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# Histochemical Localization of Alkaline Phosphatase Activity during Cutaneous Wound Healing In a Catfish under Acid Stress

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***Abstract-*** *Heteropneustes fossilis* (Heteropneustidae) is an air breathing freshwater catfish, has an ever increasing demand as a major source of protein and vitamin A. Acid precipitation has detrimental effect on fish population. We have investigated the activity and localization of the enzyme alkaline phosphatase (ALP), by means of histochemical study on experimental wound repair in the fish skin under acid stress. After injury there was a significant decrease in ALP activity in the cells of the migrating epidermis. There was a gradual increase in the activity of ALP in basal cells, epithelial cells of the middle layer and outer layer and club cells after epithelialization (18 h) of the wound during the early stages of healing. By 96h – 10 days, a fairly high ALP activity in the basal cells was observed. With completion of dermal repair, there was a gradual disappearance of ALP activity. The granulation tissue and the differentiating muscle bundles showed a weak reaction for ALP activity. The late appearance and activity of ALP delayed the wound repair process under acid stress condition.

***Index Terms-*** acid stress, alkaline phosphatase, histochemical, skin, wound repair.

## I. INTRODUCTION

Skin the utmost layer of all animals has an inevitable regenerative capacity. The fish skin has the ability to change in its structure in response to variety of external and internal stimuli (Blackstock & Pickering, 1982). Many researchers have paid attention to study the localization of enzymes,and alkaline phosphatase (ALP) in the various cells of the fish skin. Ross *et. al.*, (2000) showed changes in hydrolytic enzyme activities in skin mucus of native Atlantic salmon, *Salmo salar*) due to infection with the Salmo louse *Lepeoptheirus salmonis* and cortisol implantation.Kori-Siakpere *et. al.*(2010) studied the variations in AcP and ALP activity in the plasma of the African Catfish *Clarias gariepinus* exposed to sublethal concentrations of potassium permanganate. Sanchooli *et. al.* (2012) measured the ALP and lysozyme enzymes in epidermal mucus of different weights of *Cyprinus carpio*. Loganathan *et. al.* (2013) examined the lysozyme, protease, ALP and esterase activity of epidermal skin mucus of freshwater snake head fish *Channa striatus*. Enzymes are the biocatalysts of biological origin which accelerates the various cellular reactions. ALP is a hydrolytic enzyme released by lysosomes for hydrolysis of foreign materials. ALP works in basic medium and takes part in active transport in cellular membrane. ALP is suggested to have a protective role in fishes during first stages of wound healing (Iger & Abraham 1994). There is a growing recognition of the role of ALP in the regenerative processes. The marked quantitative and qualitative alternations in ALP as a result of injury and subsequent regeneration have been the focus of extensive investigation in higher vertebrates, but little attention has been paid to such possible

changes in fishes. Rai & Mittal (1983) reported the activity of ALP in the various tissue components of the regenerating skin of the catfish *Heteropneustes fossilis*. No literature have been found available pertaining to the activity and localization of ALP in different types of cells of fish skin during cutaneous wound healing under acid stress condition. Due to acid rain and acidic industrial effluents the pH of the aquatic medium is lowered which may be lethal or sublethal to an organism which may create any possible toxic effect to the organism. Therefore, the experiment was designed to study the localization and activity of ALP in the skin during wound healing in *Heteropneustes fossilis* under acid stress.

## II. MATERIALS AND METHODS

Adult live catfish, *Heteropneustes fossilis* (approximately, 17-18cm in length irrespective of sex) were collected from freshwater ponds at Dibrugarh, Assam and acclimatized to laboratory conditions for 7 days before setting the experiment. The fishes were fed with goat liver for maintaining fish in a healthy state (Dheer, 1978). Groups of fish were transferred to different aquaria containing acid water with pH 4.0 by adding $H_2SO_4$. This pH was maintained by changing acid water six hourly. The entire experiment was carried out in between pH 4.0 to 5.0, a sublethal pH range for this fish. Water pH (4.0 to 5.0) was maintained during the experimental period. Water of all the aquaria were changed regularly each at 6h intervals when the concentration of $H_2SO_4$ was adjusted to counteract slight pH drift due to release of excretory product and build up of any free $CO_2$ in the experimental aquaria. Free CO2 is toxic and also changes the pH of the medium (Doudoroff & Katz 1950; Lloyd & Jordan 1964; Eddy, 1974).

Before the creation of wound, fishes were anasthesized with 0.02% clove oil (Matin *et. al*., 2009). Incised wound of approximately 5mm in length and 3mm in depth parallel to the longitudinal axis of the body was made with a sharp scalpel blade between the dorsal fin and the lateral line canal in each fish. The fishes were returned immediately to the aquaria until sampling. After anasthesizing the fish, skin fragments each of which contained a wound were removed at various intervals (30min to 30 days) and were fixed in 10% neutral formalin at $4^oC$ for 15 – 24h. For each experimental intervals 5 samples were taken in five different aquaria. Frozen sections were cut at 10 - 30µm using an American optical cryocut. ALP activity was visualized using calcium cobalt method (Gomori, 1952) and coupling Azo-dye method (Pearse, 1968). In coupling Azo dye method for both ALP sodium – alpha-naphthyl-phosphate was used as substrate. Fast Red TR was employed as coupling diazonium salts for ALP. Control slides were prepared by incubating the sections in the absence of the substrate and in the presence of the substrate after inactivation of the enzymes by 10 minutes treatment with boiling water (Barka & Anderson, 1965).

## III. RESULTS

### I. EPIDERMIS

#### a. BASAL CELLS AND EPITHELIAL CELLS (Table I )

***After 30 min-2h -*** Immediately after the infliction of the wound, the ALP of epithelial cells of both in the middle layer and in the outermost layer and of the basal cells in the region I (wound gap) showed a sudden depletion and could not be located during this period in these cells but in the region II (surrounding area of wound gap) basal cells showed weak reaction for ALP whereas the epithelial cells

of the middle layer remained very strong upto to the end period and cells of the outer layer exhibited moderate weak reaction throughout the experimental period (Fig. 1).

***After 4-18h -*** At about 4h, small amount of ALP appeared in the basal cells and in the epithelial cells of the middle and outer layer of the region I (Fig. 2) whereas in region II, the basal cells showed a moderate increase in ALP activity at 8h to 18 h. However there was a slight decline in the enzyme activity in the middle layer of region II.

***After 24-96h -*** After completion of epithlialization at 18h, there was a gradual increase in the activity of ALP in the basal cells in the both regions I and II. This condition continues without any significant change in the activity upto the end of this period (Fig. 3). The epithelial cells of middle and outer layer of region I showed a significant increase in the ALP activity which increase gradually and became more intense by the end of this period. Whereas the region II epithelial cells of middle layer showed a gradual decrease in the ALP activity which is comparatively opposite to region I activity but the outer layer did not show any change in the ALP activity.

***After 5 – 30 days -*** At 5 days, the basal cells showed maximum ALP activity in both regions I and II, however there was a gradual decline in the intensity of the reaction after 5 day throughout this period. In the epithelial cells of middle and outer layer of both region I and II there was a gradual increase in ALP activity after 5 days and by the end of 30 days the activity of ALP becomes similar to that of the normal appearance, however the activity of ALP in the outer layer, epithelial cells of region II showed a constant reaction throughout the period (Fig. 4).

**b. Club cells (Table 1)**

***After 30 min – 18 h -*** No ALP activity can be detected in the club cells present in the epidermis at region I. However, after 2h ALP gave weak reaction till the end of this period. In region II, there was a slight decline in ALP activity in the club cells than the normal skin.

***After 24 – 72h -*** After epithelialization at 18 h, significant increase in the ALP activity was observed in club cells of epidermis in region I and II and remained constant upto the end of this period (Fig. 2).

***After 84h – 30 days -***In region I, the club cells showed dramatic increase in the ALP activity till 10 days and by the end of 30 days these cells of both regions I and II showed the same pattern of enzyme distribution as was observed in normal club cells (Fig. 4).

**c. Mucous cells (Table I )**

Mucous cells donot show any activity of ALP during the wound healing process after acid stress.

**Table I. The distribution of ALP activity during the different stages of healing of skin wounds in *Heteropneustes fossilis* after acid stress.**

| Time of infliction | Region I | | | | | Region II | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Basal cell | Club cell | Mucous cell | Epithelial cell | | Basal cell | Club cell | Mucous cell | Epithelial cell | |
| | | | | Middle layer | Outer layer | | | | Middle layer | Outer layer |
| Normal | ++ | + | - | +++ | ± | ± | + | - | +++ | +± |
| 30min | - | - | - | - | - | ± | ± | - | +++ | +± |
| 1hr | - | - | - | - | - | ± | ± | - | +++ | +± |
| 2hr | - | ± | - | - | - | ± | ± | - | +++ | +± |
| 4hr | ± | ± | - | ± | ± | ± | ± | - | +++ | +± |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 8hr | ± | ± | - | ± | ± | +± | ± | - | +++ | +± |
| 12hr | ± | ± | - | ± | ± | +± | ± | - | ++ | +± |
| 18hr | +± | ± | - | + | ± | +± | + | - | ++ | +± |
| 24hr | +± | + | - | + | ± | ++ | + | - | ++ | +± |
| 36hr | +± | + | - | + | ± | ++ | + | - | ++ | +± |
| 48hr | +± | + | - | + | ± | ++ | + | - | ++ | +± |
| 60hr | ++ | + | - | ++ | ± | ++ | + | - | + | +± |
| 72hr | ++ | + | - | ++ | + | ++ | + | - | + | +± |
| 84hr | ++ | +± | - | ++ | + | ++ | + | - | + | +± |
| 96hr | ++ | +± | - | ++ | + | +++ | + | - | + | +± |
| 5days | +++ | +± | - | +++ | + | +++ | + | - | + | +± |
| 10days | +++ | +± | - | +++ | ++ | ++ | + | - | ++ | +± |
| 15days | +++ | + | - | +++ | ++ | ++ | + | - | ++ | +± |
| 25days | ++ | + | - | +++ | ++ | ++ | + | - | +++ | +± |
| 30days | ++ | + | - | +++ | ++ | ++ | + | - | +++ | +± |

*ABBREVIATION USED: Region I – wounded region, Region II – adjacent area of the wounded region, '-'–negative reaction, '±' – weak reaction, '+' – moderate reaction, '++' – strong reaction, '+++' – very strong reaction.

### II. Dermis and subcutis (Table II)

***After 30 min – 96h*** **-** No reaction for the enzyme ALP was observed in subepidermal tissues. Only the RBC and WBC showed ALP activity. WBC being weak at the early hour gradually increased with strong reaction at 60h and becoming moderate to the end of the period whereas the appearance of activity of ALP in RBC started after 24h becoming strong to the end of this period.

***After 5 – 30 days*** **-** The newly formed granulation tissues, near the cut edges of the dermis, showed moderate reaction for ALP (Fig. 4) activity at 5 days. After 15 days, the activity of this enzyme finally disappeared by the end of 30 days. At 96h, fibroblast cells showed weak reaction for ALP activity. While the intensity of enzyme in the fibroblast increased slowly from 5 days in the wound gap, the enzyme showed a strong reaction only till 15 days and disappeared by the end of this period with the maturation of collagen fibre bundles. Whereas, the activity of enzyme in the dermis and subcutis remained strong only for a few days starting from 5 days and gradually disappeared to the end of the period whereas in the subcutis it remained moderate till the end of the period.

### III. Muscles

Following injury, no reaction for ALP could be observed in the muscle bundles lying in wound area during the entire process of their complete degeneration. At about 96h, a weak reaction for ALP could be observed in the differentiating muscle bundles developing at the level of normal muscle bundles, lying immediately below the dermis.

**Table II. The distribution of ALP activity in various subepidermal tissues during different stages of healing of skin wounds in *Heteropneustes fossilis* after acid stress**

| Time of infliction | Amorphous substances | Granulation tissue | RBC | WBC | Fibroblast | Dermis | Subcutis | Dedifferentiating muscle bundle | Differentiating muscle bundle |
|---|---|---|---|---|---|---|---|---|---|
| 30min | - | - | - | - | - | - | - | - | - |
| 1hr | - | - | - | ± | - | - | - | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 2hr | - | - | - | ± | - | - | - | - | - |
| 4hr | - | - | - | ± | - | - | - | - | - |
| 8hr | - | - | - | ± | - | - | - | - | - |
| 12hr | - | - | - | ± | - | - | - | - | - |
| 18hr | - | - | - | ± | - | - | - | - | - |
| 24hr | - | - | ± | ± | - | - | - | - | - |
| 48hr | - | - | ± | ± | - | - | - | - | - |
| 60hr | - | - | + | ++ | - | - | - | - | - |
| 72hr | - | - | + | + | - | - | - | - | - |
| 84hr | - | - | + | + | - | - | - | - | - |
| 96hr | - | - | + | + | ± | - | - | - | ± |
| 5days | - | + | + | + | ++ | + | - | - | ± |
| 10days | - | + | ± | ± | +++ | ++ | ± | - | ± |
| 15days | - | + | - | ± | +++ | ++ | + | - | ± |
| 20days | - | - | - | - | - | + | + | - | ± |
| 25days | - | - | - | - | - | - | + | - | ± |
| 30days | - | - | - | - | - | - | + | - | ± |

*ABBREVIATION USED: '-'–negative reaction, '±' – weak reaction, '+' – moderate reaction, '++' – strong reaction, '+++' – very strong reaction.

## IV. CONCLUSION

The most immediate enzymatic change noted just after the wound in the skin of Asian stinging catfish *Heteropneustes fossilis* after acid stress was a significant decrease in ALP activity. These may be correlated with the significant increase in the activity of ALP in various cellular components of the migrating epithelium just after infliction of wound in the skin of Asian stinging catfish *Heteropneustes fossilis* (Rai & Mittal 1983, 1991).The low level of ALP indicated that the anabolic events in those cells are probably taking place at a very slow rate and these may be the reason for delay in epithelialization period after acid stress which completes within 18h instead of 4h in normal process of cutaneous wound repair (Mittal *et. al*., 1977). The involvement of ALP in active transport (Denielli, 1972), glycogen metabolism (Gupta & Rao, 1974), protein synthesis (Pilo *et. al*., 1972), synthesis of some enzymes (Sumner, 1965) and secretory activity (Ibrahim *et. al*., 1974) were reported. Thus, any alteration in the activity of ALP affects the process of epithelialization.

Rai & Mittal (1983) while studying the ALP activity during cutaneous woud healing of normal skin of Asian stinging catfish *Heteropneustes fossilis* reported that after epithelialization there was a gradual loss on ALP activity in the epidermal cells upto 24h was observed and they suggested that the anabolic events in these cells are probably taking place at a very slow rate. The present investigation revealed that after acid stress the activity of ALP in basal cells, epithelial cells of the middle layer and outer layer and club cells gradually increased. This is attributed to the anabolic events in these cells which are probably taking place at a very high rate. In normal skin wound healing of Asian stinging catfish *Heteropneustes fossilis* a fairly high ALP activity in the basal cells between 2 to 6 days was observed by Rai & Mittal (1983) which showed that this enzyme plays a significant role in cellular division and differentiation. The role

of ALP in cell division has also been emphasized by Mori *et. al*., (1960) and Mittal & Banerjee (1975 a). Whereas in the present experiment after acid stress high ALP activity in the basal cells was observed between 5 and 15 days. It indicates that the wound healing process is taking place at slow rate as cell division and differentiation is delayed compared to the normal process of wound repair. In normal skin of Asian stinging catfish *Heteropneustes fossilis* strong ALP activity has been observed in the middle layers of epithelial cells which has been correlated with the synthesis of mucopolysaccharides (Rai & Mittal, 1983). This enzyme is believed to be involved in the synthesis of mucopolysaccharides (Kroon, 1952; Banerjee *et.al*., 1976). Howerver, in the present observation after acid water treatment, high ALP activity was found in the epithelial cells of the middle layer and outer layer and in the club cells. It is also found that after epithelialization mucopolysaccharides synthesized more during wound repair in the epidermis. This is attributed with the high activity of ALP in the synthesis of mucopolysaccharides, required during wound repair after acid stress. Further, the mucopolysaccharides synthesized may be utilized in the formation of connective tissue. It is believed that ALP activity is associated with collagen fibre synthesis (Bradfield, 1951; Fell & Danielli, 1943) and has some function in phagocytosis and degradation of collagen (Ten Cate & Syrbu, 1974). In the present experiment the cut edges of dermis in the wounded region showed gradual increase in the ALP activity from $7^{th}$ day rather than $5^{th}$ day in normal repair. This is suggested that the activity of this enzyme have been involved in the synthesis and remodelling of the dermal connective tissue during repair which is occurring at a slow rate than normal cutaneous healing in this fish. The gradual disappearance of ALP with the maturation of the collagen fibre indicated that synthesis of collagen fibres have been ceased with the completion of dermal repair.

Rai & Mittal (1983) observed that the muscle bundles did not give a positive reaction for ALP under normal conditions, showed no significant activity throughout the degenerative and regenerative phases of the wound repair. The present investigation, however revealed that after acid stress, the dedifferentiating muscle bundles did not show any ALP activity whereas differentiating myoblast showed weak reaction for ALP. This may be attributed with the acute need of some metabolites during the regeneration of muscle bundles under acid stress condition. Vorbrodt (1959) has reported that ALP is an important enzyme of animal metabolism, which plays an important role in the transport of metabolites across the membranes, it also plays an important role in protein synthesis (Pilo *et. al*., 1972) and involved in the synthesis of certain enzymes (Sumner, 1965). During stress conditions, fishes need more energy to detoxify the toxicants to overcome stress. Since, fish have a very little amount of carbohydrates; the next alternative source of energy is protein to meet the increased energy demand. The depletion of protein fraction in liver and muscle tissues may have been due to their degradation and possible utilization of degraded product for metabolic purposes (Tiwari & Singh, 2003). This may be correlated with the present result that there is an increase in energy requirement by the differentiating myoblast cells under stress conditions, so these cells of the muscle bundle showed a weak reaction for ALP during the wound healing process after acid stress.In the present experiment, activity of the enzyme ALP involved in the wound repair process was delayed than the normal wound repair. The wound healing process was completed within 30 days after acid water treatment; the process was delayed than the normal repair process which is completed in 25 days (Rai & Mittal, 1983, 1991). Therefore, present investigation revealed that due to acid stress the process of cutaneous wound repair is delayed and correspondingly appearance and disappearance of enzyme ALP was also delayed.

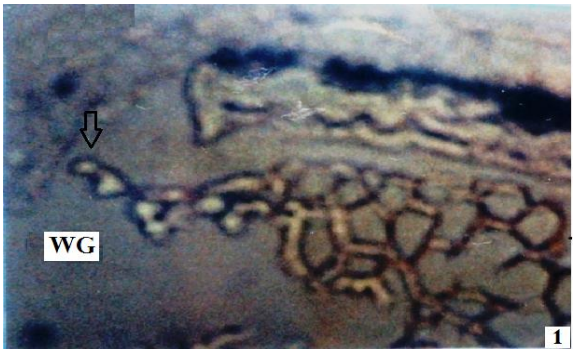


Figure 1. Coupling Azo dye for ALP. A view of the wound gap showing very weak reaction for ALP in the migrating epithelium (arrows) (2h, after acid stress, 800X) WG wound gap

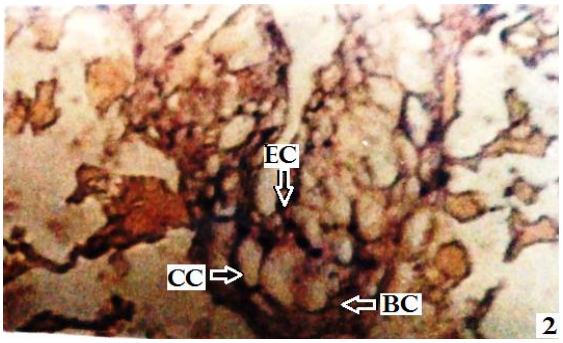


Figure 2. Coupling Azo dye for ALP. After complete epithelialization of wound, strong ALP reactions in the basal cells, club cells and epithelial cells (arrows) (18h, after acid stress, 800X). BC basal cells, CC club cells, EP epithelial cells

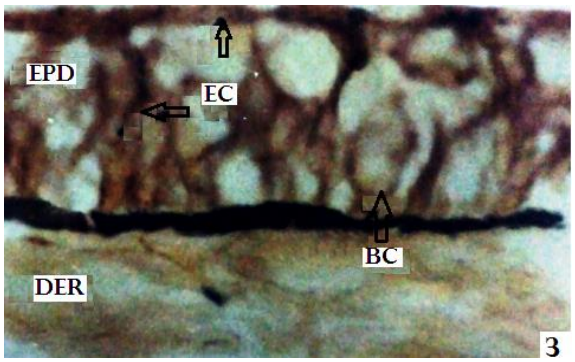


Figure 3. Coupling Azo dye showing more ALP activities in basal cells and epithelial cells (arrows) (60h, after acid stress, 800X). BC basal cells, EC epithelial cells.

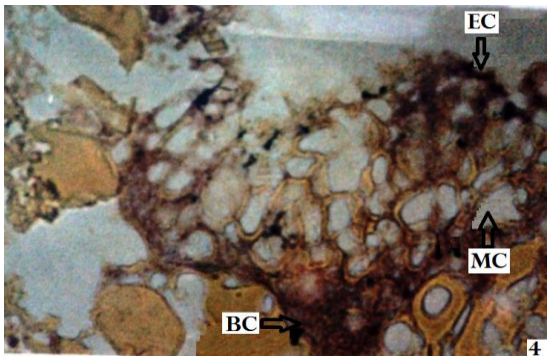


Figure 4. Coupling Azo dye showing ALP activities in the basement membrane, basal cells, club cells and epithelial cells (arrows) (5 days, after acid stress, 200X).BM basement membrane, BC basal cells, CC club cells, EC epithelial cells.

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