CHAPTER 4

EVIDENCE FOR THE INVOLVEMENT OF PROSTAGLANDIN E_2 IN DIFFERENT CELLULAR ACTIVITIES DURING EPIMORPHIC REGENERATION

INTRODUCTION

The mechanisms underlying regeneration of the amputated tail have been studied far more extensively than those responsible for autotomy itself. The most likely reason for these extensive studies could be the possible applications of regeneration in the promotion of tissue repair and regrowth in mammalian species, particularly in humans (Clause and Capaldi, 2006). The urodele amphibians known for their remarkable regenerative abilities (Mescher, 1996) and perfect tissue repair provide important model systems for studying the mechanism behind the most alluring phenomena of regeneration. If regeneration in all higher vertebrates utilizes similar mechanisms and proceeds in a similar manner, then investigations of these organisms can likely provide greater understanding of the general process of regeneration that can then be applied across species (Bellairs and Bryant, 1985).

Extensive work on urodeles have revealed several critical features which are now believed to be necessary for successful regeneration in all higher vertebrates, the first of these critical features is the formation of a specialized wound epithelium over the site of amputation, providing the proper subepidermal environment required for later regeneration. Some of the necessary steps for the formation of developmental outgrowth are-dedifferentiation of cells at the site of injury, the subsequent re-entry into these cells in cell cycle along with their adequate and appropriate innervation, and the re-initiation of original patterning programs (Muller *et al.*, 1999)

Innervation is crucial for successful regeneration and may be the first step required for its initiation. If a nerve is not present from the onset, standard wound and epidermal repair occurs without subsequent regeneration (Gardiner *et al.*, 2002; Endo *et al.*, 2004), suggesting that certain neural peptides or growth factors provided by the nerve are required for the

triggering of regenerative processes. In particular, interactions with position-specific fibroblasts derived from the blastema are thought to provide a second signal that guides the developing and differentiating cells into the particular pattern needed to produce the completely regenerated structure by forming a specific type of connective tissue scaffold (Endo *et al.*, 2004).

Still, it remains unclear to what extent the newly generated cells inherit a particular positional identity from their differentiated precursors or if these newly regenerated cells rely on extrinsic cues from the cellular environment (Brockes, 1997). For certain cell types at least, extracellular cues appear to play an important role, for example-cells that were artificially repositioned within the regenerating limb responded to their new location and consequently did not develop according to the usual program. This response demonstrates a high level of sensitivity to neighbouring cells that provide contrasting positional information (Bryant *et al.*, 1987). From these studies, it was clear that some extracellular signals are induced by injury and that there are at least two parallel and distinct pathways that are activated in the muscle cell to execute cell dedifferentiation. Thus, to unravel these extrinsic cues from the cellular environment the role of prostaglandin E_2 (PGE_2), one of the possible cues for initiation and maintenance of regeneration, is being explored.

There are two modes by which a muscle can be regenerated-the tissue mode and the epimorphic mode. Regeneration from the same muscle is the tissue mode as seen in amphibians and the other is epimorphic. Key to epimorphic regeneration is the formation of blastema, with the associated tissue interactions and control mechanisms inherent in the process of blastema formation and development. Early work (Carlson, 1970) has demonstrated that a hind limb muscle in the axolotl could also regenerate after mincing by the tissue mode of regeneration, i.e., the process of muscle regeneration that commonly takes place in mammals. The regeneration of an entire muscle by the tissue mode does not involve the formation of blastema, but rather involves the direct formation of muscle fibres from cellular elements remaining after tissue damage.

A detailed histological description of the reactions of the damaged muscle fibres after limb amputation in larval *Amblystoma* has been published (Thornton, 1938). From his studies, Thornton concluded that during the phase of dedifferentiation, myonuclei, surrounded by a thin rim of cytoplasm, break off from the damaged muscle fibres and migrate distally to join

the regeneration blastema (Figure 4.1A). In later studies, involving both light and electron microscopy, Hay (1959, 1962) and Lentz (1969a, b, c) made similar observations. Further Alibardi (1995) also worked on the finer aspects of histological alteration occurring in epidermis and also the keratinization of the skin (Figure 4.1B). This stage is a prelude to the period of tissue dedifferentiation, during which the tissues underlying the amputation surface largely loose their mature differentiated characteristics and a population of mesenchymal cells begins to accumulate in that area forming apical epithelial cap (AEC). Dedifferentiation is followed by the formation of a regeneration blastema (Figure 4.2). In absence of data concerning the histology of the regenerate it becomes important to uncover the histo-architectural modification occurring in the regenerate of the tail with the administration of a cyclooxygenase (COX-2) specific inhibitor (etoricoxib).

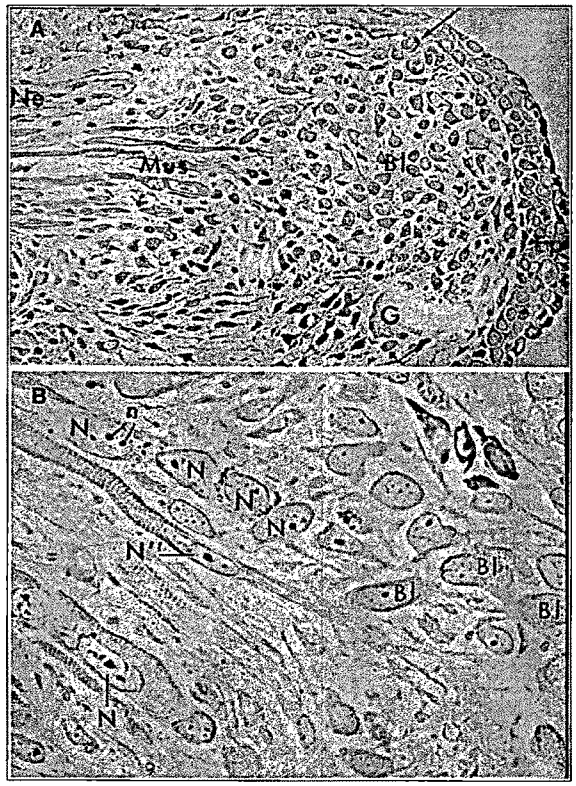


Figure 4.1

A) Section through a regenerating larval limb at 5 days (*Ambystoma punctatum*). On the left is the mature tissue, containing muscle (Mus) and nerve (Ne). The right part of the tissue is the regenerating blastema (Bl) containing mononucleate cells, and covered by the wound epidermis (Ep), G denotes an osteoclast.

B) Histological evidence for muscle cell dedifferentiation. Enlarged view of the transition zone between the ends of muscle fibers and the blastema. N˝, N′, and N mark are apparently successive stages in the dedifferentiation process. A row of dedifferentiating cells with rounded nuclei (N) are seen budding at the ends of fibres. The cytoplasm in these cells has lost the myofibrillar structures characteristic of differentiated muscle (Hay, 1959).

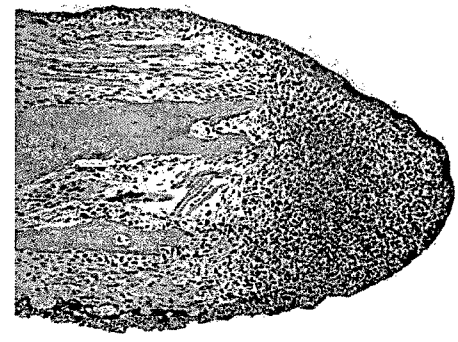


Figure 4.2
Regenerating forelimb of an adult newt 25 days after amputation. A well-developed regeneration blastema is present (distal region). The proximal region shows the portion of the original bones and muscle fibres (Carlson, 2003).

The process of new muscle formation during caudal regeneration results in a lot of protein amendments (Chapter 3). These changes which were observed with the blockage of PGE_2 could further affect the process of myogenesis and results in dynamic changes of cytoskeleton proteins. Desmin is a type III intermediate filament found near the Z line in sarcomeres and was first purified in 1977, however, its gene was characterized in 1989, and the first knock-out mouse was created in 1996 (Costa *et al.,* 2004). Desmin is only expressed in vertebrates, however homologous proteins are found in many organisms (Bar *et al.,* 2004). It is a 52 kDa protein that is a subunit of intermediate filaments in skeletal muscle tissue, smooth muscle tissue, and cardiac muscle tissue (Li *et al.,* 1997). Desmin is one of the earliest protein markers for muscle tissue in embryogenesis as it is detected in the somites of myoblasts (Bar *et al.,* 2004). Although it is present early in the development of muscle cells it is expressed at low levels and increases as the cell nears terminal differentiation. The muscle cell matures only if desmin is present. Desmin is present in higher amounts after differentiation of cells. This suggests that there may be some interaction between the two in determining muscle cell differentiation. Desmin is also important in muscle cell architecture and structure since it connects many components of the cytoplasm (Li *et al.,* 1997).

The sarcomere is a component of muscle cells composed of filaments of myosin motor proteins which allow the cell to contract. There is some evidence that desmin may also connect the sarcomere to the extracellular matrix (ECM) through desmosomes. Desmin may thus be important in signalling between the ECM and the sarcomere and may further be regulating muscle contraction and movement (Shah *et al.,* 2004). Myosin is one of the two major proteins, responsible for contraction of muscle. In muscle cells myosin is arranged in long filaments called thick filaments that lie parallel to the microfilaments of actin. During muscle contraction, filaments of actin alternately chemically link and unlink with myosin filaments in a creeping or sliding action. The energy for this reaction is supplied by adenosine triphosphate. Myosin and actin also function in the motility of diverse non-muscle cells. In slime molds, for example, although present in much smaller quantities and forming shorter filaments, the interaction of the two proteins is employed to change cell shape and permit some movements. Many convincing evidence are found for the presence of both myosin and desmin in *Xenopus* and in lizards (Quax *et al.,* 1984; Yaoita *et al.,* 1999). Thus, it was thought worth evaluating the expression of desmin and myosin in the regenerating muscle of lizards. Further, to understand whether these proteins are under the regulation of

PGE_2 the lizards were subjected to a specific cyclooxygenase-2 (COX-2) inhibitor and the expression of desmin and myosin was studied.

One of the fundamental processes in wound repair is the formation of new blood vessels, known as angiogenesis. The prostaglandins PGE_1 and PGE_2 and the related compounds have been reported as angiogenic (Bradbury *et al.,* 2005). Angiogenesis is initiated by multiple molecular signals including haemostatic factors, cytokines, growth factors, inflammation and cell matrix interaction. Vascular endothelial growth factor (VEGF) family and its receptors have been identified as the regulators for endothelial progenitor cells (EPC) recruitment in angiogenesis (Ferrara and Gerber, 2002). Expression of the inducible COX-2 enzyme during inflammatory stage of wound healing also leads to the production of VEGF and other promoters of angiogenesis. Therefore, it was thought pertinent to investigate the changes in the process of angiogenesis when the production of PGE_2 was blocked by inhibiting the key enzyme in the synthesis of prostaglandins.

All these studies gave rise to many questions and hypothesis. For instance- could a cell originating from dedifferentiated muscle redifferentiate into a chondrocyte or could a cartilage-derived cell redifferentiate into muscle? Although these questions were the subject of great speculation for several decades, new approaches had to be devised before there was a chance of obtaining a definite answer. Over many years, various research groups have used a variety of techniques, including monoclonal antibodies and retroviral and dye markers to demonstrate that cultured newt myotubes can break up into mononucleated cells when implanted into blastemas of limb (Kintner and Brockes, 1984; Lo *et al.,* 1993; Kumar *et al.,* 2000).*In vitro* studies conducted by them have also shown that nuclei of multinucleated myotubes of newts are capable of re-entering the S- phase of cell cycle under different circumstances, such as inactivation of the retinoblastoma protein by phosphorylation, in response to high concentrations of serum (Tanaka *et al.,* 1997). Recent research has showed that prostaglandin is one of the most potent factors for cell proliferation either in cancer cells or regenerating tissue (Appukuttan *et al.,* 1993). Thus, in order to determine the role of prostaglandin in cell proliferation various cellular markers are being used. The experimental and control animals were tagged *in vivo* with intraperitoneal (i.p.) injection of bromodeoxyuridine (BrdU) to label the cells undergoing DNA replication during cell proliferation.

The cell cycle is a ubiquitous, complex process involved in the growth and proliferation of cells, organismal development, regulation of DNA damage, repair, tissue hyperplasia as a response to injury, and diseases such as cancer. The cell cycle involves numerous regulatory proteins that direct the cell through a specific sequence of events culminating in mitosis and the production of two daughter cells. Central to this process are the cyclin-dependent kinases (cdks) and the cyclin proteins that regulate the cell's progression through the stages of the cell cycle referred to as G_1, S and G_2 phases (Figure. 4.3). The cell cycle can be morphologically subdivided into interphase and stages of mitosis (M) *viz.* the prophase, metaphase, anaphase, and telophase. Interphase encompasses G_1, S, and G_2 phases (Crews and Shotwell, 2003).

The G_1 and G_2 phases of the cycle represent the "gaps" in the cell cycle that occur between the two obvious landmarks, DNA synthesis and mitosis. In the first gap, G_1 phase, the cell is preparing for DNA synthesis. S-phase cells are synthesizing DNA and therefore have aneuploid DNA content between 2N and 4N. The G_2 phase is the second gap in the cell cycle during which the cell prepares for mitosis or M-phase. G_0 cells are not actively cycling. G_0 was originally used to indicate cells in quiescent state but with the potential for division, such as hepatocytes. G_0 has since been loosely and probably incorrectly used to also include terminally differentiated cells, such as those of the outer layers of the epidermis and adult neurons (Crews and Shotwell, 2003).

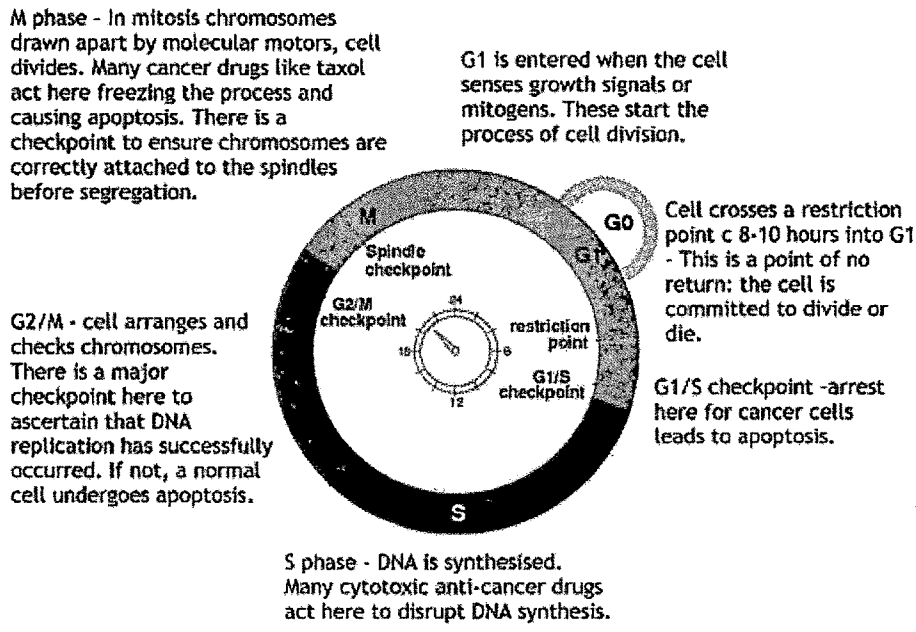


Figure 4.3: Diagrammatic representation of cell cycle (http://www.physiomics-plc.com).

Therefore, in order to investigate any changes occurring in the cell cycle of the regenerating tail of the animals they were subjected to COX-2 enzyme inhibitor resulting in inhibition of PGE_2 synthesis. Changes in the cell cycle were monitored by measuring the intensity of fluorescence emitted by BrdU labelled cells.

MATERIALS AND METHODS

Animals

A total of 50 lizards of both the sexes were selected and acclimatized in the animal house, at 30 ± 2^0 C, for a week prior to experiments. The animals were divided into two groups of twenty five each (See Materials and Methods for details).

Experimental Design and Drug Dosage

Group I: This group of animals served as control to the experimental groups and injected with vehicle (Tris Buffer of pH 8.8).

Group II: The animals were injected with Etoricoxib (25 mg/kg body weight).

The treatment in each group started four days prior to amputation and was continued till the animals reached differentiation stage (fifteen animal each). The drug / vehicle were administered every alternate day. The animals from the groups reaching the different stages of epimorphic regeneration were selected and sacrificed. The regenerate was processed for further cytological analyses.

HISTOLOGY

Histological analysis is used as one of the marker to visualize any alteration in regenerate of tail with the blockage of PGE_2. Longitudinal sections (5μm) of tails from three animals per regeneration group were stained with Harris's haematoxylin and eosin (H&E). The histological structure of the tissues on the slide were visualized at 40X using Leica DMRB Microscope (utilizing LEICA QWin software) and pictures captured using JVC video camera. Location of images was standardized to the central third of the regenerating tail between the cartilage tube and the epidermal layer.

IMMUNOSTAINING OF THE MUSCLE MARKER PROTEIN (DESMIN AND MYOSIN)

Following an initial tissue processing (as in case of MMP-2 and MMP-9) the sections were incubated with primary antibody against desmin (A generous gift from Dr. Jyotsna Dhawan, Center for Cellular and Molecular Biology (CCMB), Hyderabad, 1:300 dilution of Anti

Desmin) and against myosin heavy chain (A generous gift from Dr. Jyotsna Dhawan, CCMB, Hyderabad, 1:300 dilution of Anti Myosin) for 18 hours overnight inside a moist chamber. The moist sections were subsequently incubated with fluorescent conjugated secondary antibodies (goat anti-rabbit Alexa Fluor 488 , 1:500 dilution).The sections were observed under a fluorescent microscope (Leica , DMRB) using a blue filter.

IMMUNOHISTOCHEMSITRY OF VEGF

As in case of Desmin and Myosin the sections were incubated with primary antibody (A generous gift from Dr. Gopal Kundu (National Centre for Cell Science (NCCS), Pune), 1:50 dilution of Anti VEGF) for 16 hours overnight inside a moist chamber. Following overnight incubation the sections were incubated with FITC-conjugated secondary antibody (1:100). The sections were observed under a fluorescent microscope (Leica, DMRB) using a blue filter.

CELL PROLIFERATION ASSAY

Nucleic acid Localization

Fresh frozen sections (5 μm thickness) were stained with acridine orange (AO) (concentration as shown in Material and Methods). Acridine orange is a tricyclic amine that forms highly fluorescent complexes with nucleic acids. Because of differing wavelengths of maximum absorption of ultraviolet light and maximum emission of visible light, AO complexes with ribonucleic acid (RNA) are perceived as orange-red in colour, but those formed with deoxyribonucleic acid (DNA) are seen as yellow-green (Bertalanffy and Bickis, 1956; Kasten, 1967). These sections, stained with AO, were visualized under epi-fluorescence microscope (DMRB, Leica) with filter settings of 440 nm excitation filter and 510 nm barrier filter and images captured by CCD camera

BrdU injection

The lizards at blastema and differentiation stage were injected the thymidine analog 5-Bromo-2'-deoxyuridine (BrdU) (Sigma Aldrich, St. Louis, Missouri, USA): aqueous solution of 10:1 ratio. The injection was performed 24 hours before fixation. The tissues were harvested and processed for immunohistochemistry (See Materials and Methods for details).

HISTOIMMUNOFLUORESCENCE STAINING

5-Bromo-2'-Deoxyuridine (BrdU) is a uridine derivative that selectively incorporates into DNA at the S phase of cell cycle and is used for identification of DNA synthesis in tissue

sections. Fluorescent-conjugated antibody specific for BrdU, to monitor incorporated BrdU. The detailed procedure is described elsewhere (Material and Methods).Thus with the number of cells exhibiting fluorescence the intensity of cell proliferation is being assayed and the change is examined by comparing the control slides with the experimental ones. The images were analyzed with a Leica fluorescent microscope equipped with UV lamp and filters. Images were taken with a colour CCD camera.

RESULTS

HISTOLOGICAL CHANGES

a) At wound epithelium (WE) Stage

Histological feature of control tail sections showed cornified and keratinized epithelium, small size promuscle and numerous blood capillaries. Central neural tube was evident (Figure 4.4a), whereas in PGE_2 blocked sections no such definite structures were observed (Figure 4.4a and Figure 4.5a).

b) At Blastema (BL) Stage

Control animals showed thickened and active epidermis. An increase in the quantity of blood capillaries and number of promuscle bundles was observed. A thick apical epidermal cap (AEC) and an increase in number of myomeres were seen. However, in treated animals, samples demonstrated a relatively decreased rate of myogenesis, less number of promuscle cells and a reduced amount of myoblast fusion (Figure 4.4b, and Figure 4.5b).

c) At Differentiation (DF) Stage

This stage accounts for the complete growth of the tissue with the definite structures like more number of blood capillaries, melanocytes, muscle bundles and neural tube growth in control animals. Whereas, in treated animals a highly thickened and keratinized epidermis with thickened muscle bundles were found. The proportion of myogenic cells was found to be less than that of control animals which may be responsible for the observed hampered tail regeneration (Figure 4.4c and Figure 4.5c). Thus regenerate showed a fast differentiation with enhanced myogenesis as compared to treated animals. The rate of tail growth dramatically slowed down and tails appeared complete in etoricoxib treated animals (Figure 4.4c, and Figure 4.5c).

LOCALIZATION OF DESMIN AND MYOSIN

The observed delay in myogenesis was further confirmed by localizing desmin (a smooth muscle marker) and muscle protein myosin. Desmin is known to be expressed in skeletal

myocytes (green-cytoplasmic localization). It was observed that desmin filaments labelled with fluorescent dye were intermingled with those of myofibrils at blastema stage of regeneration (Figure 4.6a and Figure 4.6b). Desmin filaments were found less compact and sparsely distributed in treated animals. Complex network of desmin was localized in the regenerate at the differentiation stage (Figure 4.6a and Figure 4.6b). The resolution of immunofluorescence microscopy is limited, however, and it is impossible to conclude from these results anything about the filamentous state of the desmin, or the detailed ultrastructural relationship of desmin to the Z-band and to other structures in the muscle fibre.

The localization of myosin in the tail section of treated animals also showed an apparent reduction in the fusion of promuscle and therefore was seen as thin bands (Figure 4.7a and Figure 4.7b).

DETECTION OF VEGF

VEGF was intensily stained in the cytoplasm of inflammatory regenerative epithelium of control animals (Figure 4.8a, 4.8b.), while it was weakly stained in COX-2 inhibited animals (Figure 4.8a and Figure 4.8b). In addition, fibroblasts and vascular endothelial cells were also weakly stained. Moreover, numerous vascular structures were observed in the regenerate of control animals to that of experimental animals. The staining of the tissue was very less in proliferative zone and was comparatively more in the muscle forming regions. In some regions diffused matrix staining was also observed. However, a differential pattern of expression of VEGF was found in the regenerate at different stages of its regeneration.

ACRIDINE ORANGE STAINING

a) At wound epithelium (WE) Stage

Histofluorescence localization of the nucleic acid at wound epithelium stage suggested that the intensity and in turn the amount of both DNA and RNA fluorescence was less in the caudal sections of COX-2 inhibitor injected lizards to that of control animals as per the intensity difference between control and treated sections of the regenerate. This, may have lead to a delay in time required for wound closure due to decreased rate of cell division as evidenced by less number of cells showing green fluorescence (measure of DNA quantity) (Figure 4.9a, 4.9b).

b) At Blastema (BL) Stage

The intensity of green fluorescence of DNA was much less in the tail of COX-2 inhibited lizards with respect to the controls. However, it was observed that the number of cells

exhibiting green fluorescence was more in the mesenchymal region of the regenerate. The occurrence of green fluorescent cells was less in the apical region compared to the region closer to the stump (Figure 4.9c, 4.9d).

c) At Differentiation (DF) Stage

The relative intensity of RNA staining was found higher at DF stage as compared to other stages. A lot of protein synthesis activities were seen in the sections at this stage of regeneration however, the intensity of fluorescence was much less in the regenerate of etoricoxib treated lizards compared to group administered with the vehicle alone (Figure 4.9e, 4.9f).

CELL PROLIFERATION STUDY

In order to explore the possibility that the COX-2 induced PGE_2 affects the rate of cell proliferation during lizard tail regeneration, the mitotic marker BrdU was administered in the animal at blastema and differentiation stage (where the maximum rate of cell proliferation is encountered) and tissue was harvested after 24 hrs of BrdU administration. In this method, only cells in S-phase, between the time of injection and the time of killing incorporates BrdU.

a) Blastema (BL) Stage

A marked decrease in number of proliferating cells was observed in treated animals. Moreover, the BrdU labelled nuclei was largely confined to the cell layers below the apical epithelial cap, preferentially in the matrix of the regenerate (Figure 4.10). The results also showed that the number of cells entering the DNA synthesis phase of the cell cycle were much more in control sections of the regenerate at the blastema stage.

b) At Differentiation (DF) Stage

BrdU incorporation in the lizards of etoricoxib treated animals exhibited marked suppression compared to the BrdU labelled myocytes of the controls (Figure 4.11). The histological appearances as well as the immunohistochemical patterns of desmin and myosin from control and etoricoxib treated animals showed less number of muscle bundles and hence, localization of desmin and myosin was altered (Figure 4.6b, 4.7b). The results show abundant proliferating myocytes in the regenerate treated with vehicle and a markedly reduced number of proliferating cells in lizards treated with etoricoxib. Further, it was also observed that the entire epidermis of the vehicle treated animals appeared intensely labelled. Thus, the proliferation of cells in both blastema and differentiation stage were adversely affected in treated animals.

DISCUSSION

Regeneration proceeds by the local reversal of differentiation of adult tissues to provide the proliferating mesenchymal or epithelial progenitor cells to the regenerate (Wallace, 1981; Okada, 1991; Brockes, 1994). Recent reports have suggested the possibility that some of the fatty acids, precursors in the biosynthesis of prostaglandins and phospholipids of the plasma membrane, could control the proliferative capacity of cells (Hortwiz *et al.,* 1974; Holley *et al.,* 1974). To determine whether prostaglandins function as part of this signalling pathway for cell proliferation during epimorphic regeneration, PGE_2 synthesis was suppressed in autotomised northern house gecko by *in loco* administration of Etoricoxib a COX-2 antagonist and the progression of tail regeneration was followed. The goal of the present study was to understand whether the stress induced production of PGE_2 play a key role in epimorphic regeneration or not. The results of the current study vividly indicate that the regenerating system in the current animal model is dependent on COX-2 induced PGE_2 for the formation of the wound epithelium and the proliferation of blastemal cells.

Experimental animals showed a significant delay in attaining various stages of regeneration *viz.* Wound Epithelium and Blastema stage as compared to vehicle treated controls (Chapter 1). This delay of attaining the specific stages prompted further histological studies to unearth the reason behind such a delay. Histological results showed that formation of muscle was delayed in PGE_2 blocked animals and also a decrease in number of fibres was observed (Figure 4.4a-4.4c and Figure 4.5a-4.5c). Similar results were obtained by McLennan (1991a) that inhibitors of prostaglandin synthetase decrease the number of fibres in chicken embryo skeletal muscles and opined that PGE_2 is one of the important stimulants in formation of muscles. Longo *et al.* (1998) found that with the usage of COX-2 inhibitors [N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide] (NS-398) and 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide (SC-58125) the rate of muscle cell proliferation was reduced. Thus it could be possible that the in the present study, the PGE_2 suppression might have been the reason for the observed poor myofibre formation in the regenerate of the treated animals. The thicknesses of these muscle fibres were also found to be altered. This observation was further confirmed through desmin and myosin localization (Figure 4.6b-4.7b). Therefore, it is possible that PGE_2 might be playing a role in the synthesis of these cytoskeleton proteins (Shen *et al.,* 2005). COX-2 pathway plays an important role in muscle healing and that prostaglandins are key mediators of the COX-2 pathway. The presence of desmin in the regenerate is one of the known markers of early myogenesis (Jankowski *et al.,* 2002). Differential expression of myosin cytoskeleton protein

leads to altered muscle fibre morphology and PGE_2 is known to regulate the phosphorylation of myosin for fibre morphology (Kreisberg *et al.,* 1985).

The appearance of blood vessels was also found to be delayed and the components of extracellular matrix too were affected. It is well known that angiogenesis of blood vessels and matrix degradation are one of the important steps in regeneration (Alvarado and Tsonis, 2006, Stoick-Cooper *et al.,* 2007). The vascular endothelial growth factor (VEGF) is a 45-kDa heparin-binding homodimeric glycoprotein that is an important growth and survival factor for endothelial cells (Ferrara and Henzel, 1989). VEGF plays a critical role in physiological and pathological angiogenesis in most biological systems (Ferrara, 2000). Thus, in order to demonstrate that VEGF like molecule(s) do exist in the gecko and that they might play important roles in regulating blood vessel formation throughout tail regeneration. Moreover, a close correlation between the expression of PGE_2 and VEGF is being recognized (Hitoshi *et al.,* 2004; Miura *et al.,* 2004). Hence, localization of VEGF was performed. The results here indicate that COX-2 dependent PGE_2 release in turn stimulates autocrine or paracrine VEGF release during epimorphic regeneration (Figure 4.8a,b). Usage of COX-2 inhibitor etoricoxib leads to hampered and delayed angiogenesis at the wound epithelium and subsequent stages. Similar observation was reported by Ma *et al.* (2002) with usage of celecoxib, a selective COX-2 inhibitor during gastric ulcer healing. The change in the expression of angiogenic factor VEGF in PGE_2 blocked lizards confirms that for the induction of VEGF, PGE_2 is required. Bradbury *et al.* (2005) suggested that PGE_2 increases VEGF transcription and is involved in the Sp-1 binding site via a cAMP dependent mechanism involving EP2 and EP4 receptors. Therefore, the present finding of reduction in the VEGF in treated animals are in agreement with the known role of COX-2 products in angiogenesis (Gately, 2000; Gately and Li, 2004).

A major question since the beginning of research in regeneration has been which tissues contribute cells to the blastema and by what mechanism. Experiments tracing the origin of the blastema through transplantation of marked tissues showed that the blastema derives from multiple tissues, including dermis, peripheral nerve, bone and muscle (Crescenzi, 2002). In order to form the lost structure a lot of cellular changes take place and the augmentation of dedifferentiated cells by cell division is one of the major mechanisms in lizard caudal regeneration. Therefore, it was reasonable to investigate whether the inhibition of COX-2 products affects the rate of cell proliferation and also the nucleic acid contents of the regenerate. The present study of COX-2 inhibition resulted in marked reduction on the pool of cells in the S-phase of the cell cycle at blastema stage, as evident by reduction in

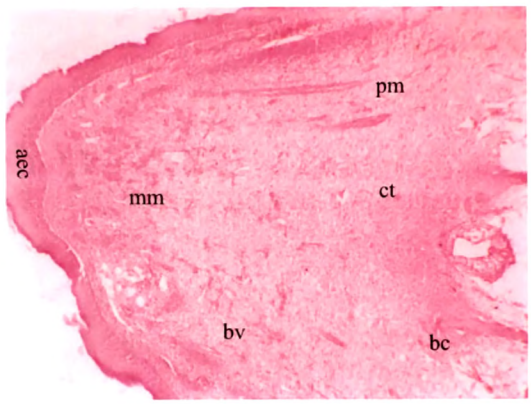
BrdU labelled cells (Figure 4.10). These findings support the hypothesis that the cyclooxygenase-2 derived PGE_2 are involved in cell proliferation. Intensity of fluorescence and also the number of BrdU labelled cells were much less in etoricoxib treated lizards suggesting that number of cells in the S-phase are less and more number of cells are under growth arrest (G_0 phase of cell cycle) possibly due to the absence of the autocoid PGE_2 in the system. Observation made by Appukuttan *et al.* (1993) points to the possible involvement of prostaglandin metabolism during cell aggregation period in the regenerating blastema and resultant cytodifferentiation of blastema in regenerating appendage of lizard. Further, in case of liver regeneration it was observed that PGE_2 production in the liver increased biphasically during hepatic regeneration and that the PGE_2 increased the proliferation of hepatocytes by a specific receptor-mediated process, which is largely associated with cAMP-dependent process (Tsujii *et al.,* 1993). An increase in DNA synthesis with the increase in PGE_2 concentration was also observed (Tsujii *et al.,* 1993). Moreover, less pool of cells was observed in the apical region of the regenerate in the experimental animals because of the inhibition of PGE_2 which is known to regulate subsequent down-stream effects including the stimulation of cell migration and cell proliferation.

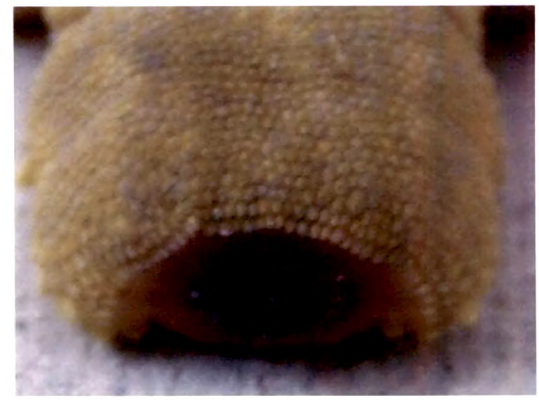
It has been observed that prostaglandin E_2 induces increase in proliferation, migration, and invasiveness of colorectal carcinoma cells (Sheng *et al.,* 2001). Moreover, in the present study it was observed, with the acridine orange staining and BrdU labelling of cells, that the cells that initiate migration belong to the intermediate layers (Figure 4.9a-f and Figure 4.10, 4.11). The basal and the superficial layer cells remain in position. This creates space through which cells from other regions can migrate towards the wound and later the largest area of the section is covered by healing epidermis. It depicts that the AEC receives a continuous input of cells from the neighbouring proliferative lateral surfaces. Similar observation was made by Santos-Ruiz *et al.* (2002) to uncover cell proliferation during blastema formation in the regenerating teleost fin. Santos-Ruiz *et al.* (2002) with the help of BrdU labelling, found that the migration of cells takes place not just to bring cells around the stump nearer to the cap but to bring cells located far from the amputation plane into the vicinity of the cap.

In conclusion, present results show that tail regeneration involves the activation of the cell cycle in the epidermal and in the mesenchymal compartments, apparently in an independent manner. Nevertheless, cells of mesenchymal origin, located far from the amputation plane, give rise to blastemal cells. Elongation of regenerate takes place through proliferation and redifferentiation of these cells which eventually help in further restoration of the lost part. However, it was observed that PGE_2 suppression leads to reduced rate of proliferation as

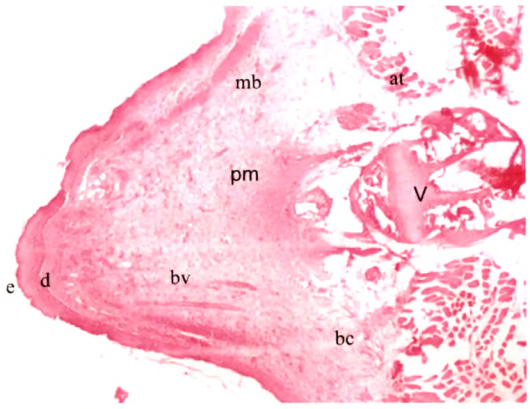
evident from the low turnover in the cell cycle. This together with its adverse affect on the cytoskeleton protein and on angiogenesis might have hampered process of epimorphosis. Therefore, it is logical to surmise that PGE_2 is one of the key modulators of epimorphic regeneration.

Figure 4.4 Histological features of tail regenerate in control animals

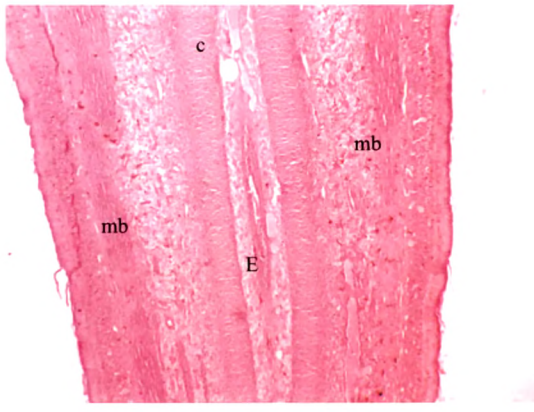




Wound epithelium stage(a)



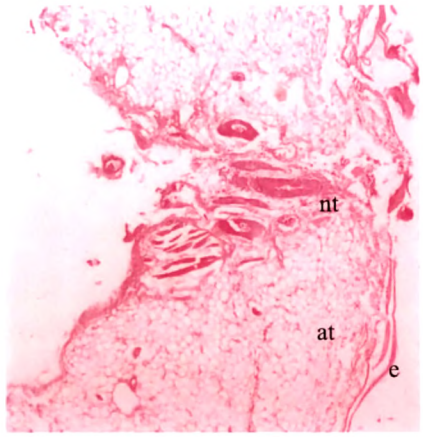
Blastema stage(b)



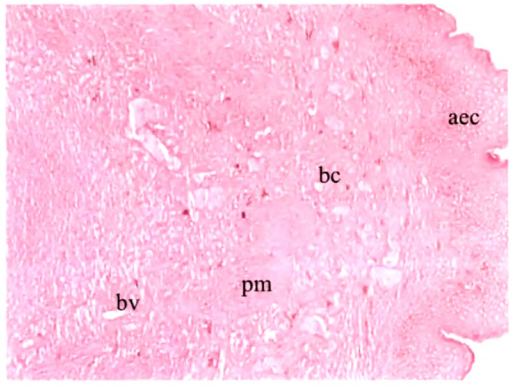
Differentiation stage(c)

at=adipose tissue; aec=apical epithelial cap; bc=blood capillary; bv=blood vessel; c=cartilage; ct=connective tissue; d=dermis; e=epidermis; E=Ependyma; nt=neural tube; mb=muscle bundle; mm= muscle myomere; pm= promuscle; V=vertebrae

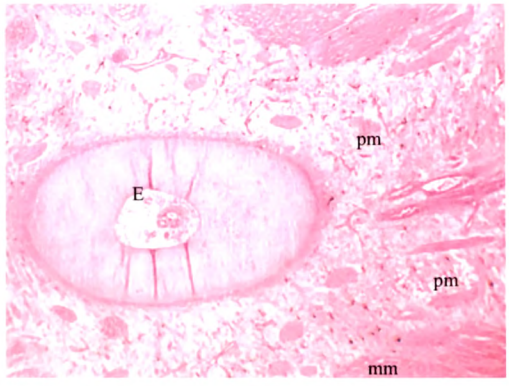
Figure 4.5 Histological feature of tail regenerate in etoricoxib treated lizard



Partial Wound Epithelium Stage (a)



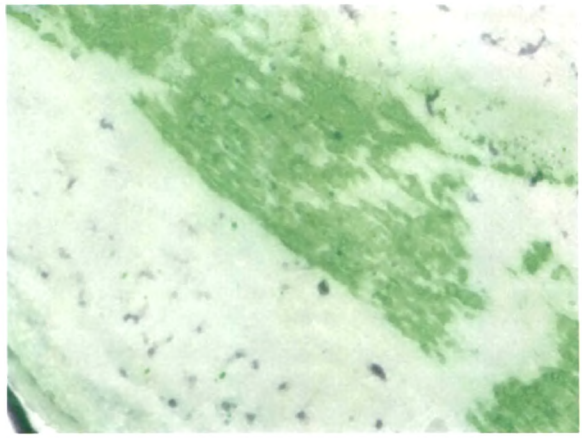
Blastema Stage (b)



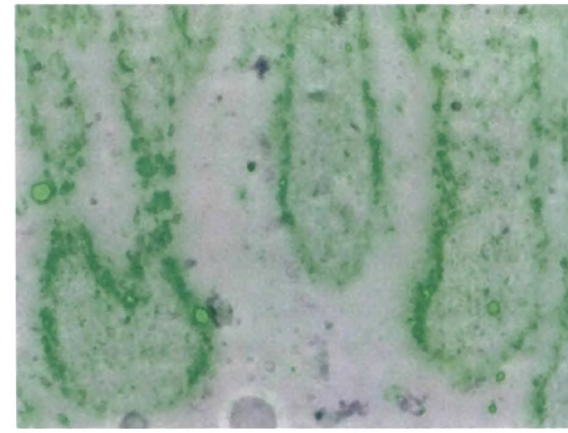
Differentiation Stage (c)

aec=apical epithelial cap; at=adipose tissue; bc=blood capillary; bv=blood vessel; ct=connective tissue; e=epidermis; E=Ependyma; nt=neural tube; mm= muscle myomere; pm= promuscle; V=vertebrae

Figure 4.6 Histofluorescence of Desmin in the regenerates of control and treated animals

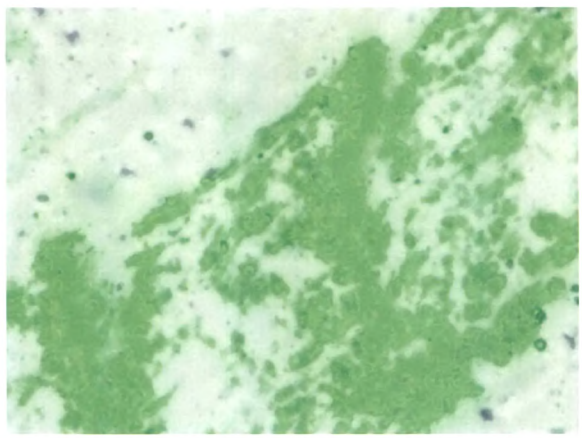


Control (a)

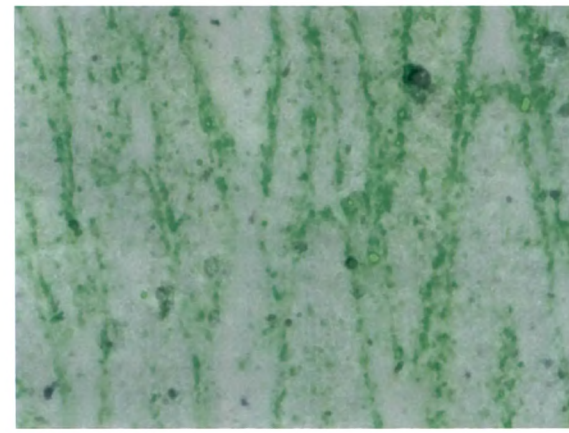


Experimental (b)

Figure 4.7 Histofluorescence of Myosin in the regenerates of control and treated animals

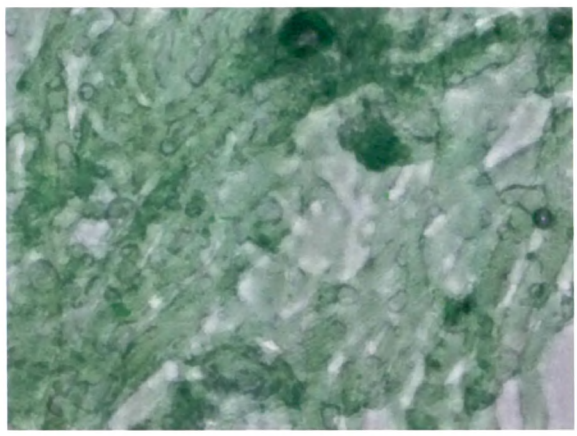


Control (a)

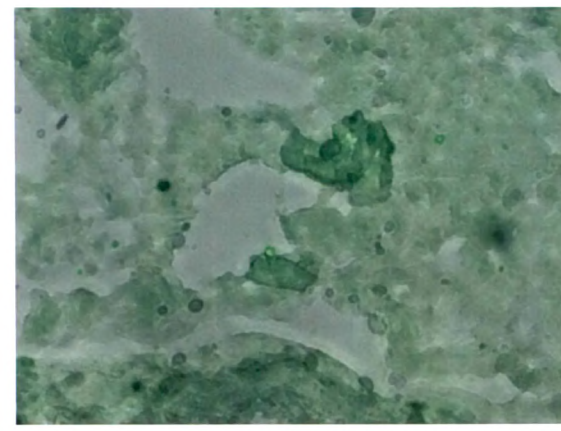


Experimental (b)

Figure 4.8 Histo fluorescence of VEGF in the regenerates of control and treated animals

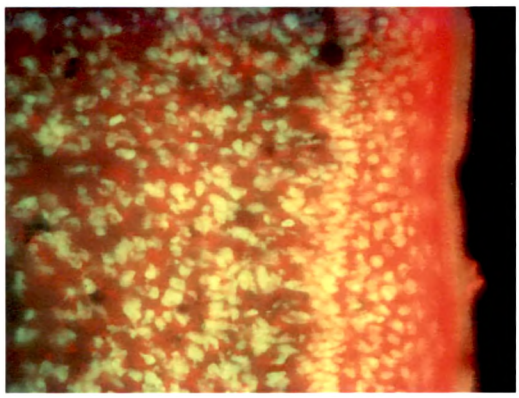


Control (a)

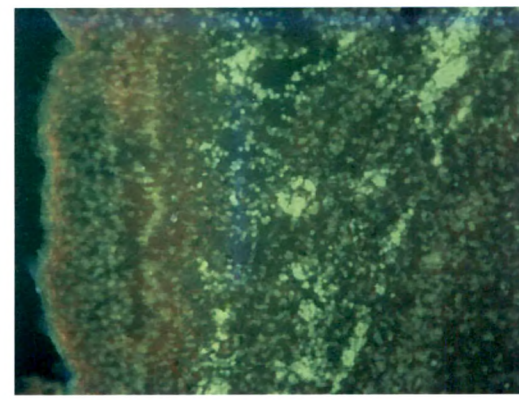


Experimental (b)

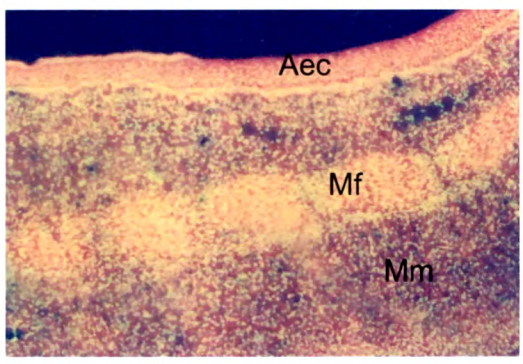
Figure 4.9 Nucleic acid localizations(Acridine Orange Staining) in regenerates of control and etoricoxib treated lizards at defined stages of epimorphic regeneration AO : DNA Green Fluorescence, RNA: Flame Red Fluorescence



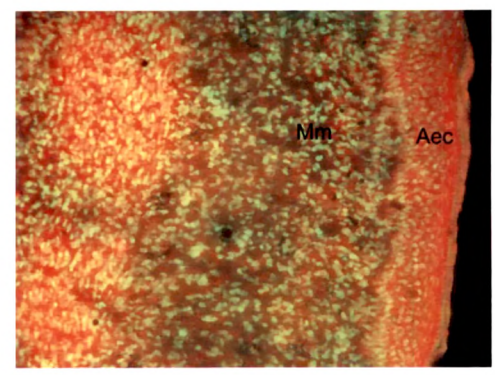
Control at WE Stage (a)



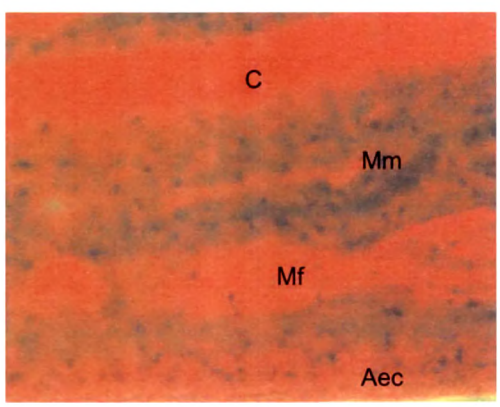
Experimental at WE Stage(b)



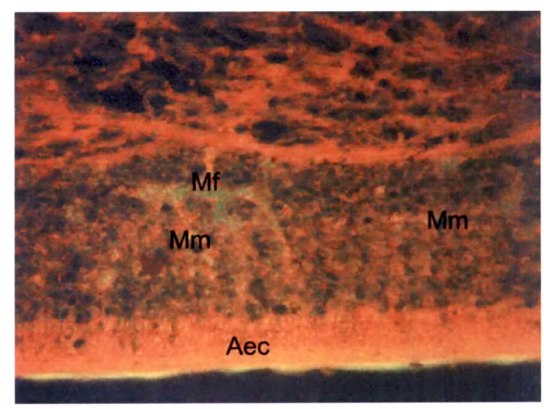
Control at BL Stage (c)



Experimental at BL Stage (d)



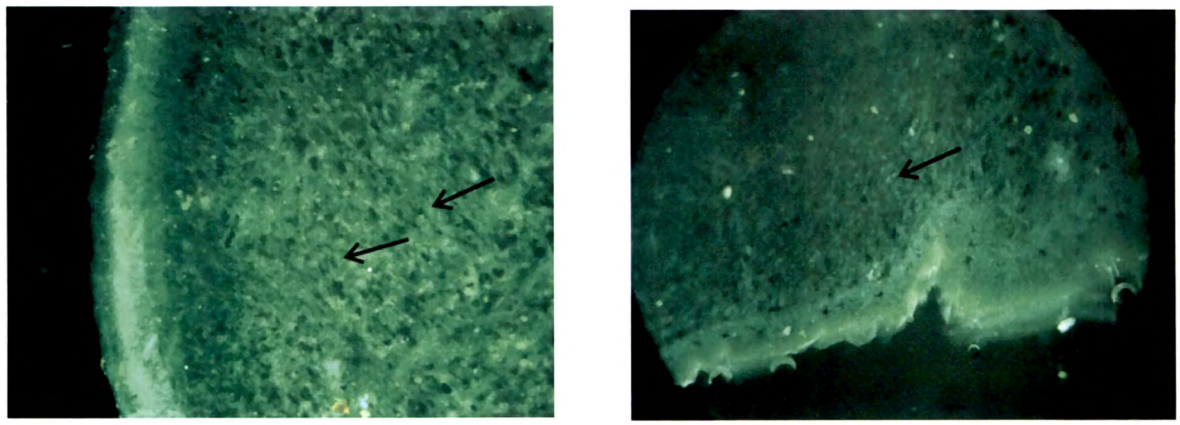
Control at DF Stage (e)



Experimental at DF Stage (f)

Aec-Apical epithelial cap; C-central tube; Mf-Myofiber ; Mm- Myomere;

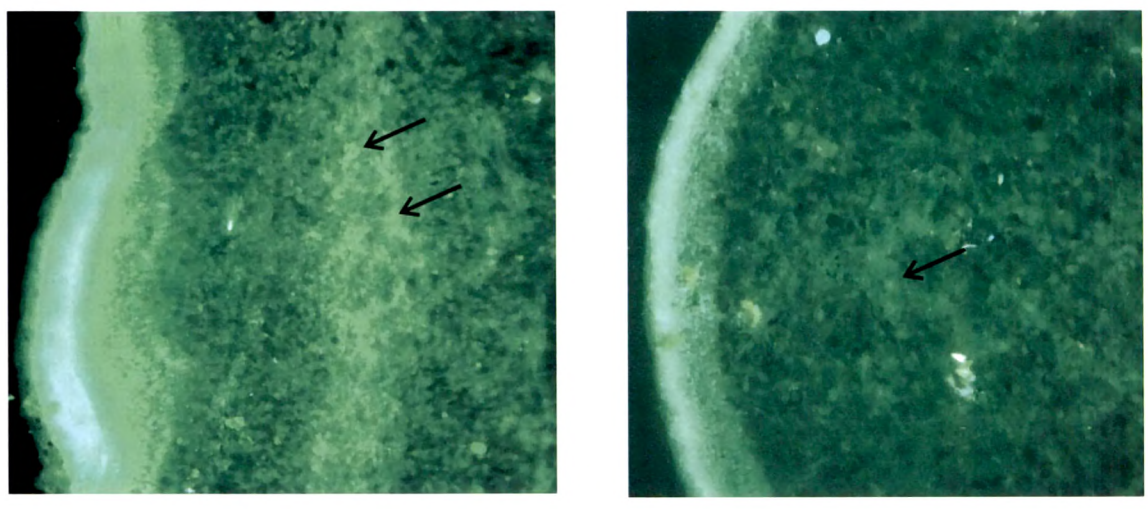
Figure 4.10 BrdU incorporation (⟵)in Lizard at blastema stage



Control (a) Experimental (b)

Blastema Stage

Figure 4.11 BrdU incorporation (⟵) in Lizard at differentiation stage



Control (a) Experimental (b)

Differentiation Stage