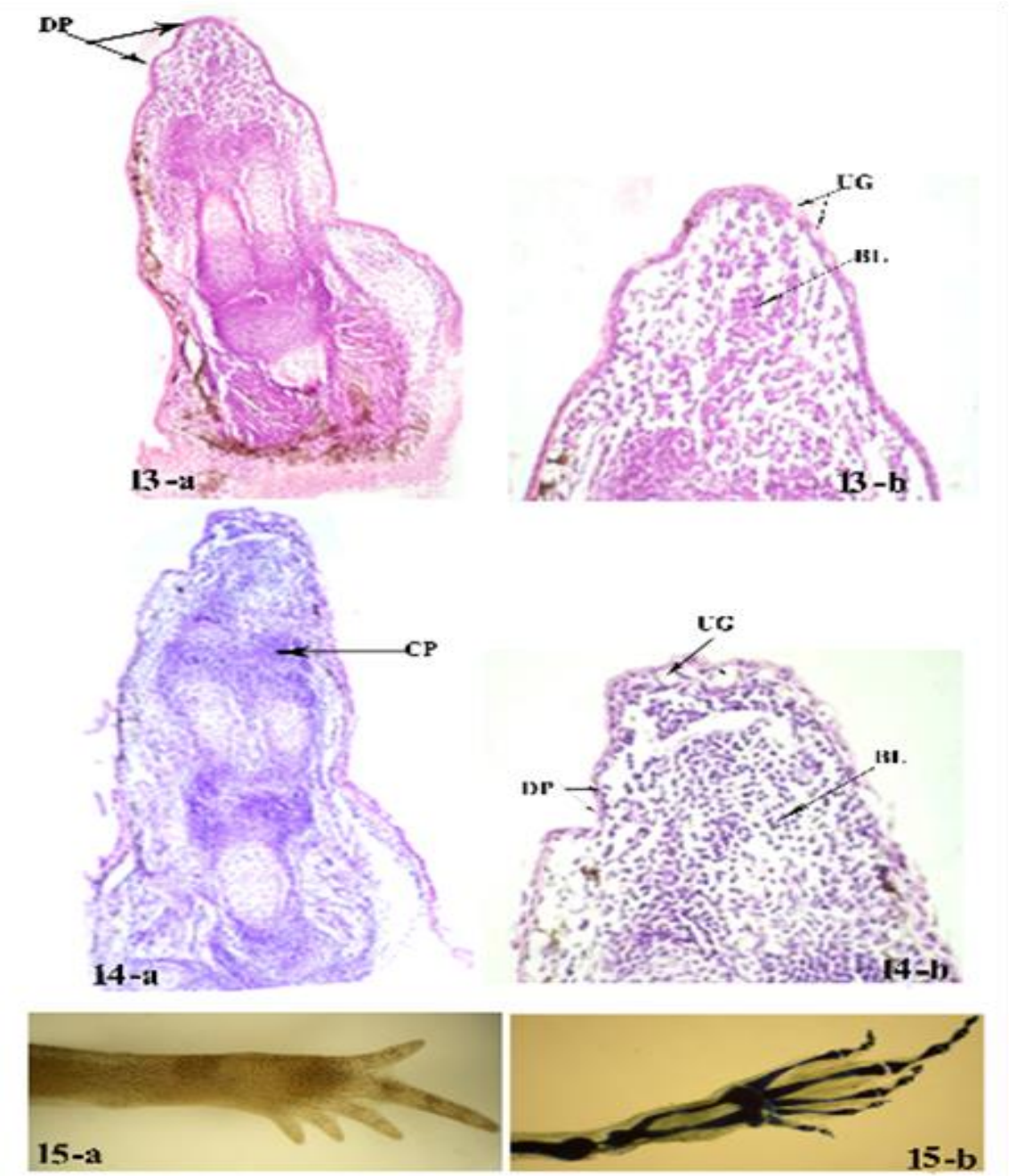
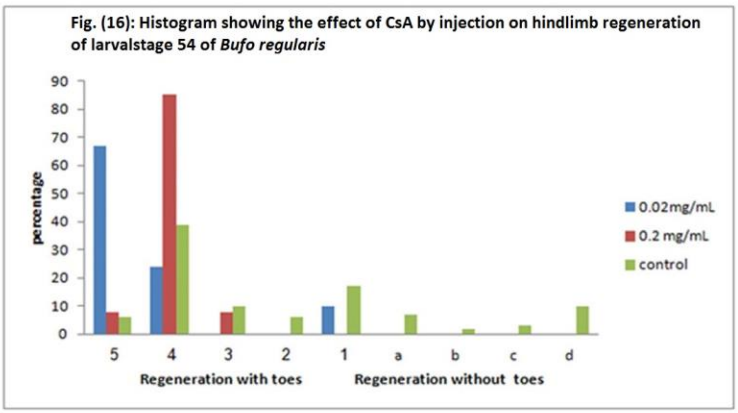


Fig (13): A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 5 days after amputation.(By immersion 0.2 mg/ml, a-X100& b-X400)
Fig (14) : A photomicrograph of a longitudinal section through a regenerate of left hind limb, fixed 7 days after amputation(By immersion 0.2 mg/ml, (a-X100& b-X400)
Fig (15-a&b): A photomicrograph of a regenerate of left hind limb ending with 5 toes, fixed 23 days after amputation. (14-b)Skeleton was stained with Victoria blue(By immersion 0.5 mg/ml, X120).



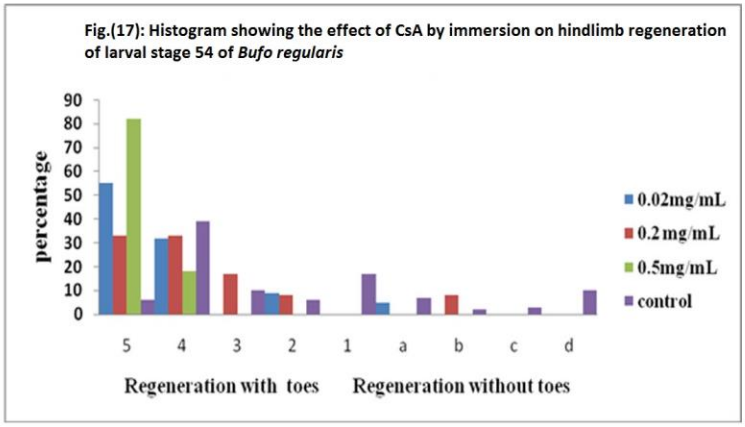


Table 1: Summary of CsA treated and control final cases of stage 45 after transection of the hindlimb at the shank level

CsA		Numbers of regenerates with toes 5-1					Number and categories without toes			
Kinds of exp.	Conc.	5	4	3	2	1	A	B	C	D
Injection	0.02mg/mL	66.7%* (14)	23.8% (5)	-	-	9.5% (2)	-	-	-	-
	0.2mg/mL	7.7% (1)	84.6%* (11)	7.7% (1)		-	-	-	-	-
Immersion	0.02mg/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)	8.3% (1)	-	-	8.3% (1)	-	-
	0.5mg/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 and D: Negative cases and * $p<0.05$ vs controls

b- General morphological characteristics of final cases (see Table 1 and Fig. 17):

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. One case restored shank region and part of foot ending with two toes. One case restored shank region only.

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.

i- 0.5 mg/mL

General morphological characteristics of final cases (see table 1 and fig. 17):

Most of the treated cases weren't able to complete their lives during the experiment. 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. 15-a) with prehallux, two had bent toes or had of empty distances between toes. Two cases restored the entire regenerate ending with four normal toes.

Demonstration with Victoria blue transparencies indicated the skeltal structure of one case (Fig. 15-b) which restored the tibiofibula, all foot and digital skeletal elements.

4. Disscussion

The immune response is one of the important factors that influences 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 inhancement of the regenerative ability of the amputed limbs compared with the normal limb regeneration.

Several studies suggest 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.

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, splenectomFor example, splenectom (Fini and Sicard, 1980), X-irradiation (Sicard and lombard, 1990), Cobra venum factor and anti-lymphatic serum (Sicard, 1981 a&b), and skin allograft callenge itself delayed regeneration when presented at the time or before amputation (Sicard *et al*. 1986). While 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.

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 forelimb regeneration of the adult newts *Notophthalmus viridescens*. Fahmy (1993) and Fahmy and Sicard (2002) found that certain doses of CY inhibited regeneration of hindlimbs 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 Sicared, 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 (Ferns *et al*.,1990). In another study, the effect of CsA was either stimulatory or inhibitory, dependent on the dose of CsA (Tavares *et al*., 1998). In a third experiments, CsA was without any effect on arteries smooth muscle cells in rats (Jonasson *et al*., 1988). Finally, in 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).

Collectively, the effect of CsA in inhibiting or delaying the process of regeneration in different animals suggests that Csa might suppress hindlimb regeneration in *Bufo regularis* tadpoles. However, in the present study, CsA effect were unexpected; it significantly promoted regeneration in both kinds of experiments whether the treatment with CsA was by injection or immersion ($p<0.05$). in addition, the effect of CsA on limb regeneration at this 54 stage was dose-dependent. As the concentration of CsA increased the regeneration outcomes were enhanced.

Administration of CsA to tadpoles by immersion 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) of the same kind of treatment, 66.6% of the regenerates ended with either five or four toes, 25% 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 fivee or four toes, 9% of cases ended with two toes, and 4% off cases restored the shank and foot region without toes.

Whereas administrating CsA by intraperitoneal injection, enhancement of regeneration was also significantly observed ($p<0.05$). In the higher concentration (0.2 mg/mL) (table 1), 92.3% of treated cases ended with five or four toes, and only the remaining 7.7% ended with three toes as compared with 45% control cases ended with five or four toes, and the rest of control cases had all different regenerates starting from three toes to regenerates without toes. In lower concentration (0.02 mg/mL), 92% of the treated cases had regenerated ended with either five or four toes, and 9.5% of the cases ended with one spike-like toe.

Thus, in the present study, the immune system apparently had an effect on epimorphic regeneration, and that CsA had positive infuence on the enhancement of hindlimb regeneration at that stage. Therefore, he present study was anatagonistic with the ealier mentioned researches that observed that immuonosuppressant drugs inhibit regeneration, e.g. Sicard, 1981a&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, the most frequently used immunosuppressive drug, had been found to induce cancer by a cell-autonomus mechanism. Irintchev *et al.* (2002) showed that high numbers of fiber profiles in CsA treated-regenerated muscle, CsA treatment had a hyperplastic effect on regenerating muscles, and drug induced phenotype alteraion are 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 necrosisi, and that this carcinomas in rats fed a CsA diet.

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 therelation between the concentration variables and the result observed. As the concentration of CsA increased, either by injection or immersion, the enhancement and promotion of hind limb regeneration increased. The dose-dependent effect of CsA on regeneration (Table 1, Figs. 16 and 17) suggests that the immune system might influence the regeneration processes, and that it might exert a growth-promoting influence on the dedifferentiating cells.

Histological observation supported the gross regenerating tadpoles hindlimb showed accleration in dedifferentiation, differentiation and histogenesis process after CsA treatment, in both kinds of experiments, 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 and in both kinds of CsA administration. 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. The dermis-free wond epithelium which migrates across the amputation surface from the cut edge of the epidermis promotes the continued proliferation of the cells in the area of injury. Delaying the dedifferentiation causes accumulation of blastema (Tassava and Mescher, 1975; Mescher,1976; Globus *et al*., 1980). So the early formation of dermis could prematurely inhibit regeneration. Thus, it can 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 (Goss and Holt 1992 and Rose, 1970a).

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 (Fig. 2) at day three post amputation. This observation was noticed in all concentrations of CsA used except at the lower concentrations (0.02 mg/mL), when the tadpole were immersed in CsA solution. On contrary, no signs of dedifferentiation process were noticed in the control cases untill the third day post amputation, where accumulation of mesenchyme cells occurred forming a poorly-formed blastema. Once blastema is formed, regeneration seems to 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, and 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 only took place, and on the seventh day, histogenesis of the foot and toes regions took place.

Thus it is clear that CsA accelerated all processes of regeneration of tadpoles hindlimb at stage 54. Fahmy (1993) found that the effect of the least concentration used of Cyclophosphamide on tadpoles enhanced regeneration. The general immunosuppressant CsA is 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 and Kahan, 1989). IL-2 was reported to inhibit liver regeneration in hepatomized rats (Wadamori *et al*., 1996). Therefore, the present work suggests that CsA had blocked the production of IL-2 and thus arrested its

inhibitory effect in regeneration of *Bufo regularis* tadpoles hindlimbs.

The present results also showed that CsA administration by injection enhanced and accelerated the regeneration process of tadpoles hindlimbs more than by administrating CsA by immersion. The effect of CsA on regeneration was obviously depending on the route of administration and the circulating level of the drug. Ibarra *et al*. (1996) showed that CsA pharmaco kinetics was impotantly altered, depending on the route of administration.

As a general, the present work achieved its major objectives. The effect of immunomodulator CsA treatment to the tadpole larve of *Bufo regularis* was surperising and unexpected. CsA significantly promoted and accelerated regeneration of their hindlimbs after amputation at the mid shank level.

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