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Exogenous administration of EGF augments nucleic acid biosynthesis and cell proliferation in the regenerating tail of wall lizard

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ABSTRACT

A number of growth factors are known to play indispensible roles in the process of tail regeneration in lizard. However, studies on the role of Epidermal Growth Factor (EGF) are few. The current study was aimed at specifically studying the effect of exogenous administration of EGF on the proliferative and synthetic activities of cells in the regenerating tail of adult wall lizard Hemidactylus flaviviridis. The lizard restores an amputated tail through three major stages of growth, viz., Wound Epithelium, Blastema and Growth and Differentiation. These stages, particularly the blastema stage, involve large scale proliferation of cells, which is naturally accompanied by extensive DNA replication and protein synthetic activities. We have used biochemical estimations and histofluorescence analysis to show that externally applied EGF enhances the process of early growth and repair in an amputated tail. The synthesis of nucleic acids and proteins was also found significantly elevated. These results suggest a definite role of EGF as a protagonist of healing and proliferation of an amputated tail in adult wall lizard. Further studies identifying the cellular events which are being regulated by the timed expression of EGF are required to draw a better understanding of the mechanism underlying epimorphic regeneration.

Keywords: Epidermal Growth Factor, Lizard Tail Regeneration, Wound Epithelium, Blastema.

Abbreviations: *EGF- Epidermal Growth Factor, WE- Wound Epithelium, BL- Blastema, DF- Differentiation*

INTRODUCTION

Though epimorphic regeneration has been extensively studied in amphibians, the mechanisms that regulate tail regeneration in lizards are largely unknown. Earlier studies have revealed that the lizard tail regeneration is influenced by several neuroendocrine factors [1, 2]. However, recently the focus of research has shifted to the role of growth factors in epimorphic regeneration. Many growth factors like VEGF, FGF-2, EGF, TGF, NGF, etc. have been shown to influence the process. The role of (Fibroblast Growth Factor-2) FGF-2 in lizard tail regeneration has already been worked out [3]. An isolated study has demonstrated the role of epidermal growth factor (EGF) in epimorphic regeneration [4]. However, an understanding of the stage specific influence of EGF on lizard tail regeneration is still at large.

The process of epimorphic regeneration is characterized by three destined stages namely wound epithelium, regeneration blastema and differentiation. Immediately after autotomy of the tail, the first process to occur is the migration of epithelial cells to cover the wound at the site of amputation. After attaining functional maturity, wound epithelium helps in recruiting a pool of pleuripotent blastemal cells in the progress zone. These cells proliferate and differentiate to compensate the lost tail [5]. It is therefore prudent to presume that several putative factors should be acting in conjunction, in a specifically timed pattern, to successfully attain each of these stages of regeneration.

EGF is suspected to be involved in the healing cascade, which involves inflammation, wound cell-migration, mitosis, neovascularization and regeneration of the extracellular matrix. Healing of a variety of wounds in animals and patients is in fact known to be enhanced by treatment with various factors of plant and animal origin, an important one being EGF [6, 7]. Combined application of recombinant human EGF (rhEGF) with recombinant human FGF-2 (rhFGF-2) is found to be beneficial to wound repair [8]. Systemic administration of EGF promoted hematopoietic regeneration in mice exposed to lethal dose of radiation [9]. EGF application to cultured mouse intestinal epithelial cells dramatically increases migration into a wounded area [10] and similar results have been reported with rabbit duodenal organ cultures [11] as well as cultured human colonic cell lines or epithelia [12-14]. Furthermore, a clinical study indicates that topical EGF application is effective in treatment of ulcerative colitis [15], suggesting that the promotion of wound healing by this factor *in vitro* effectively models the *in vivo* response.

Apart from its role in wound healing, EGF is known as a proliferation inducing factor [16, 17]. It has been shown to be a mitogenic agent *in vitro* as well as *in vivo*. EGF stimulates mitogenesis of cultured neuroprogenitor cells [18-20], helps in efficient liver regeneration [21] and also plays an important role in cell proliferation during embryogenesis [22]. The activities of epidermal growth factor and its receptor the EGFR have been identified as key drivers in the process of cell growth and replication [23, 24]. Heightened activity of the EGF receptor, whether caused by an increase in the concentration of ligand around the cell, an increase in receptor numbers or receptor mutation can lead to an increase in the drive for a cell to replicate. Epidermal growth factor is also thought to play a role in tumor development and metastasis. EGF receptor overexpression has been detected in a variety of human breast cancer cells [25]. EGF has potent growth-promoting effects in mammary epithelium, stimulating DNA synthesis and cellular proliferation [26].

From the above review it is clear that EGF with its proven influence in wound healing as well as cell proliferation in diverse *in vivo* and *in vitro* models should be playing a definite role in one or many of the stages in lizard tail regeneration. Hence, the present study was planned to evaluate the mitogenic potential of EGF during various stages of tail regeneration in wall lizard, *Hemidactylus flaviviridis*.

MATERIALS AND METHODS

Animal Maintenance:
Adult wall lizards, *Hemidactylus flaviviridis*, of both sexes, with intact tails, weighing 10 ± 2 gm were collected from nearby households. All animals were screened and the healthy ones were acclimatized to the standard laboratory conditions before the commencement of experimentation. The lizards were housed at 30 ± 2ºC and 12:12 hour light to dark cycles. The animals were fed with in-house reared cockroach nymphs twice a week and purified water was given daily, *ad libitum*. The experimental protocol followed in the current study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC). All procedures of amputation and treatments were done under hypothermic anesthesia [27] in strict compliance with the ethical guidelines of CPCSEA, India.

Chemicals:
EGF was purchased from Sigma Chemicals Co., St. Louis, MO, U.S.A. All other chemicals were of AR grade and procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India; Qualigens Fine Chemicals, Mumbai, India.

Morphogenetic Progress of regeneration:
A total of 42 animals were acclimatized in the animal house for 4 days, prior to the experiment. *Series 1:* 12 animals were used for the first part of the experiment, divided into 2 groups of 6 each. The Control and Treatment groups were administered 0.6% Saline and EGF (10µg/kg body weight in 0.6% saline) respectively, injected locally at the second segment of tail from the vent. Treatment went on for 4 days, followed by autotomy of tail. *Series 2:* From the remaining pool of animals, 12 animals that attained WE stage on the same day were selected for series 2. These were divided into 2 groups of 6 each and treated in the same way as series 1. *Series 3:* From the remaining pool of

animals, 12 animals that attained BL stage on the same day were selected for series 3. These were divided into 2 groups of 6 each and treated in the same way as the earlier series. The length of regenerates was measured in each animal with a digital vernier caliper (Mitutoyo, Japan).

Histofluorescent Localisation of Nucleic Acids:
Animals from the above experiment were used for histofluorescent analysis staining of nucleic acids. Tail regenerates from each stage of regenerating tissue were collected under hypothermic anesthesia and transferred to cryostat microtome at –20^{o}C. Tissues were mounted in cryo-embedding medium (Tissue-tek, Sakura Finetek, USA) and sectioned at 10µm. The sections were exposed to 0.1% Acridine Orange in 0.1M phosphate buffer (pH-6.0) and observed under fluorescence microscope (Leica, DM2500) with 410 nm excitation filter and 510 nm emission cut off filter. Acridine Orange induces specific yellow emission for DNA and flame-red emission for RNA in the ultraviolet range [28].

Biochemical Estimations:
A total of 24 lizards were acclimatized for 4 days in the animal house and divided into 2 groups of 12 animals each. The Control and Treatment groups were treated as in the previous experiment. Treatment began 4 days prior to autotomy and continued till the end of the experiment. Regenerating tail tissue from 6 animals of each group which attained Blastema stage was collected under mild hypothermic anesthesia and 20% homogenate was prepared in PBS. Similarly, regenerating tissue at the differentiation stage was collected from 6 animals of each group and processed in the same way. Nucleic acids were estimated according to the method of Schneider [29] and total protein was estimated using the method described by Lowry *et al.* [30].

Statistical Analysis:
The quantitative data were subjected to Bartlett test for homogeneity and the significant variation in the means between the treatment groups with control group was evaluated through Student's 't' test with 95% confidence limit. The values are expressed as either Mean ± SE or as Mode with range in parenthesis. All statistical analyses were done using SPSS v11.5 (IBM, USA).

RESULTS

Morhpogenetic changes during regeneration:
Administration prior to autotomy: Administration of EGF prior to autotomy in the lizard, *Hemidactylus flaviviridis*, was found to enhance the process of tail regeneration. In EGF treated lizards, the wound was healed four days in advance to control lizards. Blastema formation was also faster. Length of regenerates revealed a boost in growth in the treated animals. The growth rate of the regenerate from 2-12mm and also from 12-24mm was found significantly increased in the treated group (Figure 1A and 1B). A 72.72% increase in the rate of growth of regenerate was recorded in the EGF treated lizards (Table 1). The onset of differentiation stage in regenerates of the EGF treated animals was also earlier than that in control animals.

Administration at WH stage: Animals treated with EGF at WH stage developed blastema faster than control animals (Table 1). Proliferative activities in the regenerates of EGF treated animals were significantly ($p \leq 0.01$) higher in comparison to control animals (Figure 1A and 1B). The initiation of differentiation also took place earlier in the EGF treated animals. The percent rise in the rate of growth was 30.81% at this stage.

Administration at BL stage: Treatment with EGF at BL stage increased the rate of growth of regenerate during initial events (Figure 1A and 1B), but later on, there was no significant increase in the growth rate of regenerate (Table 1).

Nucleic acid content in tail regenerates:
Large scale proliferation of cells begins from the Blastema stage and continues to the end of regeneration. Therefore, nucleic acids were estimated from the Blastema stage onwards. The DNA content was found to be significantly ($p \leq 0.01$) higher in blastema of EGF treated animals, when compared to that in control (Figure 2A and 2B). This result indicates heightened DNA synthetic activity in response to EGF administration. RNA content was also significantly ($p \leq 0.01$) higher in the treatment group, showing increased transcriptional activity and increased protein content after EGF treatment (at $p \leq 0.05$) showed increased translational activity (Figure 2A and 2B).

DF stage: At differentiation stage, a significant ($p \leq 0.01$) hike was observed in the DNA content of tail regenerates in EGF treated animals as compared to that in control lizards. However, RNA and protein levels at this stage were comparable in both the groups (Figure 2A and 2B).

Histofluorescent staining of nucleic acids:
Acridine orange staining was used for localization of nucleic acids in the growing tail tissue at early blastema, late blastema and differentiation stages. Intense yellow staining of the epidermal region at all stages is indicative of the extensive proliferation occurring in the epidermis to cope with the growing mass of tissue underneath it. The early and late blastema stages both show greater intensity of yellow fluorescence in the sections from treatment group (Figure 3A and 3B). Towards the late blastema phase, the intensity of red fluorescence increases and reflects the increase in protein synthetic activity as cells begin to progressively mature (Figure 4A and 4B). In sections from tissues collected during the differentiation stage, more areas are stained red, which could be a result of elevated expression of a variety of genes associated with the differentiation of proliferating cells into specific cell types. Cells of the ependyma region are stained red, probably owing to their differentiation into cartilage. Muscle bundles can also be seen in these sections, growing as bundles while getting differentiated. The sections from control and treatment groups at this stage do not show any major difference in the staining intensity or pattern, and certainly not as pronounced as that seen in the blastema (Figure 5A and 5B). Overall, throughout the process of regeneration, there is high RNA synthetic activity in the mesenchyma, while DNA synthetic activity is majorly seen in the epidermis.

DISCUSSION

The present study revealed a positive influence of EGF on tail regeneration in *Hemidactylus flaviviridis*. Administration of epidermal growth factor to the animals accelerated the process of wound healing after autotomy of the tail. This could be due to the rapid closure of the wound by the epithelial cells. Thus, EGF may be stimulating epithelial cell proliferation during the early healing process. The proliferation of epithelial cells at the amputation site is accompanied by migration over the wound surface. Various studies have shown that activation of the epidermal growth factor (EGF) receptor following ligand binding may play an important role in epithelial repair processes by inducing cell migration, proliferation, and differentiation [31]. Xu *et al*. [32] have also shown that epithelial wound healing is, at least in part, mediated in an autocrine fashion by epidermal growth factor receptor (EGFR)–ligand interactions.

The healing of wound is followed by proliferative activities in cells of the newly formed blastema. Blastema from animals treated with EGF showed heightened cell proliferation. *In vitro* studies have previously shown that EGF has the capacity to trigger cell proliferation [33, 34] and to rescue cells from apoptosis [35]. Though EGF has been shown to be mitogenic for epithelial cells [36-38], in the regenerating blastema it might be promoting proliferation of the blastemal cells, which are pleuripotent in nature. The observed accelerated growth of the blastema in EGF treated animals can be credited to the influence of exogenous EGF. Therefore, lizards treated with EGF showed an early attainment of blastema and differentiation stages. Geimer and Bade [39] demonstrated that EGF is a potent mitogen for most cultured cells and has previously been shown to induce the migration of rat liver epithelial cells. The involvement of EGF in cell proliferation was confirmed by nucleic acid quantification studies. There was a significant increase in the DNA content in the regenerates at the blastema and differentiation stages in lizards treated with EGF. Histofluorescence studies showed an obvious increase in intensity of staining of nucleic acids in the apical epithelial cap of EGF treated lizards (Figures 3 and 4), indicating a rise in DNA synthetic activity in this region.

The proliferative role of EGF is effected by binding with its receptors. EGF binding to the EGFR rapidly initiates a number of signal transduction pathways, including Ras/Raf/MEK/ERK, phospholipase C-γ/PKC, and PI 3-kinase/Akt,that regulate cellular function [40]. Various studies have demonstrated the involvement of the EGF receptor and the MAP kinase signaling pathway in epithelial cell proliferation [41, 42]. The observed rise in the levels of DNA in EGF treated animals explains the high rate of proliferative activities induced by EGF. Hyldahl [43] showed that EGF stimulates the initiation of DNA synthesis in the corneal endothelial cells and also increases cell division in lenses in culture [44].

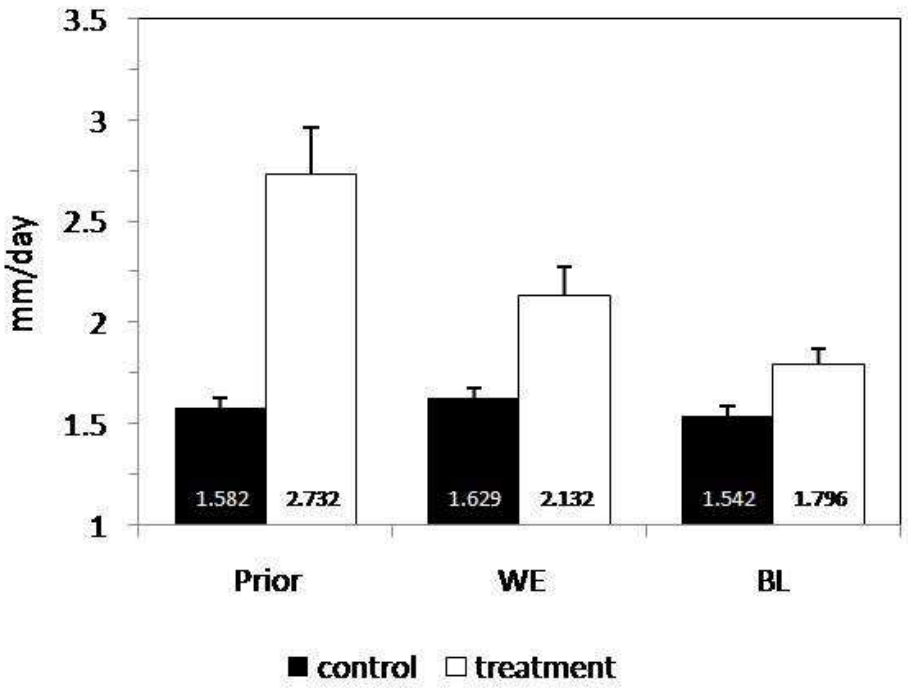
Along with its role in cell proliferation and growth, EGF was also found to be involved in the synthetic activities during tail regeneration. The regenerates of lizards treated with EGF showed an increase in RNA and protein levels

during early growth of the regenerate. Histoflourescence studies also showed a significantly higher localization of RNA in the regenerates of treated animals at the blastema stage (Figures 3 and 4). RNA staining was prominent in the lower margin of the AEC, indicating that as the proximal cells of the AEC are engaged in proliferative activities, the distal ones start the synthetic processes. Moreover, EGF is known to enhance transcriptional and translational activities, as has been shown earlier by many *in vitro* studies [36, 45-47]. During differentiation stage, the DNA levels in the regenerates of the EGF treated animals were higher than those in control lizards. However, no such increase was observed in the RNA levels.

In summary, the administration of exogenous EGF enhanced the process of tail regeneration in *Hemidactylus flaviviridis*, particularly during the early stages. There was a positive correlation between EGF and the DNA levels in the regenerates of lizards. EGF administration shortened the time taken by the animals to attain the WE and BL stages, but had little influence on the differentiating tail. Thus, EGF, as is FGF-2, might be involved in the early events of tail regeneration. However, further study in this respect needs to be carried out to gain deeper understanding of the mechanisms by which epidermal growth factor influences the regeneration of tail in *Hemidactylus flaviviridis*.

Table 1: Number of days taken by the animals to attain various stages of regeneration and the percentage increase in growth in the treated versus control lizards

Treatment schedule	Experimental groups	Number of days taken to attain a stage			% increase in rate of regeneration	
		Wound Epithelium	Blastema	Differentiation	From 2-12 mm	From 12-24 mm
Prior to autotomy	Control	7	9	17	-	-
	EGF	4	6	14	72.72	32.74
At WE stage	Control	-	10	17	-	-
	EGF	-	7	14	30.81	24.10
At blastema stage	Control	-	-	16	-	-
	EGF	-	-	15	16.50	4.92



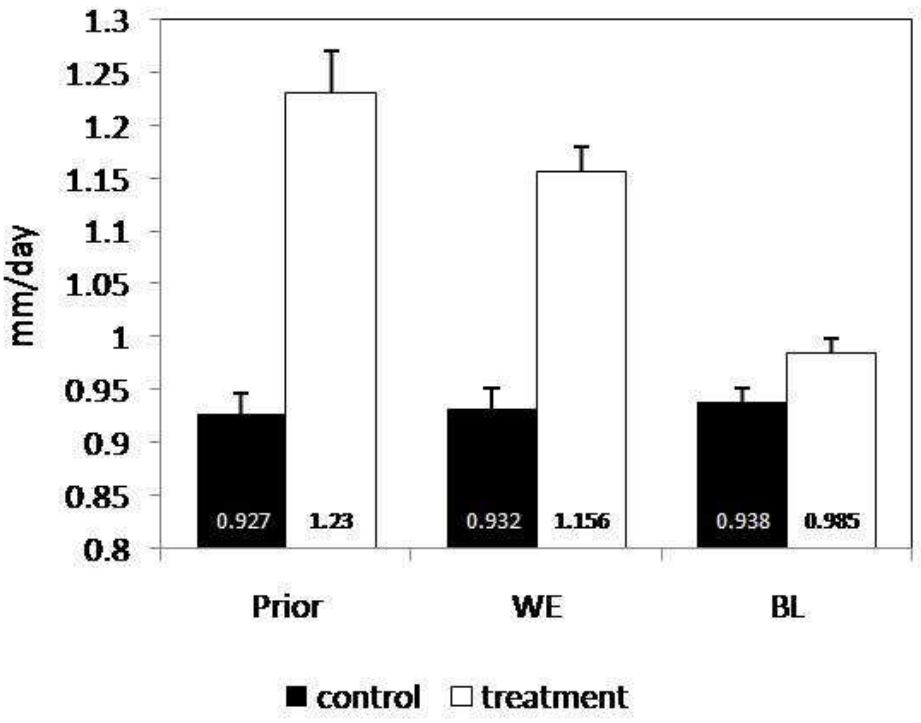
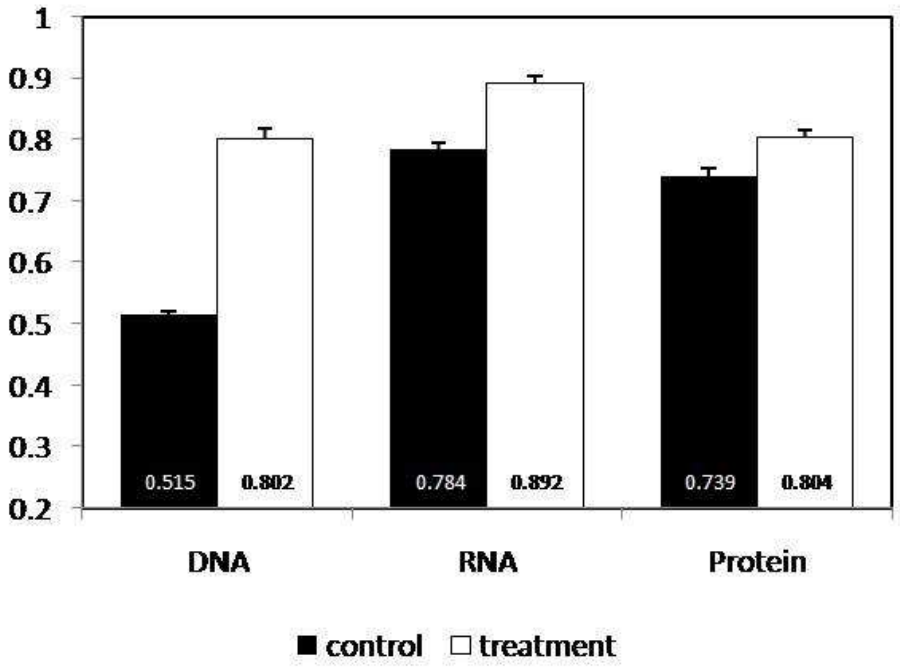


Figure 1: The rates of growth of regenerates from (A) 2-12mm and (B) 12-24mm for three series of animals where treatment began prior to autotomy, at WE stage or at BL stage



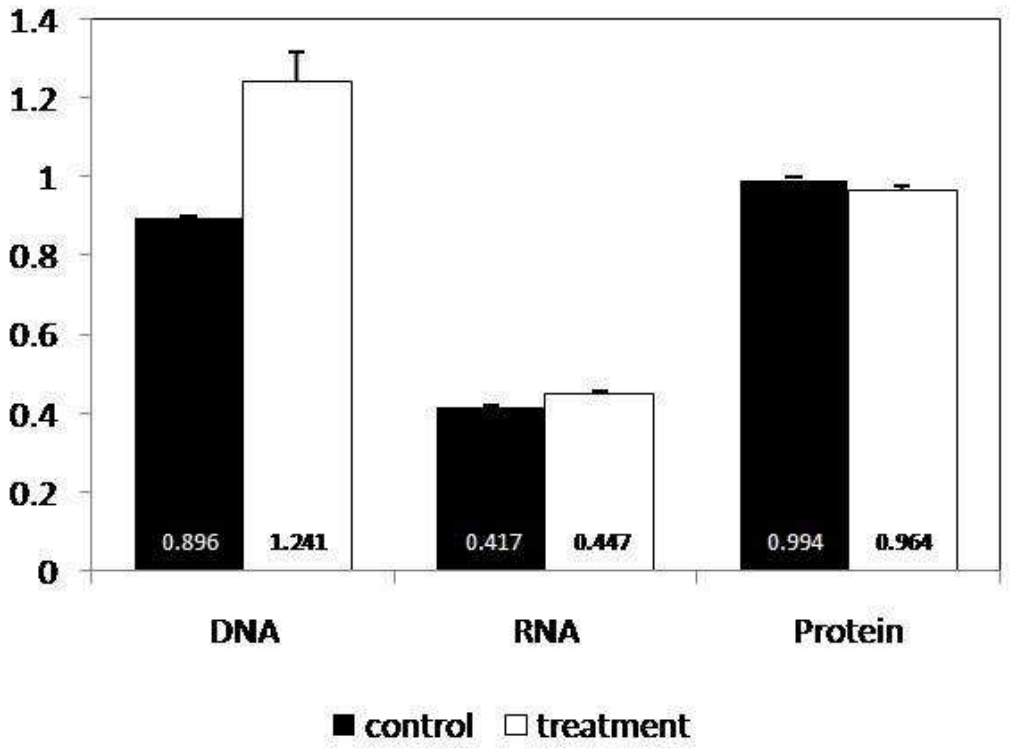
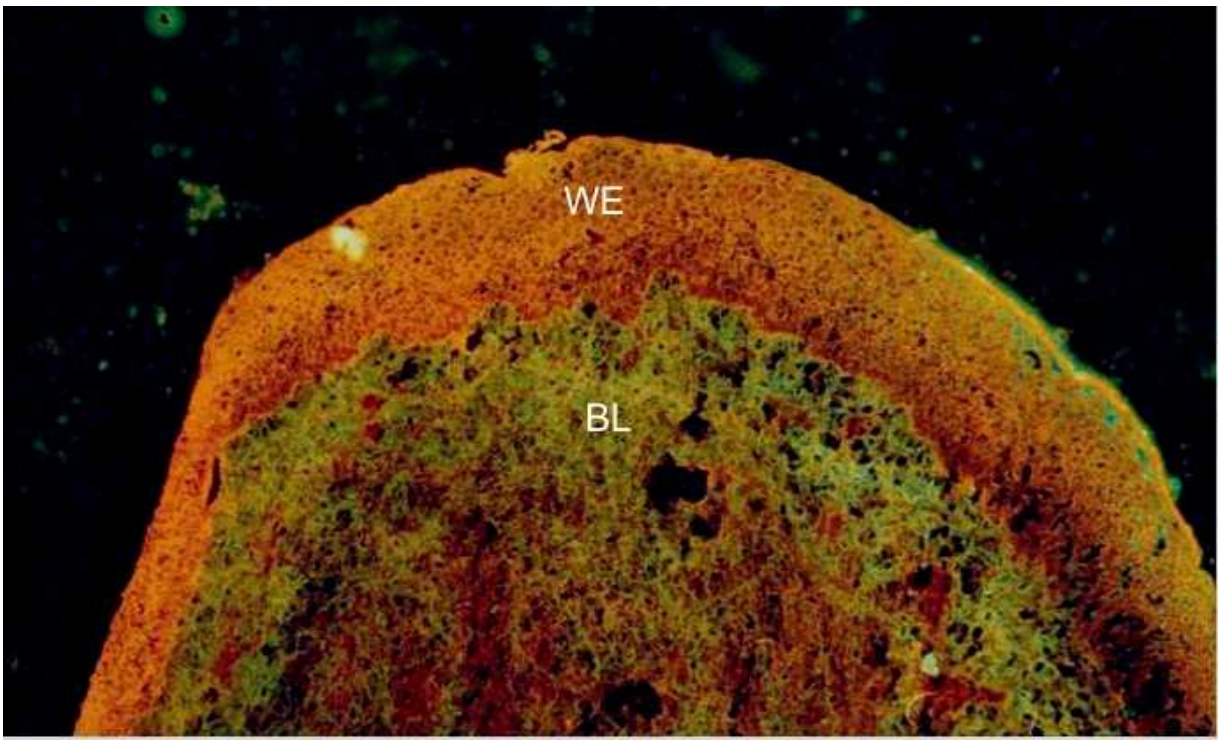


Figure 2: Nucleic acid and protein content in tissue regenerates of experimental animals at (A) Blastema stage and (B) Differentiation stage. DNA and RNA values are in µg/100mgprotein and Protein content is in mg/100mg tissue



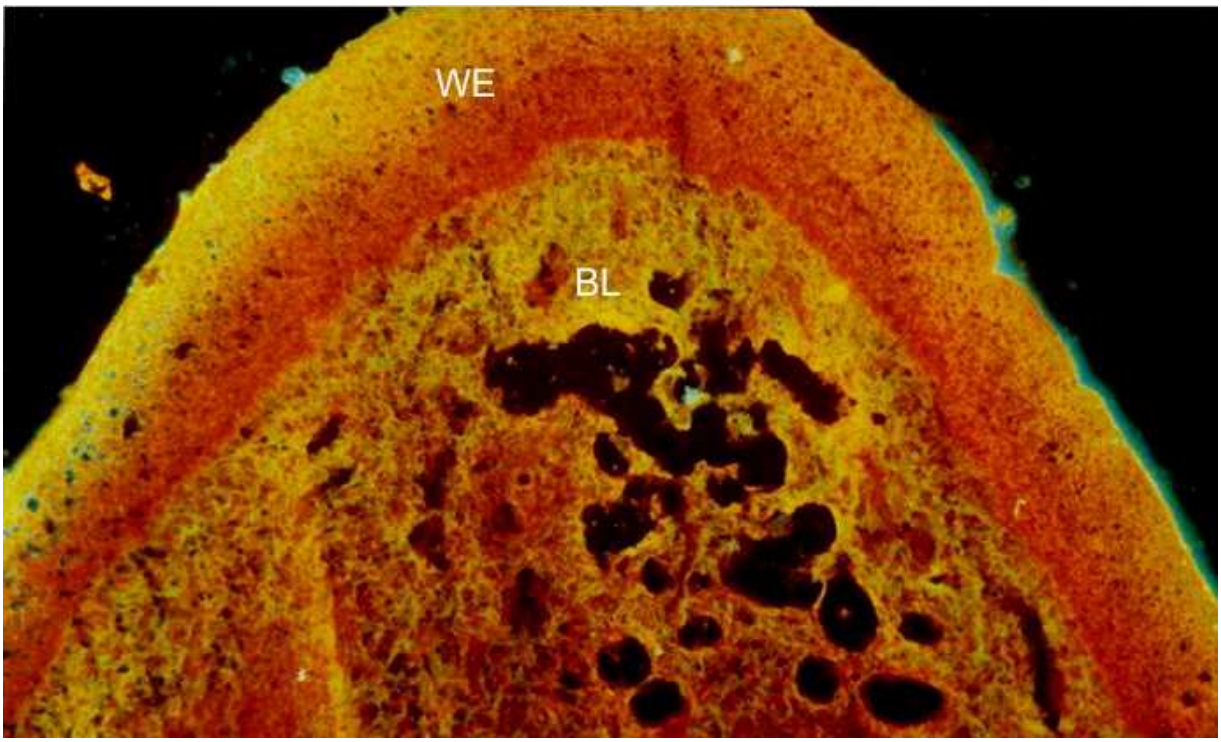
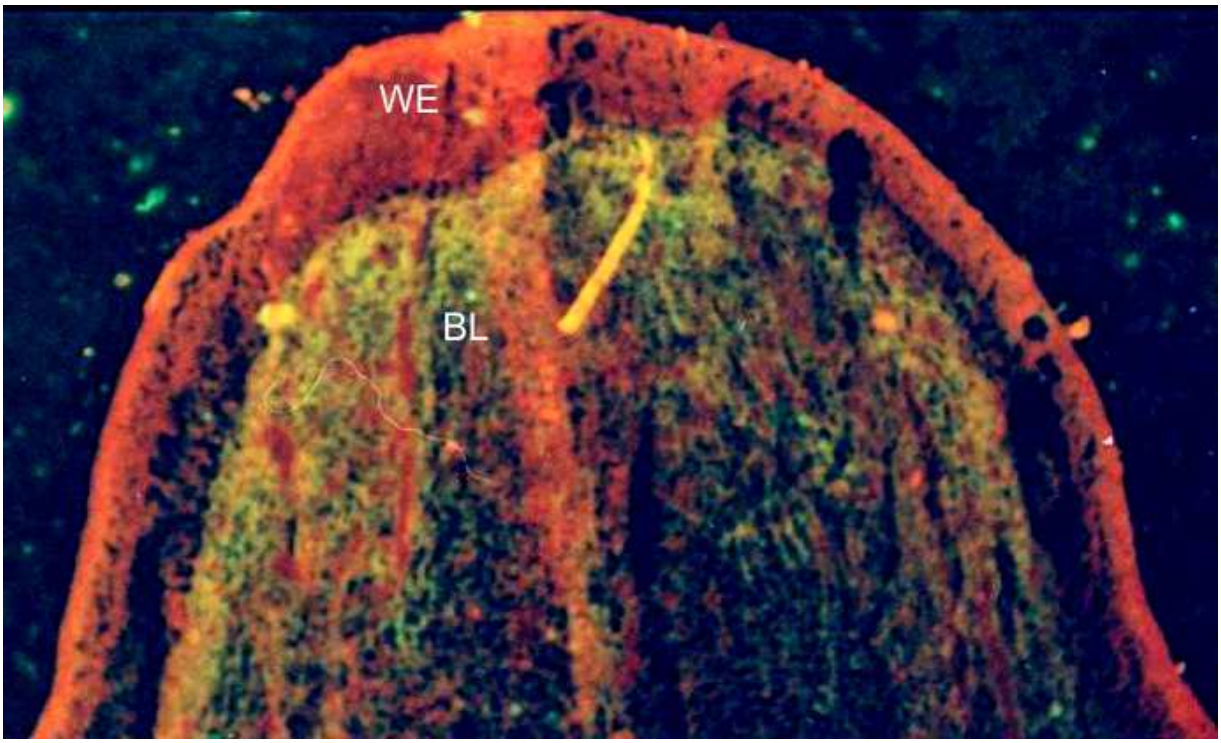


Figure 3: Nucleic acid localization in regenerates of (A) Control and (B) EGF treated lizards at early blastema stage. Yellow fluorescence shows DNA localization and red fluorescence shows RNA localization. WE: Wound Epithelium, BL: Blastema



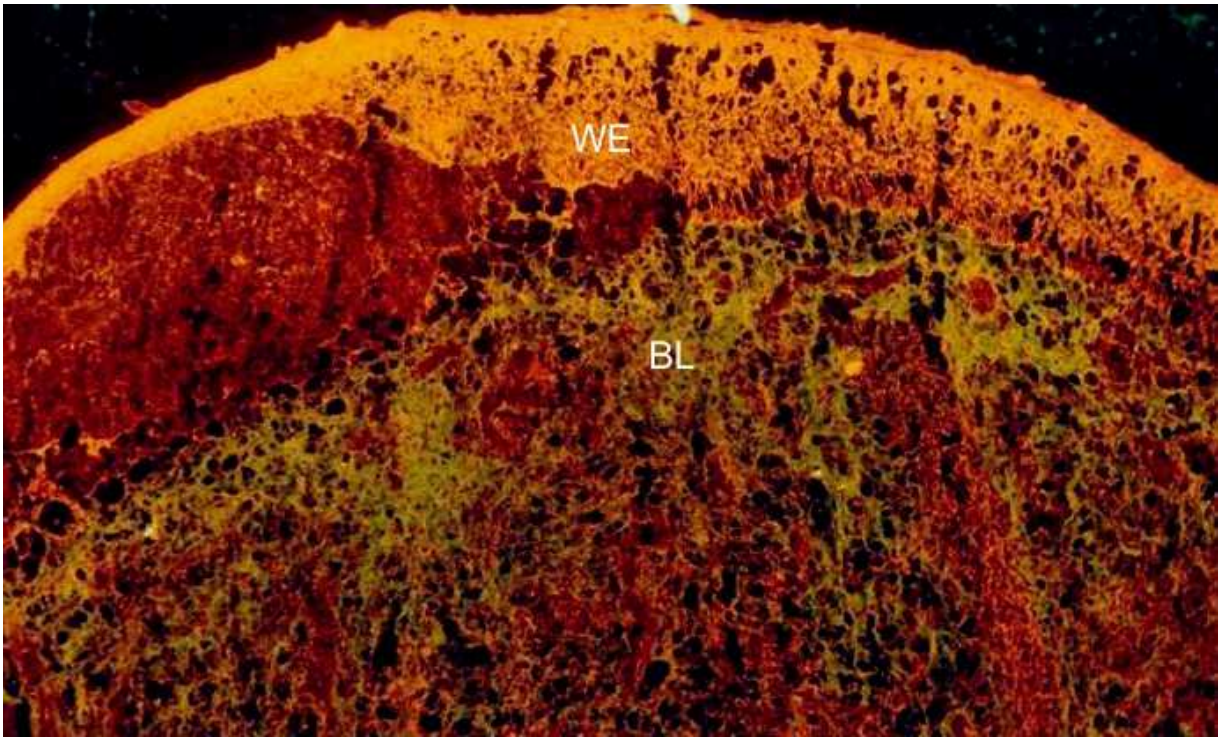
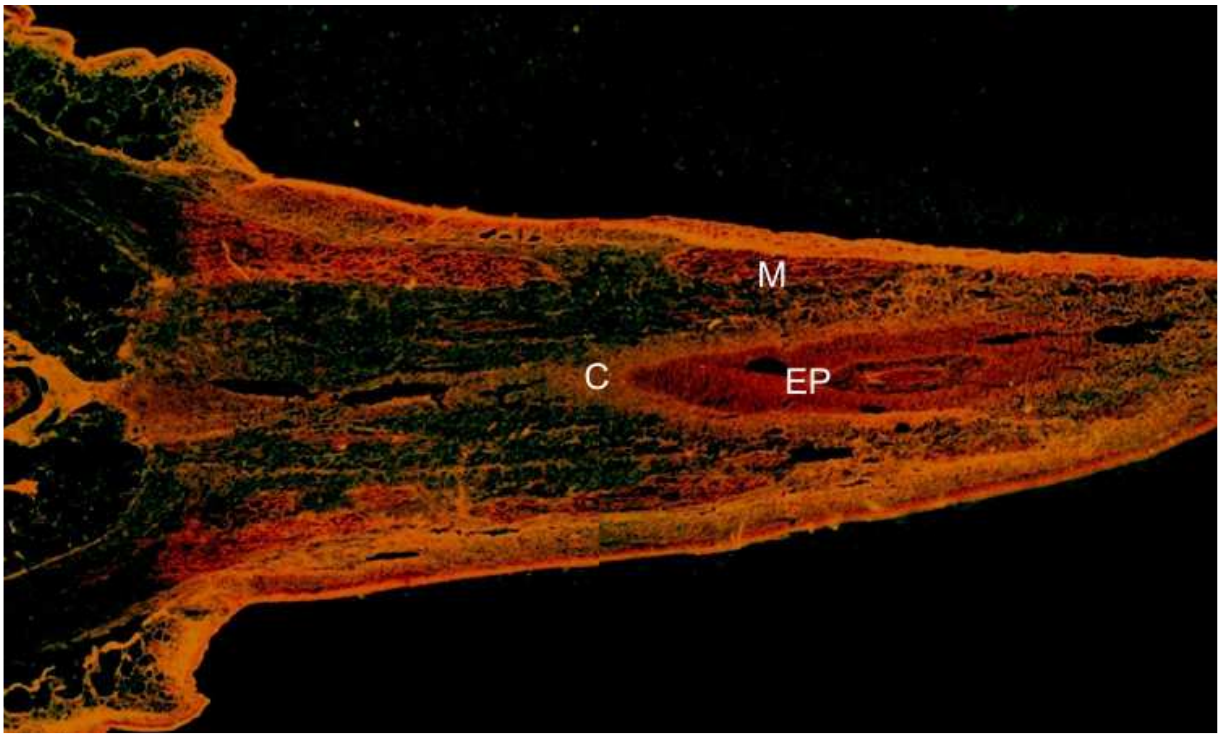


Figure 4: Nucleic acid localization in regenerates of (A) Control and (B) EGF treated lizards during late blastema stage. WE: Wound Epithelium, BL: Blastema



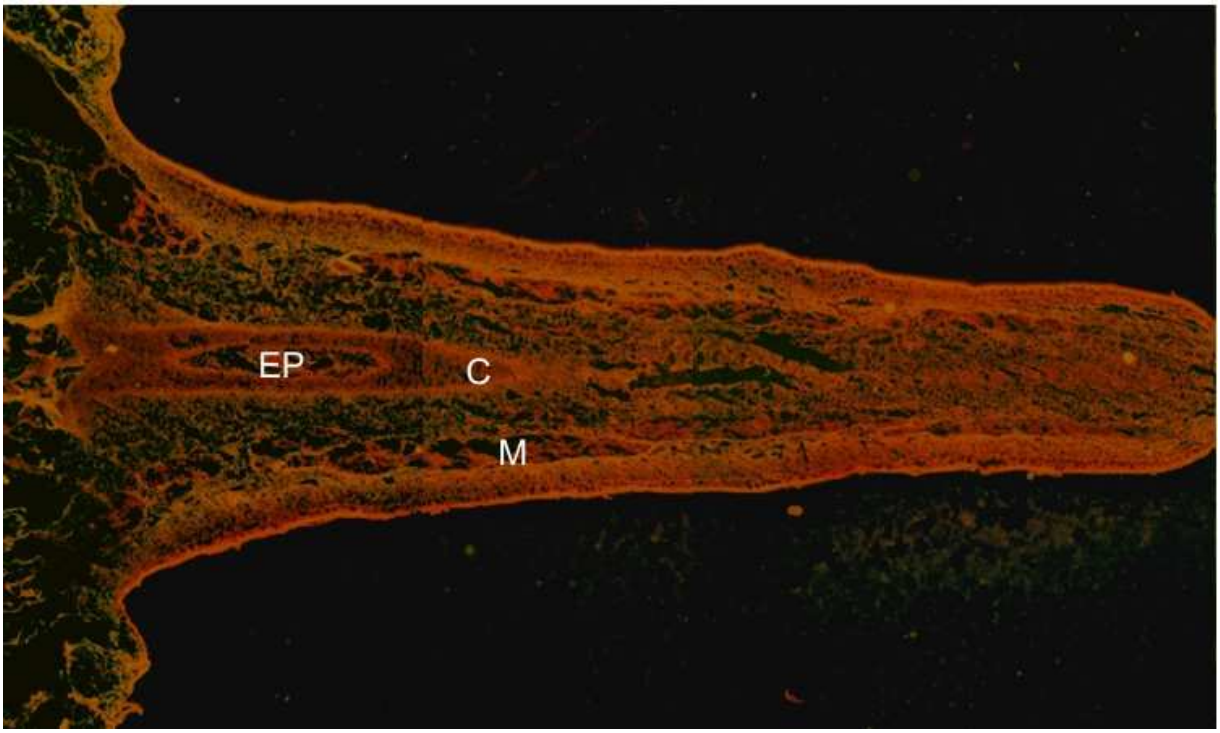


Figure 5: Nucleic acid localization in regenerates of (A) Control and (B) EGF treated lizards during differentiation stage. M: Myomeres, EP: Ependyma, C: Cartilage

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